CONTINUOUS FLOW DIGITAL PCR FOR ABSOLUTE QUANTIFICATION OF NUCLEIC ACIDS

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Declaration

The substance of this thesis is the original work of the author, and due reference and acknowledgement has been made, where necessary, to the work of others. No part of this thesis has been submitted in canditure for any degree.

__________________________________________
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__________________________________________
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Abstract

Digital polymerase chain reaction (PCR) has emerged as an extremely powerful technique for quantification of nucleic acids. Digital PCR offers superior quantification accuracy, precision and sensitivity compared to quantitative PCR – the current gold standard in nucleic acid quantification. The quantification capabilities of digital PCR permit new applications such as non-invasive prenatal diagnosis and rare mutation detection in cancer diagnostics. The technique also offers improvement in viral load quantification for HIV monitoring and residual minimum disease quantification for monitoring disease progression. Digital PCR quantifies the number of targets by partitioning a biological sample into many individual reactions, performing PCR and directly counting the number of positive reactions in an experiment. Quantification is highly accurate and precise since the starting quantity of target molecules is not derived from standards or C_q values as with quantitative PCR. As digital PCR quantification is absolute, very small copy number ratios can be precisely measured. The thousands of reactions used in a digital PCR experiment permits single molecule sensitivity, a fundamental characteristic of digital PCR. The commercially available digital PCR instruments employ microfluidic arrays or droplets to partition samples into thousands of individual reactions. These instruments require expensive consumables, greatly increasing the experimental cost and limiting experimental design. Continuous flow PCR is a novel technology that generates, thermal cycles and fluorescently detects droplets in a continuous system that does not require any consumables - facilitating low cost digital PCR.

A continuous flow digital PCR instrument was designed and developed at the Stokes Institute. These droplet based instruments operate by delivering droplets through the temperature zones required for PCR. The flowing droplets are fully wrapped and separated by an immiscible carrier fluid that prevents contamination on the device. Two droplet production techniques were characterised. Liquid bridge dispensers were employed in this thesis as they were shown to produce highly consistent droplets as small as 45nL in volume. Carryover contamination at the liquid bridge dispenser was identified and countered using wash steps. A proof of concept amplification study demonstrated the devices capability of amplifying a DNA target in a flowing droplet. A GAPDH and RNase P target was amplified on the device in two separate experiments and verified using gel electrophoresis.

Digital PCR was successfully performed on the continuous flow platform. This is the first time absolute quantification using digital PCR has been performed in a flowing system. Droplets were stable, highly consistent and results demonstrated that there was no cross over contamination on the instrument. Amplification of a single RNase P target molecule in a flowing droplet was achieved, demonstrating single molecule sensitivity. Singleplex digital PCR was used to quantify various concentrations of RNase P target molecules in gDNA, ranging from 178 – 6100 copies/mL. The singleplex quantification results correlated extremely well with the theoretical copy number calculations. Duplex digital was performed on the instrument, examining the copy number ratio of RNase P to SRY. The digital copy number ratio quantification results deviated from the expected RNase P to SRY ratio of 2:1. A quantitative PCR study confirmed that this was due to poor optimisation of the duplex assay and was not related to instrument performance. Continuous flow instruments are a highly desirable alternative to current digital PCR platforms. The technology is best placed to perform digital PCR for applications requiring quantification of rare targets in large sample volumes. The flexibility in droplet production permits processing of large sample volumes quickly and at a low cost versus commercial digital PCR platforms which require numerous consumable chips.
Acknowledgements

I would like to offer a sincere thank you to Dr. Tara Dalton for giving me the opportunity to pursue this research and facilitating an excellent learning environment. Your guidance and support made this research possible. Also thank you to Prof. Mark Davies for his advice and support throughout the project.

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Thanks to all of my colleagues and friends in Stokes who have made my time as a postgraduate some of the best years of my life!

I want to thank my parents and family for their love, support and encouragement in all my endeavours. Special thanks to Ciara for her support over the years and who has yet to figure out ‘what is going on in those bubbles’!
Dedication

This work is dedicated to my loving family for their continuous support and encouragement.
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<tr>
<td>$A$</td>
<td>Inverse calibration matrix</td>
<td>-</td>
</tr>
<tr>
<td>$c$</td>
<td>Speed of light</td>
<td>ms$^{-1}$</td>
</tr>
<tr>
<td>$C$</td>
<td>Calibration matrix</td>
<td>-</td>
</tr>
<tr>
<td>$Ca$</td>
<td>Capillary number</td>
<td>-</td>
</tr>
<tr>
<td>$C_d$</td>
<td>Network droplet capacity</td>
<td>-</td>
</tr>
<tr>
<td>$C_q$</td>
<td>Quantification Cycle</td>
<td>-</td>
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<tr>
<td>$D$</td>
<td>Capillary diameter</td>
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<tr>
<td>$E$</td>
<td>Energy of a photon of light</td>
<td>J</td>
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<tr>
<td>$f$</td>
<td>Droplet frequency</td>
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</tr>
<tr>
<td>$h$</td>
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</tr>
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<td>Kg</td>
</tr>
<tr>
<td>$n$</td>
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<td>-</td>
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<tr>
<td>$n_{bp}$</td>
<td>Genome size in base pairs</td>
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<tr>
<td>$N_c$</td>
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<td>-</td>
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<td>$N_{total}$</td>
<td>Total number of target molecules</td>
<td>-</td>
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<tr>
<td>$P$</td>
<td>Probability</td>
<td>-</td>
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$\Delta P_{bp}$ Biphasic pressure drop Pa

$\Delta P_{bp-t}$ Total biphasic pressure drop Pa

$\Delta P_{sp}$ Single phase pressure drop Pa

$Q$ Volumetric flow rate $m^3 s^{-1}$

$Q_a$ Aqueous volumetric flow rate $m^3 s^{-1}$

$Q_o$ Oil volumetric flow rate $m^3 s^{-1}$

$Q_{out}$ Outlet volumetric flow rate $m^3 s^{-1}$

$Q^*$ Volumetric flow rate fraction -

$Re$ Reynolds number -

$R_{ii}$ Inlet internal radius m

$R_{io}$ Outlet internal radius m

$R_{sp}$ Single phase fluidic resistance $N s m^{-5}$

$R^2$ Coefficient of determination -

$r_i$ Internal radius m

$r_d$ Droplet radius m

$T$ Threshold -

$t_a$ Time to answer s

$t_c$ Thermal cycling time s

$T_m$ Melt temperature °C

$t_p$ Droplet processing time s

$U$ Velocity $m s^{-1}$

$v$ wavelength m

$V_d$ Droplet volume $m^3$

$V_l$ Volume lead droplets $m^3$

$V_t$ Total volume droplets $m^3$

$w$ Confidence interval width -

$x_t$ Unit size m

xxi
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon$</td>
<td>Polymerase chain reaction efficiency</td>
<td>-</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Expected average number of occurrences</td>
<td>-</td>
</tr>
<tr>
<td>$\hat{\mu}$</td>
<td>Estimated average number of occurrences</td>
<td>-</td>
</tr>
<tr>
<td>$\mu_a$</td>
<td>Aqueous viscosity</td>
<td>Pa s</td>
</tr>
<tr>
<td>$\mu_o$</td>
<td>Oil viscosity</td>
<td>Pa s</td>
</tr>
<tr>
<td>$\mu_f$</td>
<td>Viscosity</td>
<td>Pa s</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Density</td>
<td>Kg m$^{-3}$</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Interfacial tension</td>
<td>N m$^{-1}$</td>
</tr>
<tr>
<td>$\sigma_i$</td>
<td>Variance</td>
<td>-</td>
</tr>
</tbody>
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**Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>AB7900HT</td>
<td>Applied Biosystems 7900HT instrument</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral treatment</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cf</td>
<td>Cell free</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CNC</td>
<td>Computer numerical control</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dPCR</td>
<td>Digital polymerase chain reaction</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GM</td>
<td>Genetically modified</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NIPD</td>
<td>Non-invasive prenatal diagnosis</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non small cell lung cancer</td>
</tr>
<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PID</td>
<td>Proportional integral derivative</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROX</td>
<td>6-Carboxy-X-rhodamine</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 Background

Living organisms contain deoxyribonucleic acid (DNA), a nucleic acid that contains the genetic instructions required for their development and function. DNA exists as two long polymer strands. These polymer strands consist of basic repeating units called nucleotides on a sugar phosphate backbone. There are four different nucleotides bases; adenine, cytosine, guanine and thymine. The strands are complementary to each other with a nucleotide on one strand interacting with just one type of nucleotide on the other strand. The complementary base pairing forms hydrogen bonds between the strands, resulting in a double helix structure, as shown in figure 1.1.

![Figure 1.1: The DNA double helix.](image-url)
DNA is found in every nucleated cell in the body and forms the basis of human inheritance. This genetic information is carried by discrete sections of DNA called genes. The nucleotide sequence of a gene is specific to the production of a particular protein which has a distinct function in the cell. Every nucleated cell in the body contains the same set of genes. However, not all of these genes are selectively turned on or expressed. The genes that are expressed vary for different cell types, allowing the cells in the body to have different functionalities.

Gene expression, the process by which information from a gene is used create a functional gene product is a highly controlled and regulated process. The most frequently produced gene products are proteins. There are a number of steps within the gene expression mechanism, the first being transcription. The specific DNA sequence is first transcribed to an intermediate molecule called messenger ribonucleic acid (mRNA). The quantity of mRNA transcripts reflects the activity of a gene in a cell. In the final step of gene expression, mRNA is translated into one or more functional proteins. The transcription of DNA to mRNA to protein is the central dogma of molecular biology and is illustrated in figure 1.2. At the DNA level, human genome sequencing has identified mutations and genetic

![Figure 1.2: The central dogma of molecular biology.](image)

variations which have roles in many genetic disorders and diseases (Venter et al., 2001). Using this information, investigations at the DNA level permits diagnosis of these genetic disorders and diseases. However, for many diseases it is more informative to observe gene
expression levels. In addition to gene expression controlling a cell's character and function, a cell can also change its gene expression in response to internal or external signals. The quantity of proteins produced and hence the quantity of mRNA present changes in response to these internal or external cell signals - many of which are triggered by disease. Therefore, knowledge of the quantity of mRNA can give an insight into a cell's current biological state.

Although microarray technology can be used to measure whole genome expression profiles, the information provided by these microarrays is not quantitative. Quantitative polymerase chain reaction (qPCR) is considered the gold standard in quantification and can be used to identify genetic mutations or measure gene expression. The polymerase chain reaction (PCR) is a method of amplifying specific sequences of nucleic acids. This method was first described by Saiki et al. in 1985 and considered a significant milestone in molecular biology. Small, undetectable quantities of nucleic acids are amplified using PCR, facilitating the detection of the target nucleotide sequence. The amplification of nucleic acids is achieved by thermally cycling a reaction through specific PCR temperatures. There are three steps in the PCR process; denaturation, annealing, and extension. The first step of PCR is denaturation. The sample is heated, causing the two strands in the double stranded DNA (dsDNA) double helix to separate. The temperature is then lowered to allow short complementary sequences called primers to anneal to the single stranded DNA (ssDNA) target. In the final step of the PCR process, the temperature is raised to allow the Taq DNA polymerase to extend the primers, creating a new dsDNA molecule. The PCR process is illustrated in figure 1.3. The target DNA is exponentially amplified through the repetition of the PCR process, yielding millions of target DNA molecules that can easily be detected.

The PCR process can be used to amplify a genetic target to detectable levels but does not provide any information about the starting concentration. Endpoint analysis techniques such as gel electrophoresis can be used to determine the presence of amplified product but does not provide accurate quantitative information. As discussed earlier, the ability to quantify the number of genetic targets present in a sample is important. The addition of fluorescence DNA-binding dyes and hybridisation probes that bind to DNA provide a method
CHAPTER 1 Introduction

Step 1: Denaturation

Step 2: Annealing

Step 3: Extension

Finish: Amplification

Figure 1.3: Schematic illustrating a PCR cycle. Double stranded DNA is denatured (step 1), the primers anneal to the target strand (step 2) and the Taq polymerase extends along the primers, creating a new dsDNA molecule.

of determining the starting target concentration. As target DNA molecules are exponentially amplified there is a corresponding increase in fluorescence. Higuchi et al. (1993) first presented a method of quantifying the initial starting concentration of target molecules in a sample by monitoring the fluorescence signal after each cycle. This is known as qPCR. Quantification is achieved by observing the cycle number at which the fluorescence intensity of a reaction reaches a defined fluorescence intensity threshold. This cycle number is known as the quantification cycle ($C_q$). The initial quantity of target molecules can be determined through the use of standard curves. This method of quantification is known as absolute quantification. Alternatively, if the copy number ratio of two targets is desired, relative quantification can be used. Relative quantification uses the difference in $C_q$ values between two targets to calculate the relative concentration. This is possible since it is known that the number of target molecules double after each cycle and a $C_q$ difference of 1 cycle constitutes a 2-fold difference in starting concentration.
Although qPCR is considered the gold standard in quantification, its does have a number of shortcomings. Firstly, the absolute quantification accuracy is dependent on the accuracy of the standards and the assumption that the amplification efficiency of the targets and the standards are the same. Standard preparation is subject to inaccuracies and the amplification efficiencies are rarely identical, yielding uncertainty in the quantitative result. Although relative quantification does not require standards, relative quantification is limited to a 2-fold difference in starting copy number. In addition, conventional qPCR does not possess single molecule sensitivity and therefore does not perform well in applications requiring amplification of a very small number of rare targets. For some biological applications, extremely accurate and sensitive quantification is vital. The requirement for an alternative quantification approach led to the development of digital PCR, a technique which directly quantifies the initial number of target molecules by counting the number of positive reactions in an experiment.

1.2 A New Digital Age: Development and Principles of Digital PCR

Sykes et al. (1992) first described a digital PCR (dPCR) based quantification method using limiting dilutions. This was the first study to describe a method of determining the number of input molecules by simply counting the number of positive PCR reactions in a limiting dilution. This plus/minus approach paved the road for what is now known as digital PCR. In 1999, Vogelstein & Kinzler coined the term digital PCR. They proposed and implemented the concept of digital PCR to quantify genetic mutations in a DNA sample. The authors achieved this by diluting a DNA sample to ensure single target molecules were isolated in the individual wells of a 96 well plate. The plate was subjected to PCR and interrogated using fluorescence detection. The number of wild-type and mutant sequences were then quantified by counting the number of positive wells. This digital PCR ‘counting’ concept is illustrated in figure 1.4 (a).

This technique permits accurate, precise quantification of the number of target molecules in a sample. Although promising, the approach outlined by Vogelstein & Kinzler was time
Figure 1.4: Digital PCR Concepts. (a) Original concept: the number of starting target molecules can be directly quantified by counting the number of positive micro-reactors after PCR. (b) Poisson digital concept: the number of starting target molecules can be quantified by applying a statistical algorithm based on the proportion of positives.

Modern digital PCR was realised with the development of microfluidic chip and droplet based technology. Digital PCR technology is discussed later in section 1.5. Chip and droplet based platforms permit thousands of miniaturised PCR reactions (micro-reactors) in a single experiment, consuming low quantities of reagent at a high-throughput.

The digital PCR concept evolved with the development of digital PCR technology.
Dube et al. (2008) proposed an approach of quantifying the number of target molecules based on the proportion of positive reactions using Poisson statistics. A positive micro-reactor contains at least one target molecule. If the sample is sufficiently dilute, the starting copy number can be quantified by directly counting the number of positives, as described earlier. However, if there is a large proportion of positive reactions resulting in more than one target molecule per well, this approach cannot be applied. In this scenario, counting the number of positives would yield an inaccurate quantitative result. A Poisson based algorithm can be used to determine the number of starting copies (and 95% confidence intervals) based on the number of positives and total number of micro-reactors (Dube et al., 2008). This digital PCR approach is illustrated in figure 1.4 (b).

Observing 1.4 (b), target DNA molecules are randomly distributed upon partitioning a sample into micro-reactors, with some reactions containing more than one target molecule. The micro-reactors are subjected to PCR and interrogated using fluorescence techniques. Using the experimental binary output of the number of positive and negative micro-reactors, the starting copy number can be quantified using statistical methods. This digital PCR approach does not require a pre-quantification step to generate a dilution suitable for directly counting the number of positives. Using this approach, the dynamic range of an instrument is greatly increased and the number of empty, ‘wasted’ wells are reduced.

1.3 Benefits of Digital PCR

Digital PCR offers superior quantitative accuracy and precision compared to qPCR. Digital PCR does not rely on data collected during the exponential phase of PCR like qPCR and does not require a standard curve to achieve absolute quantification. Therefore, the absolute quantification accuracy of dPCR is not affected by inaccurate standards or differences in amplification efficiency between the target and standards. Digital PCR copy numbers are quantified directly rather than being derived from $C_q$ values. As discussed earlier, relative quantification using qPCR is limited to a 2-fold change in starting copy number as this constitutes a quantification cycle difference equal to 1. Digital PCR can accurately discriminate extremely small copy number ratios as copy number quantification is absolute.
and independent of variations in amplification efficiency.

Digital PCR also offers increased sensitivity. In standard qPCR reactions, rare target molecules can be difficult to amplify due to the large amount of DNA background, resulting in reduced sensitivity (Zimmermann et al., 2008). In digital PCR, the relative concentration of target molecules is increased upon partitioning a sample into micro-reactors.

![Diagram of PCR Reaction, Partitioning into micro-reactors, PCR - single molecule amplification](image)

Figure 1.5: Increased sensitivity associated with digital PCR: partitioning a sample into micro-reactors increases relative target concentration.

The needle in a haystack problem is transformed to a target molecule in a much smaller volume with fewer background molecules, increasing sensitivity (Vogelstein & Kinzler, 1999). As a result, digital PCR is capable of single molecule detection. For example, if a sample mixture containing 1 molecule of a mutant target in 5,000 non mutated molecules is partitioned into 1,000 independent micro-reactors, the micro-reactor containing the single mutant target molecule now contains 5 background molecules. This 1000-fold increase in relative concentration should allow a 1000-fold improvement in detection sensitivity. This concept is illustrated in figure 1.5.

The characteristics of qPCR and dPCR are examined further in Chapter 2. In summary, digital PCR offers:

- extremely accurate, precise absolute quantification
- quantification of very small copy number ratios
- single molecule sensitivity
The quantification capabilities of digital PCR opens the door to new biological applications and offers improvement in existing applications demanding greater quantitative accuracy and sensitivity. The specific biological applications of digital PCR are discussed next.

### 1.4 Digital PCR Applications

To date, digital PCR has been employed in applications requiring highly sensitive and accurate quantification. These applications typically require quantification of rare target molecules or extremely small changes in expression between a target and internal reference. Digital PCR can perform absolute quantification for single or multiple targets in a sample. As a result, the qPCR term ‘relative quantification’ does not apply. Absolute copy number ratios are calculated using absolute quantification reads. Consequently, the biological applications that can be addressed by dPCR are divided into singleplex or multiplex applications. A brief review of the digital PCR applications studied in the literature is presented in this section.

#### 1.4.1 Singleplex Digital PCR Applications:

Singleplex or single target absolute quantification can be employed in studies where the copy number per unit mass or volume is required. It can also be used to determine copy number ratios by performing absolute quantification of two separate targets in two individual experiments, eliminating the need for duplex assay optimisation. The singleplex applications that have been reported in the literature or have the potential to be addressed by digital PCR are discussed here.

A recent study by White et al. (2009) used digital PCR to provide absolute quantification for high throughput sequencing. Next generation sequencing (NGS) platforms currently quantify sequencing libraries by mass using gel electrophoresis or ultra violet (UV) spectrophotometry. These quantification techniques require many more library molecules than what is actually required for sequencing. As much as 1-5 micrograms of DNA is needed to allow for quantification (White et al., 2009). The large input DNA requirement limits the types of samples that can analysed as microgram quantities are not available for
some forensic, environmental and clinical samples. Preamplification of these samples to bring the number of molecules into quantifiable range can distort results due to differences in amplification efficiencies. White et al. (2009) used digital PCR to perform highly accurate absolute quantification for sequencing libraries, consuming subfemtogram amounts of library material. It was shown that digital PCR reduces the sample input requirement, allowing minute samples to be sequenced on the NGS platforms without the uncertainties associated with preamplification. More significantly, this study also demonstrated that the improved quantification accuracy of dPCR justifies the elimination of titration runs on NGS platforms, reducing experimental time and cost.

A study quantifying genetically modified (GM) MON810 maize using dPCR by Corbisier et al. (2010) demonstrates the power of singleplex dPCR. Currently, GM foods are quantitatively analysed using qPCR by estimating the amount of the transgenic event relative to an endogenous gene. GM analysis requires that the amplification efficiency of the target is the same in the material analysed as the standard material used which can be difficult to achieve using qPCR (Corbisier et al., 2010). Digital PCR was proposed as a suitable alternative as it does not require standards and is capable of low copy number detection. In this study the quantity of maize MON810 and a reference gene were quantified separately using dPCR. The absolute quantification results obtained using dPCR were converted into absolute quantification ratios and were found to be in excellent agreement with the ratios determined using qPCR. The study concluded that although both qPCR and dPCR can be used to quantify MON810 maize, dPCR is advantageous as it does not require extensive optimisation and offers superior accuracy.

Viral load quantification is arguably one of the most promising digital PCR applications. Viral load quantification is essential in diseases such as human immunodeficiency virus (HIV) when initiating antiretroviral treatment (ART), monitoring disease progression, monitoring ART response and switching ART regime (Luft et al., 2011). In the case of HIV, HIV-1 ribonucleic acid (RNA) levels are quantified in plasma samples to assess a patient's infectivity. The majority of the current commercial viral load assays perform absolute quantification using qPCR to determine the viral load count in molecules per unit volume (Luft et al., 2011). Consequently, the accuracy of the quantification relies on the
accuracy of the standards. Interpretation of the viral load results is complicated by the lack of a universal standard (Yan et al., 2010). Digital PCR is capable of accurate quantification as it does not rely on standards, making it a suitable technique for viral load quantification. According to Yan et al. (2010), the use of ART has reduced plasma HIV-1 RNA concentrations to levels requiring extremely sensitive and accurate nucleic acid detection assays to monitor disease progression and guide therapeutic decisions. Although the qPCR based assays are effective at quantifying the viral load across a wide dynamic range, low HIV-1 RNA levels can be difficult to detect and quantify (Yan et al., 2010). A clinical ART goal is to achieve a plasma viral load <40-50 copies/mL - a critical level that suggests adequate virological suppression (Yan et al., 2010). This goal is also at the lower limit of quantification (LLOQ) of the current HIV-1 assays. Yan et al. (2010) showed poor concordance between each of the three qPCR HIV-1 assays around the LLOQ. This lack of reproducibility makes it difficult to interpret the low levels of viremia detected using qPCR assays and complicates clinical management (Yan et al., 2010). Digital PCR offers unparalleled sensitivity and accuracy across all levels of target concentration and has the potential to quantify viral load levels below the current assays LLOQ. Furthermore, digital PCR provides a standardised method of viral load quantification, making it a superior method to the qPCR based assays.

Recently, Goh et al. (2011) demonstrated sensitive quantification of minimal residual disease in chronic myeloid leukemia (CML) using digital PCR. The BCR-ABL fusion gene is the causative genetic event in CML, leading to expression of BCR-ABL protein tyrosine kinase, causing malignant transformation of blood cells in CML. Currently, a tyrosine kinase inhibitor called imatinib is used to treat patients with CML. qPCR is used to quantify the number of BCR-ABL transcripts to monitor disease progression and a patients response to therapy. Sustained undetectable levels of BCR-ABL is regarded as PCR negativity and is not indicative of a cure. This is due to the sensitivity limit of qPCR technology. According to Goh et al. (2011), quantitation of very low levels of minimal residual disease could help in more accurately monitoring disease progression and guiding therapeutic decisions. This study showed that digital PCR provides the required sensitivity to achieve this. A target on
the BCR-ABL gene was quantified using digital PCR. It was found that digital PCR exhibited a 2-3 log improvement compared to conventional qPCR. A number of PCR negative samples assayed by qPCR showed detectable levels using digital PCR, demonstrating its potential for residual minimal disease monitoring.

Singleplex digital PCR has been proven to be extremely valuable in next generation sequencing library quantification as demonstrated by White et al. (2009). It also has been proven valuable in single target quantification applications such as GM analysis and residual minimal disease, as shown by Corbisier et al. (2010) and Goh et al. (2011). Corbisier et al. (2010) also demonstrated its potential for calculating absolute copy number ratios without implementing multiplex digital PCR, eliminating the requirement for multiplex assay optimisation. Singleplex digital PCR could potentially be used for viral load quantitation, providing accurate and absolute quantification where quantification using qPCR fails. In summary, the potential singleplex digital PCR applications are as follows:

- Next generation sequencing library quantification
- Viral load quantification
- Residual minimum disease and GM analysis
- Applications requiring highly accurate and precise absolute quantification
- Applications requiring absolute copy number ratios without duplex assay optimisation

Although absolute copy number ratios can be determined using singleplex digital PCR, multiplex digital PCR remains the most accurate method of calculating absolute copy number ratios. Absolute copy number ratios obtained using singleplex digital PCR are subject to errors in sample preparation, significantly reducing the accuracy of the results. Duplex digital PCR is a more accurate approach to absolute copy number ratio estimation. The applications of duplex digital PCR are discussed in the next subsection.
1.4.2 Multiplex Digital PCR Applications

Duplex digital PCR offers superior accuracy in absolute copy number ratio estimation since the ratio of one target to another is independent of sample preparation errors. Duplex digital PCR targets two separate sequences in a sample volume. Subsequent absolute quantification and calculation of the copy number ratio provides the most accurate estimation of the true copy number ratio. The biological applications requiring highly sensitive and accurate absolute copy number ratio estimations will now be discussed.

Cancer Diagnostics: Mutation Detection and Copy Number Ratio Quantification

In 1999, Vogelstein & Kinzler used duplex digital PCR to detect mutant oncogenes in the stools of patients with colorectal cancer. Colorectal cancer is one of the most common forms of cancer and has shown to be curable if detected during the early stages of the disease (Dong et al., 2001). Colorectal cancer cells are shed into the stool, permitting non-invasive detection of colorectal cancer using stool DNA. There are a specific series of genetic changes that drive tumorigenesis - mutation of the K-RAS oncogene is just one of the frequent genetic changes in colorectal cancer (Vogelstein & Kinzler, 1993). Vogelstein & Kinzler (1999) used digital PCR to detect mutations by targeting mutant and wild-type molecules using two different sequence specific hybridisation probes. Dong et al. (2001) used the same digital PCR technique to detect K-RAS oncogene mutations. A more recent study by Pekin et al. (2011) used microfluidic digital PCR to detect and quantify mutated and wild-type K-RAS DNA to determine the mutant allelic fraction. As demonstrated by these studies, the sensitivity of digital PCR permits rare mutation detection from stool DNA, providing a potential method for early detection of the disease. The sensitivity of dPCR is superior to commonly used quantification techniques such as qPCR, making it suitable for rare target detection (Pekin et al., 2011). In addition to the detection of colorectal cancer, Pohl & Shih (2004) state that digital PCR can be also be employed to detect ovarian serous carcinoma and appendiceal adenomas.

Recent studies by Wang et al. (2010) and Yung et al. (2009) used digital PCR to quantify epidermal growth factor receptor (EGFR) mutations to monitor non small cell lung cancer (NSCLC) disease progression and clinical response to EGFR inhibitors. NSCLC
accounts for 80% of all lung cancers and is the leading cause of cancer deaths worldwide (Yung et al., 2009). Clinical trials have shown that reversible inhibitors of the EGFR gene effectively retard disease progression in some patients (Kris et al., 2003).

Yung et al. (2009) quantified two common EGFR mutations associated with NSCLC in tumor tissue and in plasma using digital PCR. EGFR exon 19 deletions were quantified by targeting a wild-type specific sequence (which did not show an increase in fluorescence if a deletion was present) and a reference target on the EGFR gene which indicated the presence of the molecule regardless of the mutational status. Detection of the L858R mutation was performed using a pair of primers targeting the sequence and three sequence hybridisation probes. One probe was specific to the wild-type sequence. The other two probes targeted the two variants of the L858R mutation. Successful digital PCR quantification was performed at mutant fractional concentrations as low as 0.1%, demonstrating the value of digital PCR in the detection and quantification of low abundance mutant molecules.

Wang et al. (2010) employed the same method to detect EGFR mutations. In addition, this study quantified the ratio of the EGFR target gene to an endogenous control, RPP30 using a duplex digital PCR assay to identify genomic amplification of the EGFR gene - an event associated with metastatic progression. Both of these studies demonstrated detection and quantification of rare mutated alleles in a high background of wild-type genomic DNA, owed to the increased sensitivity offered by digital PCR. Consequently, digital PCR could potentially be used to monitor disease progression and to examine treatment response in patients with NSCLC.

A study by Qin et al. (2008) proposed digital PCR as an alternative method of quantifying copy number variations. Current technologies such as comparative genomic hybridisation, single nucleotide polymorphism (SNP) microarrays and qPCR are limited in resolution and at best can discriminate a 2-fold difference in the copy number (Qin et al., 2008). This study used digital PCR to enable accurate measurement of the CYP2D6 gene, a gene which can influence the efficacy of drugs through the protein it encodes. It is therefore of great importance in therapeutic decision making (Qin et al., 2008). The absolute copy number ratio between the CYP2D6 target and an RNase P reference target was calculated using digital PCR to determine whether deletion or duplication of the gene had occurred,
indicating whether a patient is at risk of a severe toxic response or requires an increased dosage of drugs. This study by Qin et al. (2008) also quantified the ERBB2 gene in breast cancer and normal samples. The ERBB2 gene is over expressed in up to 30% of invasive breast cancer patients. This over expression is caused by gene amplification. Previous studies have shown that ERBB2 amplification is well correlated with a reduced response to chemotherapy, a high recurrence rate and short survival time. As a result, it serves a prognostic predictor for breast cancer patients. Over expression of the ERBB2 gene is currently detected using immunohistochemistry or fluorescence in situ hybridisation. Immunohistochemistry is easy to perform but inaccurate whereas fluorescence in situ hybridisation is accurate, expensive and time consuming (Qin et al., 2008). The absolute copy number ratio of ERBB2 gene to an RNase P reference gene was calculated using digital PCR in 40 breast cancer samples and 8 normal breast samples. The digital results showed an over expression of the ERBB2 gene in 35% of the breast cancer patients and normal expression levels in each of the normal samples. The breast cancer samples that did not show amplification of the ERBB2 gene were in the early stages of the disease. The study concluded that digital PCR offers excellent discrimination power and can serve to quickly and accurately verify microarray genome scan results.

Non-Invasive Prenatal Diagnostics

Prenatal diagnosis is performed to detect genetic abnormalities such as fetal aneuploidy. Current methods for prenatal diagnosis of aneuploidies involve invasive sampling or non-invasive screening. Non-invasive screening techniques such as nuchal translucency and maternal serum biochemical screening detect secondary symptoms associated with aneuploidy and cannot be used for diagnosis (Lo et al., 2007b). Definitive diagnosis requires invasive sampling from the chorionic villus or amniocentesis, posing a risk to the unborn fetus. The risks associated with these invasive procedures are often greater than the risk of a fetus having a chromosomal abnormality. As a result, invasive diagnostic testing has been limited to high risk pregnancies where the maternal age is >35 years (Hodges & Wallace, 2005). An alternative non-invasive prenatal diagnostic test would be extremely valuable as
it would eliminate the risk posed to the fetus and encourage standardised prenatal diagnostic testing regardless of age.

Lo et al. (1997) discovered cell-free (cf) fetal nucleic acids circulating in maternal plasma - a promising source of genetic material on which to perform non-invasive prenatal diagnosis (NIPD) using a standard blood sample obtained from the mother. However, according to Lun et al. (2008) the cf fetal nucleic acids comprise only a fraction (10% in the first trimester) of the total number of cell free nucleic acids. The cf fetal molecules coexist with cf maternal molecules and share many of the same sequences, complicating analysis. High sensitivity and extremely fine quantitative discrimination capabilities are required for diagnosis, making digital PCR a suitable technique for NIPD.

Numerous studies have focused on NIPD of trisomy 21 - the most frequent chromosomal aneuploidy (Lo et al., 2007a,b; Fan & Quake, 2007; Zimmermann et al., 2008; Chiu et al., 2009, 2011). In trisomy 21, there is an additional copy of chromosome 21. A study by Lo et al. (2007a) proposed an approach that could be used to detect trisomy 21 from a maternal blood sample using digital PCR. This technique, called relative chromosome dosage (RCD) determined the relative chromosome dosage of a chromosome 21 locus to a reference chromosome, chromosome 1. However, RCD is complicated by the fact that the target on the fetal chromosome 21 molecules are shared with maternal chromosome 21 molecules, diluting or drowning out the over representation of fetal derived chromosome 21 sequences. For example, say there are a total of 100 chromosome 21 molecules (90 maternal and 10 fetal) in a euploid (normal) sample. For a 10% fetal DNA concentration, there are 105 chromosome 21 molecules (90 maternal and 15 fetal) in an aneuploid sample. The ratio of chromosome 21 molecules to a reference changes from 2:1 to 1.05:1 due to the presence of background maternal DNA. Lo et al. (2007a) demonstrated detection of trisomy 21 using the RCD approach in artificial samples containing 25% fetal DNA. The total number of molecules (~8,000 chromosome 21 molecules) available from a standard blood test is simply not enough for a statistically significant diagnosis in samples containing 10% fetal DNA. According to Zimmermann et al. (2008), a fractional fetal DNA concentration of 20% would permit diagnosis of chromosomal aneuploidy from a standard 15mL blood test. It is difficult to see the digital PCR RCD approach being employed for NIPD unless
the fetal DNA concentration can be enriched. Dhallan et al. (2004) used formaldehyde to stabilise cells and minimise the release of maternal DNA into the blood. However, the enrichment of fetal molecules observed in this study has not been reproduced in subsequent studies (Chinnapapagari et al., 2005). The problems posed by the low fetal DNA concentration has led to the development of alternative approaches to NIPD.

Lo et al. (2007b) achieved NIPD through the analysis of placental expressed mRNA in maternal plasma using mass spectrometry. This study performed NIPD of trisomy 21 by determining the ratio between alleles of a SNP in PLAC4 mRNA. The targeted molecules were placental derived and distinguishable from maternal sequences, resulting in a 1:1 ratio for euploid fetuses and 2:1 for aneuploid fetuses. Another study by Lo et al. (2007a) used digital PCR to calculate the SNP allelic ratio, calling this approach digital-SNP. Since the placental mRNA molecules are fetal specific, quantification of the allelic ratio can easily be performed using digital PCR. This approach overcomes the problems associated with low concentrations of fetal derived molecules in maternal plasma. However, this approach is only applicable to heterozygous fetuses and only covers half of the population. A preliminary study by Go et al. (2007) proposed the inclusion of additional SNPs within PLAC4 to increase the population coverage. The use of multiple SNP’s could increase the population coverage to 95% (Lo, 2009). This approach could permit NIPD using digital PCR.

**Multiplex Digital PCR Application Summary**

The accuracy and sensitivity of digital PCR makes multiplex digital PCR an extremely valuable tool for the detection and quantification of mutations and copy number ratios for the diagnosis of colon, lung and breast cancers and applications with a high background of molecules such as non-invasive prenatal diagnosis. In summary, some of the potential multiplex digital PCR applications are:

- Detection and quantification of genetic mutations in colon, lung and breast cancer.
- Quantification of allelic ratios for non-invasive prenatal diagnosis.
1.5 Digital PCR Technology

Digital PCR offers increased quantification accuracy, precision and sensitivity over qPCR, as discussed earlier in the chapter. qPCR offers good quantification accuracy across a wide dynamic range, making it suitable for many applications. For most qPCR applications, the level of accuracy and precision offered by digital PCR is simply not required. In addition, the cost of digital PCR is greater than qPCR. This is discussed further in the following subsections. As a result, most current digital PCR technologies aim to address the niche applications examined in the previous sections rather than replacing qPCR.

The current state of the art in digital PCR technology is examined in this section, justifying the requirement for an alternative approach. Until recently, implementation of digital PCR in research and clinical settings has been delayed by the limitations in technology. Digital PCR was first performed by Vogelstein & Kinzler (1999) using standard 96 well plates. This micro-titer plate based technique was slow and expensive. As a result, the technique was not suitable for large, complex experiments. It was clear that smaller micro-reactor volumes, reduced sample preparation times, increased number of micro-reactors and greater throughputs were required.

The potential of digital PCR spurred significant research effort in the field and brought about the development of a number of miniaturised methods for performing digital PCR. Miniaturised formats reduce reagent consumption 100 to 1000-fold from the original plate based technique, dramatically reducing experimental costs (Sundberg et al., 2010). The advancements in lab on a chip technology made over the years were utilised and adapted for use in digital PCR. Two technologies have emerged as suitable solutions to the digital PCR problem. The instruments can be divided into two categories:

- Chip based technology
- Droplet based technology

The various commercial instruments and research prototypes based on chip and droplet technology are examined in the following subsections.
1.5.1 Chip Based Platforms

The requirement for a miniaturised digital PCR format led to the development of chip based systems which create micro-reactors using pneumatic valves (Qin et al., 2008) or surface tension (Heyries et al., 2011). Samples are automatically partitioned into individual chambers or micro-reactors, thermal cycled and fluorescently detected. These chip based technologies will now be examined.

Fluidigm Incorporated developed digital array chips that partition a sample using integrated fluidic circuits. Reactions are partitioned into thousands of individual micro-reactor chambers using flexible valves. The most recent digital array format by Fluidigm, the 48.77 Digital Array IFC consists of 48 individual panels of 770 chambers, offering a total of 36,960 micro-reactors per chip at a chamber volume of 0.85nL. The total reaction volume interrogated per array is 31.4μL. Chip priming and loading requires 48 liquid transfer steps and a time to answer of 3.25 hours. These integrated fluidic circuits provide an elegant solution to digital PCR. However, the integrated fluidic circuits are expensive consumables, greatly increasing the cost per experiment. Furthermore, the number of micro-reactors are fixed, reducing experimentation flexibility and limiting the maximum sample volume that can be processed. A large study or large sample volume may require multiple chips, greatly increasing the experimental cost.

Life Technologies Corporation manufacture a chip based quantitative and digital PCR instrument called OpenArray®. Reactions are partitioned into micro-reactors using through holes that act as individual wells. The internal surface of each through hole is hydrophilic while all other surfaces are hydrophobic. Reactions are loaded using a proprietary loader and micro-reactors are retained in the through holes via surface tension. The OpenArray® digital array format consists of 144 sub-arrays, each consisting of 64 through-hole micro-reactors. Each through-hole is 33nL in volume. The total reaction volume interrogated per plate is 304μL. The time to answer for each 9,216 well array is approximately 3 hours. Like the Fluidigm platform, the experimentation flexibility is restricted due to the fixed number of reactions and limited volume that can be processed. Furthermore, the surface coated arrays are expensive consumables, resulting in a high experimental cost.
A recent study by Heyries et al. (2011) demonstrated digital PCR using a surface-tension based partitioning chip called Megapixel dPCR. Samples are injected into a polydimethysiloxane device containing a bifurcating channel network that connects to dead end chambers. The chambers are then partitioned by injecting an immiscible silicone oil into the device. The silicone oil preferentially wets the channel walls, partitioning the sample into 10pL micro-reactors. The prototype array presented in this study consisted of 10 sub-arrays with 100,000 micro-reactors per sub-array, generating 1,000,000 micro-reactors per array. The total sample volume interrogated in a full array is 10μL. The time to answer for the device was approximately 4 hours. The megaplex digital PCR array can quickly process a large number of micro-reactors at a low reagent cost. However, small volume digital PCR requires high template concentrations for efficient analysis. Since the volume that can be processed is restricted (10μL) for this array based format, the results may not be statistically significant if the target concentration is low. There may not be enough target molecules in the sample volume processed on the chip. To overcome this large sample volumes need to be interrogated. Multiple chips are required, significantly increasing the experimental cost. This is an important practical consideration for all fixed micro-reactor number formats.

Chip or array based instruments offer quick time to answers and a relatively large number of low volume micro-reactors. The hands on time is minimal as samples are automatically partitioned. Reagent consumption is dramatically reduced in comparison to the original plate based technique employed by Vogelstein & Kinzler (1999). The short time to answer is owed to the parallelised nature of the devices; the micro-reactors are simultaneously thermal cycled and detected at endpoint. However, the manufacture of intricate microfluidic chips is costly. This is compounded by the fact that the chips are single use consumables, greatly increasing the experimental cost. There is also a high instrumentation cost associated with these microfluidic array based instruments. The instrumentation used to load, cycle and fluorescently analyse the arrays is expensive. Fixed micro-reactor arrays are disadvantageous as the total sample volume that can be processed is limited.

Droplet based digital PCR platforms offer an alternative solution to these inflexible, high consumable cost chip based formats. The droplet based technologies are discussed in
the next subsection.

### 1.5.2 Droplet Based Platforms

Chip based dPCR instruments are essentially miniaturised versions of the original well based approach adapted by Vogelstein & Kinzler (1999). Significant research has been devoted to droplet based technologies in recent years in an attempt to miniaturise PCR without the use of expensive chips. These devices typically consist of aqueous (sample) droplets in an immiscible carrier fluid, similar to an emulsion. The aqueous droplets act as independent micro-reactors - the equivalent of miniaturised wells. Surfactants and other additives are added to the emulsions to enhance stability, preventing droplet coalescence.

RainDance Technologies Incorporated developed a microfluidic platform called Rainstorm™. This technology is capable of generating droplets as small as 4pL in volume using hydrodynamic flow focusing shear based droplet generators at kilohertz frequencies (Pekin et al., 2011). Sample droplets are generated on a PDMS chip and collected in a microfuge tube. The microfuge tube containing the emulsion is then thermal cycled. The amplified emulsion is withdrawn from the microfuge tube and the droplets are serially fed past a detector contained in a second consumable PDMS chip for endpoint fluorescence detection. The Rainstorm™ digital PCR work-flow is illustrated in figure 1.6. The total run time is estimated to be approximately 3 hours for a 200\mu L reaction. The instrument offers high droplet throughputs and low reagent costs. However, each digital PCR experiment requires two consumable chips for droplet production and detection. Although much less expensive than the micro-fluidic arrays, these consumable chips increase the experimental cost. In addition, the instrument requires a number of liquid transfer steps, complicating work-flow. Droplets are produced on chip, transferred to a microfuge tube for thermal cycling and then reinjected into a second chip for analysis. Although RainDance provide flexibility in the number of droplets (and reaction volume) that can be processed, the droplet size cannot be readily adjusted using the shear based droplet generators. This means that thousands of unnecessary droplets are produced and analysed for large sample volumes containing rare targets. Flexibility in droplet size would require a number of different droplet production
CHAPTER 1 Introduction

Figure 1.6: Adapted from Pekin et al. (2011). RainDance digital PCR work-flow. (a) Sample droplets are generated using a flow focusing droplet generator. (b) The droplets are collected in a microfuge tube and thermally cycled for PCR. (c) The amplified droplets are injected into a second chip for endpoint detection.

chip designs or the ability to alter flow rates. Currently, the geometry of the droplet production chip and flow rates used by the shear based droplet generators are fixed, resulting in a fixed droplet volume.

QuantaLife Incorporated developed a droplet based digital PCR instrument using a similar work-flow to RainDance - droplets are generated on a droplet production chip, collected, thermal cycled off chip and fluorescently interrogated on a second chip. Droplet generation is accomplished using a shearing T-junction on the Droplet Digital™ platform. Reactions 20μL in volume are segmented into 1nL droplets, yielding a total of 20,000 droplets. As with the RainDance platform, the instrument design does not offer flexibility in droplet size. Following amplification in a microfuge tube, the droplets are reinjected to the endpoint detection chip which interrogates the droplets in single file at a rate of 1,000 droplets per second. The droplet production and detection chips are single use only, increasing the experimental cost. There is a high instrumentation cost associated with the platform. Furthermore, there is no thermal cycling module supplied with the instrument.
The Droplet Digital™ platform also requires a number of liquid transfer steps to and from the droplet production and detection chips, complicating work-flow. In addition, the instrument is restricted to 20,000 1nL droplets, limiting experimentation flexibility.

The current droplet and chip based instruments have been examined. These two technologies are summarised in the next subsection, outlining the disadvantages of each and justifying the requirement for an alternative digital PCR solution.

1.5.3 Technology Review

The current state of the art in digital PCR technology has been examined. Both microfluidic and droplet based technologies have disadvantages in terms of instrument cost, experimental cost and flexibility. The disadvantages of microfluidic chip based instruments are:

- high consumable cost - greatly increases the cost per experiment
- high instrument cost
- fixed number of micro-reactors - limits the volume of sample that can be processed and reduces experimentation flexibility

The disadvantages of droplet based digital PCR platforms are:

- consumable chip requirement for droplet generation and detection - increases the cost per experiment and complicates work-flow
- high instrument cost
- no flexibility in droplet size - surplus number of droplets generated and detected in large reaction volume experiments.

The use of consumables is a disadvantage of both microfluidic chip and droplet based technologies. Removing the requirement of these consumables to achieve partitioning permits low cost digital PCR. This is not possible using chip based technology since partitioning relies upon wetting of the aqueous to the microfluidic array chambers. This is possible using droplet based technology and is discussed in the next section. Clearly, uniting the
droplet production, thermal cycling and fluorescence detection modules into a single module is highly advantageous as it provides a simplified, continuous work-flow. Reducing the instrument cost is also desirable. An instrument capable of generating any number of micro-reactors is advantageous. Flexibility in droplet size is also desirable as large sample volumes can be quickly processed by increasing the droplet size, reducing the number of data points in an experiment.

It is evident that an alternative approach is needed to satisfy these requirements. Continuous flow technology facilitates low cost digital PCR as it does not use any consumables. Droplets are generated, thermal cycled and analysed in a continuous work-flow that does not require liquid transfer steps. The technology does not require complex instrumentation, reducing instrument cost. The number and size of the droplets generated can be changed as needed to suit a particular application. Continuous flow PCR is discussed in the next section.

1.6 Continuous Flow Technology

Continuous flow instruments utilise the characteristics of biphasic flow for PCR. Significant research has been undertaken in the area of biphasic flow in capillaries as it is utilised in heat transfer applications. Consequently, biphasic flow in capillaries is well understood. A liquid film exists between droplets and the flow conduit in biphasic flow. It is this liquid film that makes biphasic flow suitable for PCR. The oil film prevents the aqueous droplets from wetting the wall of the tube, preventing contamination of the flow conduit (Morris, 2008). The oil separation between aqueous droplets ensure that the droplets act as independent micro-reactors. Continuous flow technology has been patented for performing nucleic acid quantification using qPCR and dPCR (Davies & Dalton, 2010).

The aqueous (sample) droplets travel along a flow conduit separated and wrapped by an immiscible carrier fluid. The droplets are cycled through the PCR temperatures as they flow through channels within isothermal blocks. The droplets travel through the heated flow conduit for a specified dwell time which is controlled by the flow rate and tubing length within the channel. This is repeated for the desired number of PCR cycles. The droplets
are then fluorescently interrogated as the droplet train passes by a detector. A schematic illustrating the operation of a continuous flow PCR instrument is shown in figure 1.7.

Figure 1.7: Schematic illustrating the operation and features of a continuous flow PCR instrument.

Droplets are generated using either microfluidic dipping or liquid bridge dispensing. The droplet size can be varied as required using both of these methods. The droplet production techniques employed in this body of work are described in detail in chapter 4. The reaction droplets flow directly into a thermal cycler. The droplets cycle through the temperatures for PCR as they flow from the denaturation thermal block to the annealing thermal block. Since the blocks are isothermal, there is no unnecessary ramping time as seen with most PCR platforms that operate by repeatedly heating and cooling blocks. The elimination of unnecessary ramping time has the potential to reduce thermal cycling times compared to conventional thermal cyclers. Following thermal cycling, the droplets flow through a detector where each droplet is fluorescently interrogated.

The continuous fashion of this technology results in an uncomplicated work-flow. Droplets are continuously produced, cycled and detected. There are no consumable parts owed to the presence of a liquid film wrapping and separating the droplets, eliminating the high cost per experiment associated with commercial digital PCR technologies. Highly consistent, low volume micro-reactors can be generated (Forget, 2009). In addition, the
droplet size can be changed as required. Since the blocks are isothermal, the instrumentation is cheap as it does not require expensive heating and cooling elements such as Peltiers. Continuous flow technology offers flexible, low cost digital PCR on an inexpensive platform. The objectives of this thesis are set out in the next section.

1.7 Objectives

Digital PCR has the potential to be an extremely powerful tool in molecular biology due to its highly sensitive, accurate and precise quantification capabilities. This is reflected by the applications discussed earlier. However, the improvement in quantification achieved using digital PCR comes at high cost using current digital PCR technology. As shown in the technology review, partitioning a sample into thousands of micro-reactors typically requires consumables, increasing the cost per experiment.

Continuous flow instruments provide an inexpensive and straightforward approach to droplet based digital PCR. Droplets are produced, thermal cycled and detected in a continuous fashion without any consumable requirement. Consequently, continuous flow technology is a highly desirable solution to digital PCR. The objectives of this thesis are as follows:

1. To design, fabricate and characterise a continuous flow digital PCR instrument

   • Examine design theory for a continuous flow digital PCR instrument
   • Explore the statistics of digital PCR
   • Develop an endpoint algorithm capable of converting endpoint fluorescence data to a binary output signal
   • Demonstrate amplification on the continuous flow instrument
   • Characterise the droplet production techniques for continuous flow digital PCR

2. To demonstrate single molecule amplification on the continuous flow instrument

3. To perform singleplex digital PCR on the continuous flow instrument
4. To perform duplex digital PCR on the continuous flow instrument

A primary aim of this thesis is to develop a fully functional continuous flow digital PCR instrument. A theoretical design evaluation should be performed to gain an understanding of the factors governing the performance of a continuous flow instrument. In addition, a study examining the statistics of digital PCR is required to identify the parameters affecting digital PCR quantification accuracy, precision and dynamic range. An algorithm capable of converting an endpoint fluorescence signal from droplets to a binary output should also be developed. The devices amplification capabilities should then be investigated. A droplet production technique suitable for continuous flow digital PCR should then be identified.

Following device development and characterisation, singleplex and duplex digital PCR can be performed on the instrument. Successful single molecule amplification should be demonstrated within these studies. Finally, design modifications and recommendations can be proposed based on the performance of the instrument.

1.8 Chapter Closure

This chapter presented background information on the concept of digital PCR, its quantification capabilities and potential biological applications. The current state of the art in digital PCR technology has also been discussed, revealing the requirement for alternative approaches to digital PCR. The operation of a continuous flow instrument has been described, highlighting the advantageous features of the technology. The motivation behind the project has been made clear and the objectives of the thesis have been set out. The remainder of this thesis is structured as follows:

Chapter 2: Biology discusses the polymerase chain reaction process, fluorescence detection chemistries and qPCR theory. qPCR is compared to dPCR, highlighting the advantages and disadvantages of each and justifying the requirement for dPCR.

Chapter 3: Theory outlines the theory governing biphasic flow and discusses the statistics of digital PCR.
**Chapter 4: Device Design** presents the design criteria for a digital PCR instrument, examines continuous flow design theory and describes the continuous flow digital PCR instrument in detail.

**Chapter 5: Experimental Methods** details the reaction components used in the digital PCR experiments, the sample preparation techniques, the sample loading protocol and the endpoint algorithm used to convert raw fluorescence data to a binary output signal.

**Chapter 6: Device Characterisation** presents the results obtained from the device characterisation studies. The devices amplification capabilities are examined and the results from the droplet generation studies are presented and discussed.

**Chapter 7: Experimental Results** gives a detailed description of the digital PCR experiments performed on the instrument. The results obtained from the singleplex and duplex digital PCR studies are presented and discussed.

**Chapter 8: Conclusions and Recommendations** presents the conclusions from this work and provides recommendations for future work.
Chapter 2

Biology

2.1 Introduction

This chapter focuses on the biological processes relevant to this research. Firstly, the PCR process and the reaction components required for PCR are considered. Following this, the fluorescence detection chemistries used for the detection of amplified product are described. Fluorescence theory is also dealt with in this chapter. The qPCR methodology is examined, detailing the qPCR reaction phases and the techniques employed to perform absolute and relative quantification. Finally, the limitations in qPCR quantification accuracy and sensitivity are discussed - justifying the requirement for an alternative digital approach.

2.2 Polymerase Chain Reaction

The polymerase chain reaction process was previously described in chapter 1. PCR relies on repeated thermal cycling for the replication of DNA. Firstly, the dsDNA is denatured, separating the strands into ssDNA. Denaturation occurs between 90-95°C. Following denaturation, the temperature is reduced to 60°C, allowing the specific oligonucleotide sequences (primers) to anneal to the region of interest. In the final step of the PCR process, the temperature is increased to 70°C and the DNA polymerase extends the primers along the ssDNA, forming a new dsDNA strand. This process describes three temperature PCR.
Two temperature reaction chemistries have been developed, allowing annealing and extension to be performed at the same temperature - usually 60°C. Two temperature chemistries speed up the PCR process and permits simplification of PCR devices.

The core reagent components required for PCR are as follows: DNA polymerase, deoxyribonucleotide triphosphate (dNTP’s), magnesium chloride and a reaction buffer. The dNTP mix contains the four deoxyribonucleotides - deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate and deoxyguanosine triphosphate. These DNA building blocks are incorporated into the growing strand by the DNA polymerase. Taq DNA polymerase is an enzyme isolated from a thermophilic bacterium that is able to withstand the PCR denaturation temperatures. Each of these components are available separately - allowing for flexibility in sample preparation. However, it is typical for these individual components to be combined into a pre-optimised master mix, allowing for faster sample preparation and consistent, standardised PCR reactions.

The PCR reaction also requires template DNA and primers. The primer set consists of forward and reverse primers that are synthetic strands of dNTP’s specifically designed to anneal to a target sequence. These primers anneal to the target strand, allowing the DNA polymerase to synthesise a new strand by adding dNTP’s. The optimal primer length is generally accepted to be 18-22 base pairs (bp), long enough to ensure adequate specificity and short enough to bind to the template at the annealing temperature. The target template can be either complementary DNA (cDNA) or genomic DNA (gDNA), depending on whether one or two step PCR is performed. Two step PCR involves reverse transcription of RNA into cDNA, which can then be used as a template for target amplification. This research will focus on one step PCR using gDNA, which allows for direct amplification of a target sequence from gDNA template.

Traditional end-point PCR uses the aforementioned reagents to amplify the target sequence followed by an endpoint analysis step such as gel electrophoresis. Modern PCR methodologies are based on fluorescence monitoring. This is achieved by using primers in conjunction with either hybridisation probes or a DNA binding dye. In this study, Taqman® probes - a hybridisation based probe is used. The two detection chemistries used in qPCR are discussed in the next section.
2.3 Fluorescence Detection Chemistries

2.3.1 DNA-Binding Dyes

DNA-binding dyes are used in qPCR as fluorescent reporters to monitor the fluorescence signal after each cycle. In solution, the background fluorescence is low. When DNA-binding dyes bind to double stranded DNA (dsDNA), there is an increase in fluorescence. As the quantity of dsDNA increases during PCR, the DNA-binding dye binds to the generated product, corresponding to an increase in fluorescence.

A major drawback in using DNA-binding dyes as fluorescent reporters is that the dyes are not specific to the target of interest. During PCR, DNA sequences other than the target sequence may be amplified. This is known as non-specific product. Binding dyes will bind to non-specific product resulting in an increase in fluorescence and making it difficult to discriminate between the target amplicon and other artifacts such as primer-dimer. Consequently, it is possible to overestimate the quantity of the target of interest when using binding dyes in qPCR. Dissociation curves, also known as melt curves are produced to confirm that the correct product has amplified in a qPCR reaction. A dissociation curve is performed post PCR and used to identify the melt temperature ($T_m$) of the reaction. $T_m$ is the temperature at which the dsDNA denatures, resulting in an abrupt reduction in fluorescence due to the release of the DNA-binding dye molecules. The $T_m$ is dependent on the base pair content and length, allowing for discrimination between target product and other non-specific products. Although non specific, DNA-binding dyes are cheap and simple to use versus hybridisation probes and are commonly used in optimised qPCR conditions.

DNA-binding dyes were not used in this research due to their non-specificity. The non-specific nature of these binding dyes could skew the digital PCR results if there are positive micro-reactors arising from the generation of non-specific product. Producing dissociation curves for the many reactions required in a digital PCR experiment is laborious, requires additional hardware components and increases the overall complexity of the system. Consequently, hybridisation probes are used to detect amplified product in this body of work.
2.3.2 Hybridisation Probes

Hybridisation probes are a commonly used fluorescence detection chemistry - owing mainly to their sequence specific nature. These probes, which work in conjunction with the primer set, bind to a specific sequence between the forward and reverse primers.

In this research, Taqman® probes were used for fluorescent detection of amplified product. These probes are a specific sequence of oligonucleotides complementary to the target sequence with a fluorescent reporter dye attached to the 5’ end and a quencher attached to the 3’ end. The quencher is a molecule that suppresses the fluorophore reporter emission when in close proximity. The specificity offered by this detection chemistry makes it suitable for end-point fluorescence detection measurements on a digital PCR platform. Since the assay and probes are specific to the gene of interest, there is reduced uncertainty in end-point signal analysis. An increase in fluorescence corresponds to amplification of target molecules.

A schematic of how Taqman® probes function in the PCR process is shown in figure 2.1. The forward and reverse primers, sequence specific probes and dsDNA are free in solution. The dsDNA is denatured, allowing the primers and probes to anneal to the ssDNA. The quencher and reporter are in close proximity to each other when the probes are intact, separated only by the length of the probe. The fluorescent signal from the reporter dye is suppressed by the quencher, with only some background fluorescence observed. The DNA polymerase extends the primer along the target strand, incorporating dNTP’s to form a new strand. As the dNTP’s are added, the DNA polymerase cleaves the probe and displaces the probe fragments. The reporter and quencher are released. The reporter and quencher are no longer in close proximity, resulting in an increase in fluorescence. The fluorescence signal from a reaction increases as the target DNA amplifies and probes are cleaved, permitting fluorescence detection of sequence specific amplified product.
Figure 2.1: Taqman® Probe functioning in PCR. The primer set and sequence specific Taqman® probe anneal to the target sequence. The DNA polymerase incorporates dNTP’s, extending the primers and cleaving the probe. The fluorophore is released resulting in an increase in fluorescence.
There are various fluorogenic reporters available for selection, allowing for multiplex PCR - amplification and detection of two or more target sequences in a sample. The most frequently used fluorophore is 6-carboxyfluorescein (FAM) with a maximum excitation wavelength of 493nm and a maximum emission wavelength of 525nm. Taqman® Master Mixes include a passive reference dye called 6-Carboxy-X-rhodamine (ROX). ROX does not take part in the PCR reaction and is used in qPCR to normalise signals, correcting for variations in reaction volumes and fluctuations in the fluorescence detection system. The selection of reporter dyes for the digital PCR experiments on the continuous flow prototype is considered in the experimental design chapter, chapter 4.

2.4 Fluorescence Theory

The development of fluorescence based technology has accelerated in recent years with the discovery of PCR. Fluorescence based technology has applications in DNA sequencing, biomolecule assays, fluorescence in situ hybridisation and cell signalling. In this thesis, the fluorescence mechanism is used for the quantification of nucleic acids. Fluorescence theory is examined here, aiding in the design of the end-point fluorescence detection system and in the selection of reporter dyes.

Fluorescence is a three stage mechanism which occurs in fluorescent molecules (fluorophores) that absorb photons of light and emits them at a higher wavelength. The fluorescence process can be represented by an electronic-state diagram (Jablonski diagram), as shown in figure 2.2. In the first step, the fluorophore is excited by a photon of light from its ground state, $S_0$, to an excited state, $S'_1$. The energy of the photon of light is given by:

$$E = \frac{h \cdot c}{\nu}$$  \hspace{1cm} (2.1)

where $h$ is Planck’s constant, $c$ is the speed of light and $\nu$ is the photons wavelength. Since both $h$ and $c$ are constant, the photons wavelength is inversely proportional to its energy. Observing figure 2.2, the energy received from the photon excites the fluorophore to its excited state $S'_1$. In the second step of the process, the fluorophore drops to a lower energy
level $S_1$ through a process known as vibrational relaxing where excess energy is dissipated. In the final step of the fluorescence process the fluorophore returns to its ground state through the emission of a photon. Since energy is dissipated to drop the excited fluorophore energy level back to its ground state energy level, the emitted photon has less energy than the excited photon. Consequently, the emitted photon has a longer wavelength. The difference between the wavelengths of the excitation and emission photons is known as the Stokes shift.

The Stokes shift is an key consideration in fluorescence detection. The shift in emission wavelength allows emission photons to be detected in isolation from the excitation photons. The excitation-emission spectra is dependent on the chosen fluorophore. The excitation wavelength and optical filters used for detection should be carefully selected to ensure a high quality signal. Ideally a light source with a narrow excitation wavelength band should be chosen to further improve the signal to noise ratio by minimising the background signal. The wavelength of the excitation light source should be selected to excite the fluorophore at its peak excitation wavelength. In addition, the detection sensor should be as sensitive as possible. Fluorescence theory and the properties affecting the efficacy of a fluorescence detection system are taken into consideration in the design of the end-point fluorescence detection system, described in Chapter 4.
2.5 Quantitative Polymerase Chain Reaction

qPCR is considered the gold standard for accurate and fast quantification of nucleic acids. In this section, qPCR reaction theory, the reaction phases and the methods of absolute and relative quantification are discussed. The requirement for an alternative quantification approach is made clear when considering the accuracy and sensitivity of qPCR.

2.5.1 qPCR Reaction Theory

Theoretically, the starting quantity of target molecules, denoted by $N_c$, doubles after each cycle of PCR. This gives the exponential relationship between the original quantity of targets and the total amount of targets as:

$$N_{\text{total}} = N_c \times 2^n$$  \hspace{1cm} (2.2)

where $n$ is the number of PCR cycles. However, this assumes that the PCR process is 100% efficient - which is never achieved in practice. During each cycle of PCR a fraction of the targets, $\epsilon$ are amplified. Taking the efficiency of the PCR reaction into account, equation 2.2 becomes:

$$N_{\text{total}} = N_c \times (\epsilon + 1)^n$$  \hspace{1cm} (2.3)

The quantification of $N_c$, the starting copy number is of interest in a qPCR experiment. Rewriting equation 2.3 yields:

$$N_c = \frac{N_{\text{total}}}{(\epsilon + 1)^n}$$  \hspace{1cm} (2.4)

The total number of copies after a given cycle number, $N_{\text{total}}$ is proportional to the increase in the above background fluorescence signal observed in a qPCR experiment.

2.5.2 qPCR Reaction Phases

A typical s-curve is shown in figure 2.3. Fluorescence is plotted against the cycle number with the distinct reaction phases of qPCR indicated. There are four reaction phases in
qPCR: the linear ground phase, the exponential growth phase, the linear phase and the plateau phase.

Figure 2.3: A typical amplification plot. The four phases of the qPCR reaction are indicated. Fluorescence is plotted versus the cycle number.

In the linear ground phase, there is not enough product accumulated to yield an increase in fluorescence above the background noise. The fluorescence level rises above the background noise during the exponential growth phase. For this phase the PCR efficiency is at its highest with the PCR product approximately doubling at each cycle. During exponential growth, the reagent components are in abundance yielding amplification with consistently high efficiency. The threshold is set during this phase of amplification to obtain the most accurate reading. The cycle at which the sample reaches this threshold level is known as the quantification cycle or $C_q$. The reaction then enters the linear phase as reagents are consumed and the amplification efficiency is lowered. The fluorescence signal levels off in the plateau phase of the reaction. At this point, the reagents are exhausted resulting in little or no amplification, corresponding to no change in the reaction fluorescence level.

Digital PCR is an endpoint approach that uses the endpoint fluorescence signals to determine whether a reaction has amplified. Therefore, it should be ensured that each digital PCR reaction reaches the plateau phase of the PCR reaction, making it easier to discriminate between positive and negative reactions.
2.5.3 Absolute Quantification

Absolute quantification, one of qPCR’s quantification strategies, can be used to determine the absolute number of copies of a gene in a sample. Absolute quantification using qPCR requires serial dilutions of standards with a known copy number. These standards are amplified and the mean $C_q$ values plotted versus the logarithm of the starting copy number, as shown in figure 2.4. A linear fit is applied to the data points obtained from the standards.

![Quantification Cycle (C_q)](image)

Figure 2.4: Standard curve. The standard data points (solid blue circles) are plotted against the input quantity. A straight line is fitted, indicated by the blue curve. The unknown (red crosses) $C_t$ values are obtained from the experiment, allowing for estimation of the input quantity.

Reactions with an unknown starting copy numbers are run in parallel, permitting copy number calculation using the standard curve. The starting copy number of an unknown can be determined using the standard curve for a given mean sample $C_q$ value. Absolute quantification has a very large dynamic range of starting molecule determination (>5 orders of magnitude), making it an extremely power tool for many applications (Heid et al., 1996). The standard curve allows for the efficiency of the PCR reaction, $\epsilon$ to be calculated using the slope of the line. Absolute quantification relies on the assumption that the amplification efficiency of the target (unknowns) is identical to the standards. Although high quality standards can be selected and designed to closely match the efficiency of the unknowns, there is typically a small difference in amplification efficiency. This leads to a loss in accuracy. The preparation of standards for use in absolute quantification also contribute
to inaccuracies in quantification. Standards are typically quantified using spectrophotometry, a quantification technique that is also subject to error. The difference in amplification efficiency and inaccurate standards means that qPCR absolute copy number estimations are never completely accurate (D’haene et al., 2010).

It is generally accepted that extremely accurate absolute quantification is not feasible using qPCR. Furthermore, it can be difficult to amplify rare target molecules in qPCR reactions due to its limited sensitivity. An alternative approach is needed for biological applications demanding highly sensitive, accurate and precise absolute quantification.

2.5.4 Relative Quantification

Relative quantification is used to calculate the fold change concentration of a target molecule relative to an endogenous control. The endogenous control acts as an internal reference since they are constitutively expressed. This feature makes these controls suitable for normalising the qPCR data. Examples of endogenous controls include RNase P, GAPDH, β-actin and B2M.

Relative quantification is a widely used quantification strategy as it does not require the inclusion of a standard curve, circumventing the high expenditure associated with the design and production of standards. Relative expression is achieved by observing the difference in $C_q$ values between the control and unknown. Relative quantification can be calculated using:

$$R = 2^{C_q(\text{sample})-C_q(\text{control})}$$

(2.5)

There are a number of mathematical models for calculating the relative expression between the control and the sample of interest (Pfaffl, 2001; Pfaffl et al., 2002). According to Lo et al. (2007a), the discrimination of a 2-fold difference in the starting copy number is the current limit of qPCR. The relative quantification approach is limited in its ability to discriminate very small changes in the starting copy number. The sensitivity of this approach is also limited. Although relative quantification also offers a very large dynamic range of starting molecule determination, an alternative strategy is needed for applications requiring
finer degrees of quantitative discrimination.

### 2.6 Quantitative PCR Summary

This chapter has examined the qPCR methodology, bringing to light the advantages and disadvantages of qPCR. Absolute quantification accuracy is highly influenced by the accuracy of the standards. The difference in amplification efficiency between a target molecule and a standard also influences quantification accuracy. Standard preparation is expensive, increasing experimental cost. Although relative quantification does not require standards, it is limited to a 2-fold change discrimination in starting copy number. qPCR also has limited sensitivity and generally does not possess single molecule detection capabilities.

Both absolute and relative quantification use an analog fluorescence signal to perform quantification. This assumes that the amplification efficiency between the target and standard/reference is identical. This is never the case, resulting in reduced quantification accuracy. The quantification capabilities and sensitivity of qPCR are sufficient for many applications. The technique is quick, relatively inexpensive and flexible. However, some applications require greater quantification accuracy, precision and sensitivity. As discussed in the introductory chapter, digital PCR is a promising alternative as it offers superior accuracy, precision and sensitivity.

### 2.7 Theoretical Copy Number - Genomic DNA

The expression of the RNase P gene and SRY gene in genomic DNA (gDNA) is examined in this body of work. TaqMan® RNase P and TaqMan® SRY detection reagents, both of which are supplied by Life Technologies Inc. were used to achieve this. These assays are discussed further in Chapter 5. The performance of the continuous flow digital PCR instrument is investigated for various dilutions of male gDNA. This template DNA was used in this body of work since the number of target copies in a sample can be calculated for a given mass of gDNA.
CHAPTER 2 Biology

Calculation of the theoretical copy number for a given gDNA concentration is outlined here. The ability to calculate the theoretical copy number is advantageous when examining the accuracy of the experimental copy number estimations obtained on the continuous flow digital PCR prototype.

The mass of DNA per genome is given by:

\[ M_{\text{genome}} = (n_{bp}).(1.096 \times 10^{-21} \text{g/bp}) \]  \hspace{1cm} (2.6)

where \( M_{\text{genome}} \) is the mass of gDNA per genome and \( n_{bp} \) is the genome size in base pairs. The human haploid genome is estimated to have 3 billion base pairs. Therefore, substituting \( 3.0 \times 10^9 \text{bp} \) for \( n \) into equation 2.6 gives:

\[ M_{\text{genome}} = (3.0 \times 10^9 \text{bp}).(1.096 \times 10^{-21} \text{g/bp}) \]  \hspace{1cm} (2.7)

This yields a mass of DNA per genome, \( M_{\text{genome}} \) equal to \( 3.3 \times 10^{-12} \text{g} \) or 3.3 pg. To calculate the copy number, the mass of the human genome (3.3 pg) is divided by the copy number of the gene of interest per haploid genome. RNase P exists as a single copy gene per haploid genome. Therefore, 3.3 pg of gDNA contains one copy of the RNase P gene. SRY, a gene on the Y chromosome, exists as a single copy gene per diploid genome. Therefore, 6.6 pg of gDNA contains one copy of the SRY gene. The gDNA can be diluted to yield sample volumes containing various copy numbers of the RNase P and SRY genes. The mass of gDNA, \( M \) needed to yield the required copy number, \( N_c \) for an experiment can be calculated as follows:

\[ M = N_c \times M_{\text{genome}} \]  \hspace{1cm} (2.8)

where \( M_{\text{genome}} = 3.3 \text{pg} \) as calculated previously using equation 2.7. From this, the gDNA concentration needed for a starting copy number of interest can be found. Equation 2.8 can be used to calculate the mass of gDNA needed to yield sample volumes with starting copy numbers appropriate to digital PCR. These equations and calculations were central to experimental design and exploration of the platforms performance.
2.8 Chapter Close

This chapter examined the polymerase chain reaction process and discussed the reagent components in a PCR reaction. Fluorescence detection chemistries were discussed, demonstrating the requirement for a sequence specific fluorescence reporter in digital PCR. Fluorescence theory was also considered, aiding in the design of an endpoint fluorescence detection system. The qPCR methodology was examined, discussing the techniques limitations and justifying the requirement for a digital approach. Finally, the theoretical calculation of the starting copy number in a gDNA sample was described.
Chapter 3

Theory

3.1 Introduction

This chapter is divided into two parts: flow theory and the theory governing the statistics of digital PCR. Firstly, the theory governing the microfluidic flow employed in continuous flow technology is examined. Single phase and biphasic capillary flow theory is discussed. Biphasic fluid flow is investigated with particular emphasis on liquid film thickness, an important characteristic for continuous flow PCR. The pressure drop in a continuous flow system is also investigated, revealing important considerations for the design of the digital PCR instrument.

In the second part of this chapter, the statistics of digital PCR are examined. This section discusses digital PCR theory and details how an absolute quantification result is calculated based on a binary endpoint output. Following this, the accuracy and precision of digital PCR are discussed in detail as these are the key benefits of digital PCR. The relationship between precision, number of target molecules and number of micro-reactors is also examined. Lastly, the dynamic range of digital PCR is investigated.

3.2 Capillary Flow

Single phase and biphasic fluid flow theory is examined in this section. Single phase fluid flow theory is discussed, detailing the equations governing the fluid flow in the continuous
flow instrument. Biphasic fluid flow is then examined, focusing on the theory governing liquid film thickness and the biphasic pressure drop in a microfluidic network.

### 3.2.1 Single Phase Flow in Capillaries

Fluid flow in capillaries has been studied extensively over the years. In capillary flow, the viscous forces dominate over inertial forces resulting in Reynolds numbers in the laminar regime. The Reynolds number is the ratio of inertial forces to viscous forces and is given by:

\[
Re = \frac{\rho U D}{\mu_f}
\]

where \(\rho\) is the fluid density, \(U\) is the mean velocity, \(D\) is the capillary diameter and \(\mu_f\) is the dynamic viscosity of the fluid. The fluid flow in microfluidic systems is generally laminar due to the characteristic length and low flow velocity. As discussed in chapter 1, capillaries are used in the continuous flow instrument. The Hagen-Poiseuille equation defines the volumetric flow rate for viscous, steady laminar flow in a circular pipe (Massey, 1989):

\[
Q = \frac{\pi r_i^4 \Delta P_{sp}}{8 \mu_f l_t}
\]

where \(Q\) is the volumetric flow rate, \(r_i\) is the internal radius of the circular pipe, \(\Delta P_{sp}\) is the pressure drop and \(l_t\) is the capillary length. Rearranging, the Hagen-Poiseuille or single phase pressure drop is given by:

\[
\Delta P_{sp} = \frac{8 \mu_f l_t Q}{\pi r_i^4}
\]

The single phase pressure drop can also be written as

\[
\Delta P_{sp} = R_{sp} Q
\]

where \(R_{sp}\) is the single phase fluidic resistance, given by:

\[
R_{sp} = \left(\frac{8 \mu_f l_t}{\pi r_i^4}\right)
\]
The single phase pressure drop across a length of tubing is of significance in microfluidic systems. When operating under negative pressure, large pressure drops can cause pump failure, degassification and boiling at lower temperatures. Instances of droplet boiling are discussed in Appendix C. Examining equation 3.3, the pressure drop is proportional to the fluid viscosity, the length of the tubing and the volumetric flow rate. More significantly, the pressure drop is inversely proportional to the internal radius to the fourth power. It is evident that the single phase pressure drop is highly influenced by the tubing diameter and that small diameter capillaries yield large pressure drops. These characteristics are important considerations for the design of a continuous flow instrument and are discussed later.

### 3.2.2 Biphasic Flow

The microfluidic systems used in this body of work draw on the characteristics of biphasic flow. Biphasic flow can be defined as a flow where two immiscible fluids are in contact with each other. This regime creates droplets encapsulated and separated by an immiscible carrier fluid, providing conditions desirable for PCR. This is discussed further in the next subsection. A schematic illustrating biphasic flow in a capillary is shown in figure 3.1.

![Biphasic flow in a flow conduit.](image)

A complete study of the theory governing biphasic flow will not be considered here. The detrimental effect of a large pressure drop in a micro-fluidic system has been introduced in the previous subsection. Consequently, the additional pressure drop associated with biphasic flow is of significance and is examined in this section. There is an additional pressure drop associated with biphasic flow. In biphasic flow, the total pressure drop is...
equal to the Hagen-Poiseuille pressure drop plus the pressure drop due to the droplets:

\[
\Delta P_{bp} = \Delta P_{sp} + \Delta P_{bp} \times N_d
\]  

(3.5)

where \( \Delta P_{bp} \) is the total biphasic pressure drop, \( \Delta P_{sp} \) is the single phase pressure drop component examined earlier, \( \Delta P_{bp} \) is the excess pressure drop due to droplets and \( N_d \) is the total number of droplets in a system. The biphasic pressure drop is given by:

\[
\Delta P_{bp} = R_{bp}Q
\]  

(3.6)

where \( R_{bp} \) is the biphasic fluidic resistance and \( Q \) is the flow rate. The theoretical biphasic fluidic resistance is given by (Kashid & Agar, 2007):

\[
R_{bp} = \frac{8 \mu_f}{\pi (r_i^4 - r_d^4)} l_d
\]  

(3.7)

where \( l_d \) is the length of the droplet and \( r_d \) is the droplet radius. The droplet radius can be determined through the relationship:

\[
r_d = r_i - h_f
\]  

(3.8)

where \( h_f \) is the liquid film thickness.

Taheny (2010) demonstrated a good correlation between the theoretical fluidic resistance (3.7) and experimental biphasic fluidic resistance data for droplet flow. Using equation 3.5, the total pressure drop in the system can be accurately calculated and used to evaluate various continuous flow instrument designs. The next subsection details liquid film thickness theory for biphasic flow in capillaries, a phenomenon that is key to continuous flow PCR technology.

### 3.2.3 Liquid Film Thickness

The liquid film of oil formed between an aqueous droplet and the capillary wall is an important characteristic of biphasic flow in the continuous flow PCR platform. In this study, the liquid film ensures that a droplet is completely encapsulated by the carrier fluid,
thus preventing the reaction droplet from wetting the wall. This prevents carryover and cross contamination between biological sample droplets, yielding flowing droplets that act as independent micro-reactors. The theoretical calculation of the liquid film thickness will now be examined.

The Bretherton law can be used to calculate the film thickness and is given by Bretherton (1961):

\[ \frac{h_f}{r_i} \sim Ca^{2/3} \]  

(3.9)

where \( h_f \) is the film thickness, \( r_i \) is the capillary radius and \( Ca \) is the capillary number. The capillary number is the ratio of viscous to capillary forces given by:

\[ Ca = \frac{\mu_f U}{\sigma} \]  

(3.10)

where \( \mu_f \) is the dynamic viscosity of the fluid, \( U \) is the velocity and \( \sigma \) is the interfacial tension. A study by Aussillous & Quéré (2000) demonstrated that equation 3.9 is only valid for \( Ca < 0.03 \). Bretherton’s law does not accurately predict the film thickness for large \( Ca \) numbers. A plot of normalised film thickness as a function of the capillary number from this study can be seen in figure 3.2.

![Figure 3.2: Adapted from Aussillous & Quéré (2000), dimensionless film thickness as a function of the capillary number for viscous fluids. Bretherton’s law is shown as the broken line. The full circles is data from Taylor’s study. The solid line is Taylor’s law empirical fit. The open squares and open triangles are data from the Aussillous & Quéré (2000) study.](image)

It can be observed that Bretherton’s law, given by the broken line is only valid when
the film thickness is negligible compared with the tube radius. An empirical fit called
Taylor’s law is shown to be accurate across a range of capillary numbers. Taylor’s law is
given by:

\[ \frac{h_f}{r_i} = \frac{1.34Ca^{2/3}}{1 + 1.34 \times 2.5Ca^{2/3}} \]  (3.11)

Observing figure 3.2, it clear that Taylor’s law accurately models the film thickness up to a
\( Ca \) of 1.4. The capillary number calculated for the flow in this work is much less than 1.4.
Therefore, Bretherton’s law and Taylor’s law are accurate models for liquid film thickness
calculations. The film thickness separating reagent droplets from the tubing wall can be
calculated using the described equations.

The effect of droplet length on the film thickness is also of interest. Han & Shikazono
(2009) studied the effect of droplet length on the liquid film thickness. Han & Shikazono
(2009) found that the film thickness is larger for short droplets of length \( l_d < 2D \), where
\( l_d \) is the length of the droplet and \( D \) is the diameter of the capillary. In this study, the
droplet length was varied within range of \( D < l_d < 6D \) and the resulting film thickness was
measured for various capillary numbers. The effects of droplet length on the film thickness
can be seen in figure 3.3.

According to Han & Shikazono (2009), the liquid film thickness is independent of
the droplet length if the droplet length is twice the diameter of the capillary and \( Ca \ll 0.01 \).
There is no liquid film thickness data for long droplets (\( > 6D \)), making it difficult to predict
whether a liquid film is present for very long droplets.

Theoretically, a very long droplet will always form a liquid film as the Taylor and
Bretherton models do not account for the effect of droplet length on the film thickness. It is
likely that the liquid film breaks down for very long droplets as the the flow changes from
biphase flow to single phase flow, allowing the aqueous to wet the wall. This is undesirable
for a continuous flow PCR instrument as it may cause contamination. Unfortunately, it is
not possible to calculate the droplet length at which this occurs. The possibility of wetting
was considered during experimental design. Wetting of long reaction droplets is examined
during characterisation of the instrument and is discussed further in chapter 6.
CHAPTER 3 Theory

3.2.4 Section Close

The theory governing single phase flow and the biphasic pressure drop in a continuous flow system has been discussed. This theoretical study provides a method of predicting the total pressure drop in a microfluidic network, aiding in the design of a continuous flow instrument. The limitations associated with large pressure drops were considered during instrument design and are discussed further in the next chapter. Liquid film thickness theory, a fundamental phenomenon of this technology has also been examined. The statistics of digital PCR are discussed in the next section.

3.3 The Statistics of Digital PCR

3.3.1 Introduction

The concept of digital PCR has been discussed in chapter 1. A biological sample containing target molecules and reagents necessary for PCR is partitioned into micro-reactors. The distribution of target molecules in independent micro-reactors is random. The micro-reactors
CHAPTER 3 Theory

are cycled through the PCR temperatures and interrogated at endpoint using fluorescence techniques. A binary end-point signal consisting of the number of positive reactions and number of negative reactions is generated. Using this binary signal, the copy number can be estimated using statistical methods.

This section examines the statistical approaches that can be employed to compute the starting copy number from a binary signal. The three performance metrics of digital PCR are accuracy, precision and dynamic range. These characteristics are examined in detail in this section.

Firstly, the possible outcomes from a digital experiment are described. The Binomial and Poisson distributions are then explored, describing the distribution of target molecules in micro-reactors. Following this, a statistical approach to copy number estimation is discussed. The variables influencing the precision of the copy number estimations are examined. Finally, the dynamic range of digital PCR is investigated, illustrating the instrumentation requirements for a wide dynamic range.

3.3.2 Digital PCR Experimental Outcomes

There are three possible outcomes in a digital experiment. There are either no positive micro-reactors, every micro-reactor is positive or a percentage of the micro-reactors are positive. In the case of an experimental result with no positive micro-reactors, the target concentration is too low or not enough sample has been interrogated. It is not possible to estimate the target concentration.

Similarly, the copy number cannot be estimated for a digital experiment in which all micro-reactors are positive. It is not possible to infer whether there are thousands or millions of target molecules in an experiment where every micro-reactor is positive. The number of targets required to saturate or fill every micro-reactor with at least one target is called the dynamic range. The dynamic range is proportional to the number of micro-reactors and is discussed later in this section.

In most digital experiments, a percentage of micro-reactors are positive. Using statistics, the copy number can be estimated based on the number of positives and the total
number of micro-reactors. The relationship between the precision of the copy number estimation and the proportion of positive reactions is examined later. The statistics of digital PCR are discussed next.

3.3.3 Target Distribution: The Binomial and Poisson Distribution

The distribution of target molecules into micro-reactors upon partitioning follows a binomial distribution. The binomial distribution is given by:

\[ P(x) = \binom{n}{x} p^x (1-p)^{n-x} \]  

for \( x = 0, 1, 2, 3, \ldots, n \), where

\[ \binom{n}{x} = \frac{n!}{x!(n-x)!} \]

This is the discrete probability distribution of the number of successes (target molecules) in \( n \) independent yes/no experiments. In digital PCR, a target molecule occupying a droplet is a ‘success’. The micro-reactors act as independent binary experiments. The binomial distribution of target molecules in droplets is given by (Barrett, 2008):

\[ P(x) = \frac{{N_c}!}{x!(N_c-x)!} \frac{1}{N_d^x} \left( 1 - \frac{1}{N_d} \right)^{N_c-x} \]  

The probability of \( x \) successes (number of target molecules in any given micro-reactor) is a function of the total number of droplets, \( N_d \) and the total number of target molecules, \( N_c \). The sum of the \( n \) probabilities equals 1. Using equation 3.13, it is possible to find the probability of a micro-reactor containing \( x \) number of target molecules for a given number of target molecules and micro-reactors.

Histogram plots are an intuitive method of exploring the distribution of targets in droplets. Take for example, 6 biological reactions - each containing 5,000 target molecules. The starting sample volume of the first reaction is partitioned into 500 droplets. The resulting histogram plot obtained using the binomial distribution equation can be seen in figure 3.4 (a). This plot demonstrates that the binomial distribution approaches a normal
distribution for a high average target concentration. The remaining biological samples are divided into 5,000, 10,000, 25,000, 50,000 and 100,000 droplets. Observing the resulting histograms, it is clear that the distribution of targets in droplets approaches an actual binary signal where a positive droplet represents one starting molecule. Although useful in examining the distribution of targets in droplets, histogram plots do not provide a method of determining the starting copy number from an experimental result.

Matlab® was used to plot the relationship between the target concentration, \( \frac{N_c}{N_d} \) and the percentage of positive reactions in an experiment using the binomial distribution. The resulting plot can be seen in figure 3.5. The components of the percentage positive curve are also plotted in this figure. Using this curve, it is possible to estimate the target concentration in an experiment based on the percentage of micro-reactors. However, the relationship between the percentage of reactions positive and the target concentration cannot be easily described mathematically using the binomial distribution. Consequently, the Poisson distribution is used to estimate the copy number and compute the associated confidence intervals. The Poisson distribution is discussed next.

The distribution of target molecules in droplets can also be modeled using the Poisson distribution. The Poisson distribution, which can be derived as a limiting case of the binomial distribution is a discrete probability distribution with probability function:

\[
P(x) = \frac{\mu^x e^{-\mu}}{x!}
\]

where \( \mu \) is expected number of occurrences, \( e \) is the base of the natural logarithm and \( x \) is the number of occurrences of an event (the number of targets per droplet). Here, the expected number of occurrences is the target concentration. The expected number of occurrences or target concentration is the average number of targets per droplet and is given by:

\[
\mu = \frac{N_c}{N_d}
\]

Using equation 3.14, it is possible to find the probability of a droplet containing any number of target molecules given a known target concentration. The Poisson distribution, like the
Figure 3.4: Histogram plots illustrating the theoretical distribution of target molecules for increasing number of droplets.
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Figure 3.5: Plot of percentage reactions versus target concentration using the binomial probability function.

binomial distribution, can be used to explore the distribution of target molecules in micro-reactors. More importantly, the Poisson distribution can be used to estimate the starting copy number based on the number of positives and total number of micro-reactors. An approach to copy number estimation using the Poisson distribution is discussed next.

3.3.4 Digital PCR Response - Copy Number Estimation

The Poisson distribution can be manipulated to provide a method of estimating the starting copy number based on the binary output from a digital experiment. A statistical approach to estimating the starting copy number is discussed here.

Heyries et al. (2011) derived a method of estimating the starting copy number based on the number of positives in an experiment. However, this study did not provide a method of computing confidence intervals and is therefore not considered here. Groth & Fazekas
(1982) state that considering the target molecule distribution as an error free variable is incorrect. This approach may yield an incorrect numerical answer and provides a misleading error free copy number estimation ($N_e$). The estimation is based on probabilities and is therefore subject to fluctuations associated with the binomial response. Consequently, it is desirable to apply confidence intervals to the copy number estimations.

A statistical technique proposed by Dube et al. (2008) permits copy number estimation and calculation of the associated confidence intervals using the number of positive micro-reactors and total number of micro-reactors. Dube et al. (2008) presented an algorithm which can be used to compute the copy number and the associated confidence intervals. The probability of success, that is a target occupying a droplet is denoted by $p$. The true concentration of target molecules, the variable of interest is given by $\mu$. Let $\hat{p}$ and $\hat{\mu}$ denote the estimators of $p$ and $\mu$. The relationship of $p$ and $\mu$ is of interest. The number of target molecules in each droplet can be modeled as a Poisson process, as discussed earlier. Finding the probability of a droplet containing no targets gives the relationship between $p$ and $\mu$ as:

$$P(0) = 1 - p = e^{-\mu}$$

Rearranging to obtain an expression for $\mu$ gives:

$$\mu = -\ln(1 - p)$$ (3.15)

which establishes the relationship between $\mu$ and $p$. A droplet getting a target or no target is a binomial process, with success probability $p$. Consider $\hat{p} = \frac{N_p}{N_d}$ as an estimator of $p$. It is known that $\hat{p}$ is an unbiased estimator of $p$ and has expectation $p$ and standard deviation $\sqrt{\frac{p(1-p)}{N_d}}$. If $N_d$ is sufficiently large, the confidence limits are given by:

$$\hat{p}_{\text{Low,High}} = \hat{p} \pm z_c \sqrt{\frac{\hat{p}(1-\hat{p})}{N_d}}$$

where $z_c$ is the standard score. Substituting $\hat{p}$ into equation 3.15, the estimator of $\mu$, $\hat{\mu}$ can be defined as:
The starting copy number can be estimated using:

\[ \hat{\mu} = -\ln(1 - \hat{p}) \]  

(3.16)

The confidence interval \([\hat{\mu}_{\text{Low}}, \hat{\mu}_{\text{High}}]\) is directly given as follows

\[ \hat{\mu}_{\text{Low}} = -\ln(1 - \hat{p}_{\text{Low}}) \quad \text{and} \quad \hat{\mu}_{\text{High}} = -\ln(1 - \hat{p}_{\text{High}}) \]  

(3.18)

The copy number confidence intervals, \(N_e(\text{Low})\) and \(N_e(\text{High})\) can be calculated using:

\[ N_e(\text{Low}) = N_d \times \hat{\mu}_{\text{Low}} \]  

(3.19)

\[ N_e(\text{High}) = N_d \times \hat{\mu}_{\text{High}} \]  

(3.20)

Using these equations, the starting copy number and its associated confidence interval can be calculated. This approach was employed to estimate the starting copy number and confidence intervals for the digital PCR experiments presented later in this thesis. An accurate method of estimating the copy number number and associated confidence intervals for a digital PCR experiment has been described. This statistical approach was implemented in the endpoint algorithm discussed in Chapter 5. The accuracy and precision of digital PCR is examined next.

### 3.3.5 Digital PCR Accuracy, Precision and Dynamic Range

Digital PCR offers unparalleled absolute quantification accuracy. Accuracy can be defined as the percentage difference between the digital PCR copy number estimation and the actual starting copy number. The copy number estimations are extremely accurate since the copy number is calculated directly using statistical distribution theory.

Precision is typically defined as the degree of reproducibility between experimental results. This characteristic will be referred to as reproducibility or repeatability in this body
of work. The precision of each individual estimation is of interest in digital PCR. Calculation of the confidence intervals accounts for variations and uncertainties in the binomial response. Each digital PCR estimation can be represented by a statistical distribution. The confidence intervals defines the width of this distribution. The copy number estimate represents the mean of the estimation probability distribution. As a result, the width of these confidence intervals is a measure of the estimation precision. Measurement accuracy, repeatability and precision is illustrated in figure 3.6. The relationship between precision and the digital PCR response is examined next.

Figure 3.6: Schematic illustrating digital PCR measurement accuracy, precision and repeatability.

The precision of a digital PCR copy number estimation is an important consideration in biological applications requiring very accurate, precise copy number estimations. High measurement precision is vital for applications requiring discrimination of low changes in copy number ratios. Here, the precision is defined as the variance about the copy number estimation. This is given by the width of the confidence interval. The difference between the copy number estimation and its confidence interval, \( w \) is given as:

\[
w = N_e - N_e_{Low or High}
\]

Therefore, the precision is given by:

\[
Precision \pm \% = \frac{w}{N_e} \times 100
\]  

(3.21)

Ideally, the precision should be to close to zero. For highly precise estimates, the width
of the estimation distribution is narrow. This means that the copy number estimation has no uncertainties associated with it. Examining equation 3.21, it is clear the precision is inversely proportional to the width of copy number confidence interval. Wide confidence interval limits yield reduced precision. The estimation precision is influenced by the standard deviation term in the copy number confidence interval, given by:

\[ \pm z_c \sqrt{\frac{\hat{p}(1 - \hat{p})}{N_d}} \]

The width of the confidence interval reduces for increasing \( N_d \). Since the precision is proportional to \( N_d \), increasing the number of micro-reactors for a fixed copy number input yields increased precision. This is due to reduced uncertainty associated with the increase in independent ‘experiments’ or micro-reactors. For a fixed number of micro-reactors, the precision reduces for increasing \( \hat{p} \). The relationship between the estimation precision, target concentration and micro-reactor number is discussed next.

Simulated data was used to investigate the relationship between precision and number of target molecules for various micro-reactor number experiments. This study was undertaken to gain an understanding of the relationship and to determine the conditions necessary for highly precise digital PCR. Matlab® was used to perform calculations in this study. The Matlab® algorithm can be found in Appendix B. Firstly, the expected number of positives in a fixed droplet number experiment was calculated using the Poisson distribution. This was repeated for an input target copy number ranging from 1 to the dynamic range of the experiment. Calculation of the dynamic range is discussed later. The expected number of positives was then used to calculate the copy number point estimates and confidence intervals for each target input iteration. Finally, the precision was calculated. This was repeated for various reaction number experiments, ranging from 1,000 to 1,000,000 micro-reactors. The resulting precision plots can be seen in figure 3.7.

Examining figure 3.7, it is clear that estimation precision is low for small target input quantities. This arises from the width of the confidence interval being large relative to the point estimation. This is observed for a low number of target molecules in each droplet number study, as illustrated by the initial steep slope shared by each micro-reactor number curve. It is clear that each curve exhibits a peak precision. Observing figure 3.7 (a), the
Figure 3.7: Estimation precision versus number of target molecules for various reaction number experiments.
level of maximum precision is greatly influenced by the number of micro-reactors. The 1,000 reaction number curve exhibits a maximum precision of ±8%, whereas the 20,000 reaction curve offers a maximum precision of approximately ±2%. Further increasing the micro-reactor number to 1,000,000 increases the precision to ±0.24%, as shown in figure 3.7 (b). The level of precision then reduces from its maximum as the micro-reactors become saturated with target molecules. As the micro-reactors fill with targets, the proportion of positives increases, causing a reduction in precision.

These curves can be used to identify the number of micro-reactors needed to achieve a desired level of precision. The precision plots aid in experimental design when deciding on the number of micro-reactors for a given biological application. It is clear that there is a starting copy number window at which an experiment will offer its maximum precision. However, the starting target quantity is typically unknown, making it difficult to ensure maximum precision. Examining the precision plots from this study it is clear that selecting a very high micro-reactor number will always yield the highest precision, regardless of the target input quantity. Consequently, an instrument capable of processing a large number of micro-reactors is advantageous. The dynamic range of digital PCR is discussed next.

### 3.3.6 Digital PCR Dynamic Range

The dynamic range is the range of starting target molecules that can be quantified. Theoretically, digital PCR can quantify a single target molecule in a sample. The upper quantification limit is therefore of interest. Digital PCR can generate copy number estimations once the experiment has not been completely saturated with target molecules. Quantification can be performed once the fraction of positive responders is less than 1. Therefore, the dynamic range of digital PCR is the number of molecules required to fill every micro-reactor with one or more targets. Clearly, a wide dynamic range is beneficial as a platform can then accurately and precisely quantify very small or large amounts of target DNA. Heyries et al. (2011) investigated the dynamic range of digital PCR using Poisson statistics. The mathematical expression for dynamic range derived in this study will now be examined.

The theoretical dynamic range is the concentration beyond which an experiment becomes completely saturated. At very high concentrations, the occurrence of an empty well
is an extremely rare event that is independent between micro-reactors. The total number of empty micro-reactors, \(N_0\) can be modeled as a Poisson process and given as:

\[
N_0 = N_d.P(0) = N_d.e^{-\mu}
\]

Substituting \(N_0\) for \(\mu\) into the equation for the Poisson distribution, equation 3.14, gives the Poisson probability function:

\[
P(x) = \frac{(N_d.e^{-\mu})^x \cdot e^{-N_d.e^{-\mu}}}{x!}
\]

The target concentration value \(\mu\) that gives \(P(0) = 0\) is of interest. The number of copies required to fill an experiment can then be calculated from \(\mu = \frac{N_c}{N_d}\). Using equation 3.22, the probability of a micro-reactor having no target molecules is given by:

\[
P(0) = \frac{(N_d.e^{-\mu})^0 \cdot e^{-N_d.e^{-\mu}}}{0!}
\]

This becomes:

\[
P(0) = e^{-N_d.e^{-\mu}}
\]

The probability of a micro-reactor having no micro-reactors is \(10^{-4}\) (approximately zero) for \(N_d.e^{-\mu} > 10\). Taking \(10^{-4}\) as the acceptable failure rate, the maximum measurable copy number \((N_c)\) satisfies the inequality \(N_d.e^{-\mu} > 10\). Rearranging, the digital PCR dynamic range becomes:

\[
N_c < N_d \cdot \ln \left( \frac{N_d}{10} \right)
\]

Using this equation, the digital PCR dynamic range can be calculated for a given number of micro-reactors. A plot of the number of micro-reactors versus the number of target molecules can be seen in figure 3.8. Observing this figure, it is clear that a wide dynamic range requires many micro-reactors. Increasing the number of micro-reactors means more target molecules are required to completely saturate the reactions, resulting in an increased dynamic range.
Figure 3.8: Plot of number of micro-reactors versus the digital PCR dynamic Range.

A method of determining the digital PCR dynamic range has been presented. The algorithm used to generate the precision plots presented in the previous section can also be used to visually observe the dynamic range and precision levels for a given number of micro-reactors. The derived dynamic range equation was used to determine the dynamic range of the continuous flow instrument for various configurations. The dynamic range of the continuous flow digital PCR instrument is described later in the experimental results chapter, Chapter 7.

### 3.4 Chapter Close

This chapter investigated the theory governing biphasic micro-fluidic flow. The total pressure drop associated with continuous flow PCR instruments has been discussed. Micro-fluidic network design considerations have identified. The theory governing liquid film thickness, a crucial characteristic of liquid-liquid flow has also been described.

The statistics of digital PCR have been examined, demonstrating how a binary endpoint signal is converted to a copy number estimation. The statistical theory behind the copy number estimations and associated confidence intervals has been discussed in detail.
The accuracy and precision of digital PCR was thoroughly examined. The relationship between precision, number of target molecules and micro-reactor number was described. A method of determining the number of micro-reactors required for a desired precision level was described. Finally, the dynamic range of a digital PCR instrument was discussed, demonstrating the requirement for thousands of micro-reactors to achieve a wide dynamic range.
Chapter 4

Device Design

4.1 Introduction

A primary aim of this research is to develop a continuous flow platform capable of performing digital PCR. This chapter describes the design, fabrication and characteristics of the digital PCR instrument. Firstly, the design considerations for a digital PCR device are explored. Following this, the design of a digital PCR continuous flow instrument is discussed, highlighting the platforms capabilities and limitations.

The second part of the chapter describes the platform in detail. The continuous flow digital PCR instrument consists of three functional elements. Each of these elements are examined in the order in which they lie in the overall process. The first section examines the droplet production techniques suitable for continuous flow instruments. These droplet production techniques are discussed, outlining their respective advantages and disadvantages. Particular attention is given to liquid bridge dispensing, the main droplet generation method employed in this thesis. The liquid bridge segmenter design, mode of operation and dispensing capabilities are described. The second section discusses the thermal cycling module required for PCR - the biological process under investigation. The design, manufacture and thermal profile of the module is described. Lastly, the end point fluorescence detection system is examined. The optical system used to achieve end point fluorescence detection on the continuous flow instrument is discussed.
4.2 Design Considerations

The design criteria for a digital PCR instrument are described here. These design considerations are based on the digital PCR literature and the findings from the statistical and biological theory sections. The key requirements for a digital PCR instrument are presented and discussed.

4.2.1 Micro-reactor Volume

The precision of digital PCR is proportional to the number of micro-reactors for a given number of starting target molecules. A digital experiment may require anything from a thousand to a hundred thousand micro-reactors, depending on the biological application. This means that many micro-reactors are required to provide a single quantitative answer. Since many reactions are required for digital PCR, the micro-reactor size should be as small as possible to minimize reagent consumption and hence the total cost per quantitative answer. Small micro-reactors are ideal as this offers a wide dynamic range from a small sample volume and increases the sensitivity. As discussed in chapter 1, micro-reactors less than 100nL in volume are used in current digital PCR platforms. Similar sized droplets should be aimed for on the continuous flow platform. In addition, the droplets generated on the platform should be monodisperse for the digital PCR statistical assumptions to hold true.

4.2.2 Droplet Throughput and Time-to-Answer

A digital PCR system should possess a sufficiently high throughput to quickly process all of the micro-reactors in an experiment and obtain a short time to answer. The time to answer is the time from the start of the experimental run to the time the last droplet passes the end-point detection system. Micro-reactors are simultaneously cycled and fluorescently monitored for stationary chip based formats. For these systems, throughput is generally defined as the rate of number of plates or wells per unit time. The time to answer is approximately equal to the thermal cycling time. However, on a continuous flow platform,
micro-reactors are serially processed and hence the droplet throughput is the crucial parameter. The digital PCR instrument design theory and design cases, found in section 4.3, demonstrate the influence of droplet throughput in determining the time to answer.

4.2.3 Flexibility

Flexibility in the total number of micro-reactors is highly advantageous. Fixed reaction number and fixed reaction volume chip based formats may not provide the required quantitative precision or sample volume capacity. In this case, multiple chips are required for a single quantitative answer which increases the experiment cost and complicates automation and reproducibility. Flexibility in micro-reactor size is also advantageous as large droplets can be produced if large samples need to be processed. Recovery of the entire sample volume or of selected droplets is another desirable feature of a digital instrument. This permits post PCR analysis techniques such as gel electrophoresis.

4.2.4 PCR, Contamination and Fluorescence Detection

The continuous flow digital PCR instrument needs be capable of performing PCR in flowing droplets. The droplets should be subjected to the exact temperatures and cycling times specified by the relevant PCR protocol. There should be a sufficient number of cycles for successful low target or single molecule amplification.

Digital PCR relies on a binary signal from micro-reactors to generate an absolute quantification result. This requires that there is no crossover or carryover contamination on the instrument. Contamination could give false-positive signals, generating erroneous digital PCR results.

A method of interrogating each individual droplet for amplification is required to produce a binary output signal. Digital PCR can be performed using endpoint fluorescence measurements - that is detection of the droplet signal after thermal cycling has been completed. The fluorescence signal can then used to determine whether a droplet is positive or negative for the DNA target. Dual reporter detection capability is required for duplex experiments. Additionally, the system should be capable of detecting the passive reference
dye for signal normalisation. This demands that the endpoint fluorescence detection system is capable of simultaneously exciting and detecting three different fluorophores from each individual droplet.

### 4.2.5 Design Goals

The continuous flow digital PCR system should be capable of:

- Partitioning a sample volume into nanolitre droplets.
- Producing monodisperse droplets.
- High-throughput droplet processing and short time to answer.
- Performing PCR.
- Detecting fluorescence signals from flowing droplets.
- Generating a binary endpoint signal and converting it to an absolute quantification result.

The design of the digital PCR platform is discussed in the following subsections, referring to these design goals where appropriate. The throughput requirements for a continuous flow digital instrument are outlined in the next section.

### 4.3 Design of a Continuous Flow Digital PCR Instrument

Droplet throughput is a key parameter in the design of a continuous flow digital PCR instrument. A high droplet throughput is required to achieve an acceptable time to answer, permitting experimental replicates and large droplet number experiments to be processed within a short time frame. This section examines droplet throughput theory, demonstrating how throughputs are determined on a continuous flow platform and highlighting the current limitations in the technology.
4.3.1 Time to Answer

The continuous flow digital PCR platform consists of three distinct modules: droplet production, thermal cycling and end-point detection. Droplets are produced, delivered to the thermal cycler and detected. This is performed at a constant outlet flow rate. The time to answer is equal to the initial thermal cycling time plus the droplet processing time, given as:

\[ t_a = t_c + t_p \]  

(4.1)

where \( t_a \) is the time to answer, \( t_c \) is the thermal cycling time and \( t_p \) is the droplet processing time. It is assumed that the excess tubing in the system is negligible. The first droplet takes time \( t_c \) reach detection. The thermal cycling time is limited by the kinetics of PCR and therefore fixed for a given chemistry type. It takes time \( t_p \) to process all subsequent droplets. The droplet processing time is given by:

\[ t_p = \frac{N_d}{f} \]  

(4.2)

where \( N_d \) is the total number for droplets and \( f \) is the droplet frequency or droplet throughput. The droplet frequency, the number of droplets passing a point per unit time, determines the time to answer for a continuous flow platform (for a fixed chemistry type and droplet number). The time to answer approaches the thermal cycling time with increasing droplet frequency. Therefore, the frequency should be as high as possible to achieve the minimum time to answer. The droplet frequency is given by:

\[ f = \frac{U}{x_t} \]  

(4.3)

where \( U \) is the average velocity or droplet velocity and \( x_t \) is the unit size. Clearly, a high droplet velocity and small unit size will yield the highest droplet frequency. The unit size is the sum of the droplet and spacing lengths:

\[ x_t = l_d + l_s \]  

(4.4)
where \( l_d \) is the droplet length and \( l_s \) is the spacing between droplets. These repeating units are illustrated in figure 4.1. In summary, a small unit size and high bulk velocity will give a high droplet throughput and short time to answer. The limitations in droplet production frequency for this instrument are discussed in the next subsection.

![Repeating units, length \( x_i \)](image)

Figure 4.1: Schematic illustrating unit length. The unit length is equal to the length of the droplet plus the length of the droplet spacing.

### 4.3.2 Design Cases

Potential design cases for a continuous flow instrument are examined here. These design cases were explored to demonstrate the limitations in droplet production throughput for the configuration used in this thesis. The production of very small droplets at high frequencies is desirable to achieve a short time to answer. However, the pressure drop across the microfluidic network is an important design consideration. The total biphasic pressure drop in capillary flow was examined in Chapter 3. Large pressure drops can lead to undesirable events such as pump failure, air ingress, boiling and degassification. This section examines the total pressure drop for systems exhibiting various droplet throughputs, outlining the limitations in continuous flow technology.

The minimum cycling time, \( t_c \) for the reaction chemistry employed in this body of work is 3600 seconds and is discussed further in the next chapter. High droplet frequencies require high flow rates and long tubing lengths to maintain the minimum residency/dwell times. The minimum length of tubing required is therefore given by:

\[
L_t = t_c \times U
\]  

(4.5)
For example, a droplet moving at a velocity of 4mm/s would require 14.4m of tubing for thermal cycling of the reaction chemistry under investigation. Since the single phase pressure drop is proportional to the capillary length and volumetric flow rate, a high throughput will result in a large single phase pressure drop. Long tubing lengths compound the total pressure drop as more droplets can fit in the system at any time. This is referred to as the networks droplet capacity and is given by:

\[ C_d = \frac{I_f}{x_t} \]  

(4.6)

A system containing a large number of droplets moving at a high velocity results in a very large biphasic pressure drop. For high droplet throughputs, the total pressure drop is sufficiently large such that negative pressure pumping is infeasible.

Pressure drop calculations for various droplet throughputs can viewed in table 4.1. Minimum time to answer estimates are presented for each of the design cases. These calculations are based on the assumptions that the tubing internal diameter is 400μm, the unit size is 2mm and there is no excess tubing in the system. This design table offers insight into what is achievable using continuous flow technology.

Table 4.1: Time to answer and pressure drop estimations for various design cases in a 10,000 droplet experiment.

<table>
<thead>
<tr>
<th>Description</th>
<th>Symbol, units</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Throughput</td>
<td>( f ) (s)</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Throughput</td>
<td>( f ) (hr)</td>
<td>360</td>
<td>720</td>
<td>1,800</td>
<td>3,600</td>
<td>7,200</td>
<td>14,400</td>
</tr>
<tr>
<td>Velocity</td>
<td>( U ) (mm/s)</td>
<td>0.2</td>
<td>0.4</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Tubing length</td>
<td>( l_t ) (m)</td>
<td>0.72</td>
<td>1.44</td>
<td>3.6</td>
<td>7.2</td>
<td>14.4</td>
<td>28.8</td>
</tr>
<tr>
<td>Cycling time</td>
<td>( t_c ) (hr)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of droplets</td>
<td>( N_d )</td>
<td>10,000</td>
<td>10,000</td>
<td>10,000</td>
<td>10,000</td>
<td>10,000</td>
<td>10,000</td>
</tr>
<tr>
<td>Processing time</td>
<td>( t_p ) (hr)</td>
<td>27</td>
<td>14</td>
<td>5.5</td>
<td>2.7</td>
<td>1.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Time to answer</td>
<td>( t_a ) (hr)</td>
<td>28</td>
<td>15</td>
<td>6.5</td>
<td>3.7</td>
<td>2.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Single phase PD</td>
<td>( \Delta P_{sp} ) (Pa)</td>
<td>115</td>
<td>460</td>
<td>2880</td>
<td>11,520</td>
<td>46,080</td>
<td>184,320</td>
</tr>
<tr>
<td>Droplet capacity</td>
<td>( C )</td>
<td>360</td>
<td>720</td>
<td>1,800</td>
<td>3,600</td>
<td>7,200</td>
<td>14,400</td>
</tr>
<tr>
<td>Biphasic pressure drop</td>
<td>( \Delta P_{bp} ) (Pa)</td>
<td>1.88</td>
<td>2.38</td>
<td>3.29</td>
<td>4.24</td>
<td>5.54</td>
<td>7.39</td>
</tr>
<tr>
<td>Total biphasic pressure drop</td>
<td>( C \times \Delta P_{bp} ) (Pa)</td>
<td>677</td>
<td>1,719</td>
<td>5,935</td>
<td>15,293</td>
<td>39,930</td>
<td>106,498</td>
</tr>
<tr>
<td>Total pressure drop</td>
<td>( \Delta P_{bp-t} ) (Pa)</td>
<td>792</td>
<td>2,180</td>
<td>8,815</td>
<td>26,813</td>
<td>86,010</td>
<td>290,798</td>
</tr>
<tr>
<td>Total pressure drop</td>
<td>( \Delta P_{bp-t} ) (Bar)</td>
<td>0.008</td>
<td>0.022</td>
<td>0.088</td>
<td>0.268</td>
<td>0.860</td>
<td>2.917</td>
</tr>
</tbody>
</table>
The velocity required to achieve a given throughput and unit size can be calculated using equation 4.3. The minimum total tubing length is calculated using equation 4.5. The droplet processing time can then be calculated and used to determine the time to answer. The droplet capacity is determined and the total pressure drop for each design case calculated using the theory described in chapter 3. Design cases 5 and 6 offer favourable time to answers (<3 hours) but exhibit a significantly large pressure drop of up to 2.9 bar. These designs cannot be achieved using negative pressure pumping configurations. Negative pressure pumping at these pressure drops may cause degassification, boiling and pump failure. Positive pressure could be used as an alternative but complicates work-flow and integration with standard biological consumables. Observing table 4.1, a design that offers a droplet throughput close to design case 3 is optimal for a continuous flow instrument operating under negative pressure. The throughput is sufficiently high to obtain a reasonably short time to answer without the system being compromised by high pressure drops.

The study presented here demonstrates that platforms working under negative pressure are not suited to applications requiring a very short time to answer for large reaction number experiments. High-throughput can be achieved on continuous flow instruments using positive pressure pumping. However, complete automation of sample loading, droplet production, thermal cycling and detection is difficult to achieve using this pumping technique. This is not within the scope of this research. Consequently, negative pressure pumping at flow rates offering a medium droplet throughput is used in the instrument described in this chapter. The design of each of the functional elements in the system will now be discussed.

4.4 Flow Conduits and Fluidic Control

4.4.1 Tubing Geometry, Material and Carrier Fluid

The tubing geometry is critical to meeting the design requirements for a digital instrument. The internal diameter determines the minimum droplet volume that can be processed on the instrument. For biphasic capillary flow, the minimum volume is the volume of a sphere with a radius equal to the internal tubing radius. Droplets smaller than this are undesirable
as it is difficult to control the dwell times and requires that the droplets do not coalesce upon collision. Since small micro-reactor volumes are fundamental to digital PCR, small tubing geometries are needed. A plot of the minimum droplet volume versus tubing internal diameter can be seen in figure 4.2.

Selecting an internal tubing diameter of 800\textmu m permits a minimum slug volume of 270nL - a micro-reactor volume too large for dPCR due to the large reaction volume required for a large droplet number experiment. An internal diameter of 100\textmu m yields minimum droplet volumes of 0.52nL/520pL. However, a network consisting of 100\textmu m tubing is not feasible due to the extremely large pressure drop. An internal diameter of 400\textmu m was selected for the continuous flow digital PCR prototype. This geometry allows a theoretical minimum reaction volume of 33nL in a capillary with a relatively low fluidic resistance.

Polytetrafluoroethylene (PTFE) tubing was used as the flow conduit in the digital PCR instrument. PTFE is chemically inert thermoplastic polymer that is commonly used in biological applications. It has a melting point much higher than the PCR denaturation temperature. The material is hydrophobic, has good mechanical properties and offers excellent light transmission - a favourable property for fluorescence detection of the flowing
droplets. PD5 silicone oil (phenyl trimethicone) was used as the immiscible carrier fluid, separating and wrapping the droplets into individual micro-reactors that do not wet the internal tubing surface, preventing contamination.

4.4.2 Fluidic Control

Precise fluidic control is essential to achieve successful continuous flow PCR. The residence or dwell time of a droplet in a particular thermal zone is dependent on the flow rate, making flow rate control a crucial factor in instrument performance. Precise fluid flow control is also integral to the production of consistent droplets, a primary goal of the digital instrument.

There are a number of pumping techniques that can be used to achieve accurate and precise fluid flow control. For this study, HNP Microsysteme high performance gear pumps and Harvard Apparatus PHD 2000 syringe pumps were used to generate and control pressure driven flow. The HNP micro-annular gear pumps provide a flow rate by displacing fluid between an internal and external rotor. Flow rate control is achieved using a LabVIEW control algorithm. Volumetric flow rate feedback is provided using a calibrated liquid flow sensor (Sensirion), yielding accurate, pulseless flow rates even at low operating pressures. The Harvard Apparatus PHD 2000 syringe pumps provide a flow rate through the movement of a leadscrew-driven pusher block. The pumps micro stepping motor gives highly accurate and precise flow rates. The pusher block moves the plunger of a syringe, displacing the liquid at a defined flow rate. Hamilton gas-tight syringes were used in all experiments. Tubing was connected to the syringes using Leur Lock fittings and Teflon fluorinated ethylene propylene sleeves (Upchurch Scientific). The micro-annular gear pumps were shown to be superior to syringe pumps when pumping at the lower pressures associated with a high droplet number experiment. It was found that the Leur Lock fittings on the syringe pumps fail at low pressures, resulting in air ingress, flow rate fluctuation and eventual droplet size inconsistencies. As a result, the gear pumps were used to control the outlet flow rate. Syringe pumps were used to pump oil into the liquid bridge dispensers as these pumps were capable of maintaining the desired infuse flow rate.
4.5 Droplet Generation

As discussed in Chapter 1, the production of droplets for digital PCR offer numerous advantages over chip based platforms. The production of consistent, low volume droplets was a primary focus for this body of work. There are a number of methods available to segment samples into droplets, most of which operate by shearing the sample fluid. These are known as shear-induced droplet generators and exist as co-flowing, cross-junction, flow-focusing and T-junction droplet generators. These segmenters were not considered for this body of work as they require high shear rates for droplet production. As discussed earlier, high flow rates can result in undesirable pressure drops in a continuous flow system. For this study, microfluidic dipping was initially used to create droplets before moving to a fluid segmentation device based on liquid bridge technology.

4.5.1 Microfluidic Dipping

Microfluidic dipping has shown to be an extremely effective droplet production method for continuous flow PCR platforms as it provides the required flexibility in droplet generation for three way mixing. This droplet production technique has been used in continuous flow qPCR and genotyping instruments, two methodologies which emphasise arrayability rather than very small, tightly packed and consistently sized droplet trains. In this thesis, microfluidic dipping was initially used to investigate the instruments amplification capabilities before moving to liquid bridge technology. Droplets are created by moving the dipping tip from an oil overlay to the sample for a specified dip time, as illustrated in figure 4.3. Sample is withdrawn and the dipping tip moves back into the oil overlay, creating a droplet.

Although a flexible droplet generation method, it does not possess the dispensing accuracy and precision needed for dPCR. The droplet volume is determined by the dwell or dip time of the loading tip and volumetric flow rate. This means the variation in droplet size is highly sensitive to pumping fluctuations. Furthermore, the sample volume reduces as droplets are created. Incremental dipping is required to account for the reduced time the tip spends in the sample due to the reduced height level of sample. Accurate and precise
incremental dipping is difficult to achieve and requires expensive robotics and hence not considered as a viable droplet production method for continuous flow digital PCR.

A 3-axis robotic stage (Festo) was used in all parts of experimentation to access sample wells from modified well plates and to dip for droplets. The stage was used to position the dipping dip or sample inlet tubing into the sample well. An image of the robotic system can be seen in figure 4.4. LabVIEW was used to control the stages allowing the user to input the well position, dip time and dip depth. This stage setup permitted continuous sampling for large experiments.

### 4.5.2 Liquid Bridge Dispensing

Liquid bridge dispensing was first used to generate droplets for biological processing by Curran (2005). This body of work demonstrated that the instability of liquid bridges could facilitate the production of extremely small, consistent droplets - making the technology suitable for droplet generation on a continuous flow instrument.

Liquid bridge dispensers operate by continuously creating and rupturing a liquid bridge. A liquid bridge is a volume of liquid held between two opposing capillaries. This is typically achieved in a sealed reservoir of density matching carrier fluid consisting of an oil inlet, sample inlet and shared outlet. Sample is delivered through the inlet capillary at a
Figure 4.4: A schematic and image of the 3-axis robotic stage used to access wells from the 384 well plates. The sample plates were positioned onto a machined groove on the loading stage. The dipping parameters were defined using a LabVIEW control algorithm and dipping commenced. Sample is drawn, delivered to the device and segmented.
volumetric flow rate $Q_a$, forming a stable liquid bridge with the opposing outlet capillary. Oil is pumped into the chamber at a flow rate $Q_o$, causing liquid bridge instability and rupture, creating a droplet of aqueous fluid. The outlet flow rate is equal to the sum of the oil and sample flow rates. Pumping the outlet and oil inlet flow rates permits sample uptake into the sample inlet tube. The liquid bridge dispensing process is illustrated in figure 4.5.

![Diagram](image)

Figure 4.5: Schematic illustrating the main steps of the liquid bridge dispensing process. The aqueous phase is delivered through the inlet capillary, forming a droplet at a constant rate $Q_a$, as shown in (a). The droplet then bridges with the opposing outlet capillary, forming a liquid bridge (b). The liquid bridge reduces at a constant rate $Q_o$ (c) and eventually ruptures (d). The dispensed droplet is carried along the outlet tube at the outlet flow rate and the process is repeated.

The minimum volume dispensed is strongly influenced by the geometry of the liquid bridge (Curran, 2005). A complete theoretical review of liquid bridge dispensing is not within the scope of this project. However, the key variables influencing droplet size will be discussed as they aid in the design of a liquid bridge based segmentation device suitable for digital PCR.

Forget (2009) characterised liquid bridge dispensers using experimental and theoretical techniques. This research examined the effect of various geometries and flow ratios on the droplet size - a key variable in the digital instrument design. The relevant findings from this study will now be discussed. Forget (2009) describes the volume of the dispensed droplets ($V_d$) as the function:
\[ V_d = f(L, Q_o, Q_a, R_{ii}, R_{io}, \mu_o, \mu_a) \] (4.7)

where \( L \) is the capillary tip spacing, \( Q_o \) is the volumetric flow rate of oil, \( Q_a \) is the volumetric flow rate of aqueous, \( R_{ii} \) is the internal radius of the inlet capillary, \( R_{io} \) is the internal radius of the outlet capillary, \( \mu_o \) is the viscosity of the oil and \( \mu_a \) is the viscosity of the aqueous phase. The droplet size and spacing is also dependent on the fluid flow rate ratio or fraction \( Q^* \), given by:

\[ Q^* = \frac{Q_o}{Q_o + Q_a} \] (4.8)

The production of consistent, low volume micro-reactors is a primary aim for the digital instrument. Therefore, the influencing parameters in equation 4.7 are of interest and relevance to liquid bridge design. Since the tubing geometry and liquid phases were fixed for this study, the variables \( R_{ii}, R_{io}, \mu_o \) and \( \mu_a \) can be ignored.

The capillary tip spacing greatly influences the droplet volume generated. Wide tip spacings require large droplet growths before bridging of the aqueous occurs. This results in a large volume being ruptured and delivered to the outlet tube, creating large droplets. Forget (2009) showed that the dispensed droplet volume reduces for a reducing tip spacing, as shown in figure 4.6. From this, it is clear that small tip spacings should be employed to ensure generation of small droplet volumes. The volumetric flow rate fraction \( Q^* \) also influences the dispensed droplet volume (Forget, 2009). Examining figure 4.6, increasing \( Q^* \) yields smaller droplet volumes as the liquid bridge ruptures earlier due to the high oil flow rate.
Figure 4.6: Droplet volume versus Q* for various capillary tip spacings (adapted from Forget (2009)). The inlet and outlet tubing internal diameter is 400 μm. For a fixed Q*, the dispensed droplet volume is proportional to the tip spacing. For a fixed tip spacing, the volume dispensed is greatly influenced by Q*.

The maximum Q* value investigated by Forget (2009) was 0.75. The behaviour of liquid bridge dispensers above this value has not been experimentally investigated. Observing figure 4.6, it is likely that a minimum dispensed droplet volume limit is reached at high Q* values. It is evident that the minimum droplet volume that can be produced for a liquid bridge dispenser is dictated by the tip spacing. It is thought that the liquid bridge will continue to dispense this minimum droplet volume with an increasing oil spacing for an increasing Q*. This continues to a Q* value equal to 1, where there is only oil flow and no droplets are produced. Forget (2009) also demonstrated an extremely low volumetric droplet variation of ±1%, a droplet production precision far superior to micro-fluidic dipping.

The research conducted by Forget (2009) shows that a combination of a small tip spacing and a high Q* value will yield low volume droplets with negligible variation in
droplet size. These findings influenced the liquid bridge dispenser designs used in the digital PCR instrument. The design, assembly and manufacture of the liquid bridge dispenser is discussed next.

### 4.5.3 Liquid Bridge Dispenser Design

The liquid bridge dispensers used in this thesis were designed to generate small, consistent droplet volumes and to integrate efficiently with the other downstream components of the continuous flow instrument.

The segmenter was machined from transparent polycarbonate, consisting of various layers that were glued and filled using clear epoxy to seal the device. A model of the device can be seen in figure 4.7. The base consisted of a channel machined using a ball nosed cutter. This channel was designed to hold the rigid polyimide support capillaries. PTFE tubing was inserted into these rigid supports and the tip spacing was set to 200-300\(\mu\)m using a camera equipped with a zoom lens. A circular pocket hole was machined on the base to form the oil chamber. The second layer also contained a circular pocket hole with a conical section leading to a through-hole. This was to designed hold the oil inlet tube and also provided an effective method of bleeding the chamber of air. This oil infuse/air

![Figure 4.7: Model of a liquid bridge dispenser used in this thesis. Assembled and exploded views are shown.](image)
withdrawal port was positioned off centre to allow optical access for imaging. Engineering drawings of the liquid bridge dispenser can be found in Appendix A.

Different variations of this design were investigated in the experimentation. The design was altered to yield a liquid bridge dispenser with a short aqueous inlet tubing length. This design change is justified in the device characterisation chapter, chapter 6. The mode of operation was consistent for all experiments. The outlet (thermal cycler) capillary flow rate and the oil inlet flow rates were pumped. The aqueous (sample) was withdrawn at a flow rate equal to the difference between the outlet and oil inlet flow rates. This approach allowed automated, uninterrupted sampling from wells.

4.5.4 Liquid Bridge Dispenser Droplet Generation

Droplets were generated using the described liquid bridge dispenser. An image of a droplet train can be seen in figure 4.8. Since the tip spacing was fixed for a particular liquid bridge design, the droplet volumes were varied by altering the ratio of the oil inlet and sample inlet flow rates, Q*.

The smallest droplet volumes generated in this study were approximately 45nL in volume, as shown in figure 4.9. These droplet volumes were achieved for a Q* of 0.8, an inlet and outlet internal diameter of 400μm and a tip spacing of 200μm. The variation in droplet size was measured using imaging techniques. The droplets were imaged and analysed using Matlab®. Using the external diameter as a reference, the length of the droplet in pixels was converted to millimeters. The droplet volume was then calculated. This was repeated for a number of droplets and plotted, also shown in figure 4.9. The
droplet volumes were found to be consistent with a maximum volumetric variation equal to ± 2%, similar to variation of ± 1% found by Forget (2009).

The high level of dispensing precision demonstrated by the liquid bridge segmenter proves it as an excellent droplet generation method for continuous flow digital PCR. As discussed earlier, highly precise, low droplet volume droplet generation cannot be achieved using micro-fluidic dipping. The suitability of both droplet production methods for biological processing is examined later in Chapter 6.

![Low volume droplet production using a liquid bridge dispenser](image)

Figure 4.9: Low volume droplet production using a liquid bridge dispenser. (a) A 45nL droplet flowing in a 400 µm internal diameter capillary. (b) Plot of measured droplet volumes demonstrating precise, repeatable droplet dispensing.

## 4.6 Continuous Flow Thermal Cycler

The flowing reaction trains produced using either micro-fluidic dipping or liquid bridge dispensing require thermal cycling for PCR. Current digital PCR platforms cycle micro-fluidic chips or wells containing droplets on a stationary thermal block. These systems achieve PCR by repeatedly heating and cooling high heat capacity metal blocks. There is an unnecessary ramp time associated with these stationary block thermocyclers as the blocks are heated and cooled to achieve the temperatures for PCR. As a result, the cycling time is generally limited by the instrument, not the PCR kinetics.

The continuous flow nature of this technology allows the use of two isothermal
blocks to achieve PCR. The elimination of the unnecessary ramp time decreases the total cycling time. In addition the fluid elements are able to quickly attain thermal equilibrium with their surroundings as they flow though the various temperature zones on the continuous flow instrument. The elimination of the ramp time and the efficient heating of droplets reduces the thermal cycling time in comparison to stationary well based platforms. The thermocycler design and operation is examined in the following subsections.

### 4.6.1 Design

A two temperature four line thermal cycler was designed based on a single line planar thermal cycler previously developed at Stokes Research Institute. Previous designs used multiple layers of thermal blocks to run parallel lines to achieve the desired throughput. The thermal cycler used in this thesis consisted of two thermal blocks with a 800μm serpentine channel milled 4mm deep. This thermal cycler design is capable of holding four lines on a single block. This design has a smaller profile, requires less machining, requires fewer heating elements and demands less power versus a multiple layer block design. An image of the thermal cycler can be seen in figure 4.10. An engineering drawing of the thermal cycler can be found in Appendix A. A 800μm ball nosed mill bit was used to mill a 4mm deep channel on a CNC milling machine. The thermal cycler blocks were milled out and mounted on polycarbonate end plates. Strip resistance heaters were applied to the underside of the thermal blocks and thermocouples placed in specific temperature control points. The tubing was embedded into the channel to provide a continuous flow conduit that moved through the temperatures required for PCR. The reaction droplets enter the preheat zone incorporated into the 95°C block and are then cycled through the PCR temperatures as the droplets flow from the 95°C thermal block to the 60°C thermal block. These zones are indicated in figure 4.11.

The continuous flow digital PCR thermal cycler was designed to cycle the reagent droplets through 50 cycles of the PCR process. The droplets in a digital PCR experiment are typically of a low target concentration with no more than a few target molecules occupying a droplet. Therefore, a sufficiently high number of cycles are required to amplify the target to a detectable level. This is particularly important in the case of single molecule
amplification where targets amplify above background noise levels at later cycles. The large cycle number used on the instrument ensures that droplets containing single or multiple target molecules amplify and reach the plateau stage of the PCR reaction. This results in a fluorescence signal significantly above the noise of the negative signal, aiding in the discrimination between positive and negative droplets.

The continuous flow thermal cycler was designed to replicate cycle times recommended by the Applied Biosystems Taqman® Gene Expression Assay protocol. The channel length ratio was designed to yield a dwell time duration ratio of 1:4 for denaturation and annealing. A reaction droplet is initially subjected to 95°C for 10 minutes as it flows through the long channel on the 95°C thermal block. It is then held at 95°C for 15 seconds for denaturation followed by an annealing step of 60°C for 60 seconds as it flows through
the 95°C and 60°C blocks. These dwell times were achieved for a volumetric flow rate of 3μL/min.

Although only one line was used in the experiments conducted in this thesis, the four line system enables high-throughput digital PCR for experiments requiring many reactions. Running multiple lines in parallel increases the droplet of the system and hence greatly reduces the time to answer. The digital PCR instrument design also included optical detection and illumination windows for each cycle, permitting cycle to cycle fluorescence detection for qPCR.

4.6.2 Thermal Control

Accurate thermal control is important for successful PCR. Denaturation temperatures that are too high will cause the DNA to degrade. Conversely, the DNA double helix will not completely denature for denaturation temperatures that are too low, resulting in poor amplification efficiency. If annealing temperatures are too high the primers will be unable to bind to the template and the target sequence will not amplify. For continuous flow platforms it is important that the heated blocks are isothermal to ensure consistent cycle to cycle amplification efficiency. Cycle to cycle temperature variations are unacceptable for qPCR since the methodology is based on monitoring of the fluorescence signal as target molecules are being amplified. Temperature variations along the blocks are not as significant for dPCR since the fluorescence signal is interrogated at endpoint. However, it is important that the low target concentration sample droplets are subjected to the entire 50 cycles at the correct
PCR temperatures for successful amplification.

The AB7900HT, a qPCR platform manufactured by Applied Biosystems was used as a benchmark instrument. The AB7900HT specifies a temperature uniformity of ±0.5°C and accuracy of ±0.25°C. This temperature control was repeated on the digital PCR prototype since the digital experiments used Applied Biosystems chemistries and protocols. The thermal control system was designed to ensure that these specifications were met.

The two thermal blocks were heated using resistance polyimide thermofoil™ (Minco) heaters. The heaters were applied to the underside of the thermal blocks using an adhesive backing. Two heaters were applied to each block, providing full coverage along the length of the blocks. Thermocouples were used to control the temperature of each block. K-type thermocouples were fixed at centrally positioned holes using a hardening thermally conductive paste, providing block temperature feedback to the temperature control system.

Proportional-integral-derivative (PID) controllers were used to accurately control the block temperatures. PID controllers maintain the output (heater power) so that there is no difference between the process variable (measured block temperature) and the set point variable (block temperature set point). The controller looks at the absolute error and the rate of change of error and varies power output based on these readings. The heaters were powered using a AC/DC power module (Traco). The power delivered to the heaters was controlled by communication between the PID controller (Eurotherm) and a relay (Crydom). A schematic of the temperature control circuitry can be seen in figure 4.12.
The thermal control system maintained the set point temperatures of both blocks with negligible fluctuations in temperature. The temperature profile of each of the blocks was obtained using a k-type thermocouple and thermocouple reader. A calibrated thermocouple was used to measure the block temperature at the thermocouple measurement points shown in figure 4.10. Thermally conductive paste was used to enhance the thermal contact between the block and the thermocouple to ensure measurement accuracy. The temperature profile of the thermal blocks can be seen in figure 4.13.

There were some heat losses through the ends of each thermal block. Consequently, the temperatures at the end of the thermal blocks were lower than the set point, resulting in sub-optimal cycle temperatures at early and late cycles. The hot block temperature profile was found to be $95\pm1.4^\circ\text{C}$. The cool block temperature profile was found to be $60\pm0.8^\circ\text{C}$. Insulation was added to the ends of each thermal block to minimize end losses. This yielded an approximately isothermal temperature profile of $95\pm0.8^\circ\text{C}$ and $60\pm0.4^\circ\text{C}$ for the hot and cool blocks. The precise temperature control demonstrated by the thermal control system ensured consistent amplification and reproducible digital PCR results.
CHAPTER 4  Device Design

Figure 4.13: Temperature profile for each of the heated blocks with and without insulation.

4.7 End-point Fluorescence Detection System

Fluorescence based detection requires fluorescence excitation and detection of the emitted signal from the flowing droplets. The three colour detection requirement has been discussed in subsection 4.2.4. This section outlines the design of the endpoint detection system, discussing the components used for excitation and detection. Following this, the endpoint detection rig is described.

4.7.1 Light Source and Filter Selection

The Taqman® assays employed in this research incorporated either FAM or VIC based fluorogenic reporters. The passive reference ROX dye is found in the mastermix and acts as internal reference to normalise fluorescence signals. The excitation and emission profiles of the FAM, VIC and ROX dyes are shown in figure 4.14. The aim was to develop an optical detection system capable of exciting and detecting each of the three fluorophores. The
excitation spectra of each of the dyes overlap, permitting excitation of all three fluorophores using a single light source.

Ideally, a light source with a very narrow band should be used to minimise background noise. Lasers typically exhibit a narrow wavelength distribution. A 488nm laser diode (Laser 2000) was used to excite each of the fluorophores. The overlapping light distribution from the laser diode was found to yield a low signal to noise ratio, making it difficult to differentiate between a positive and negative signal. A laser line filter (Omega Optical) was added to the system and shown to improve the signal to noise ratio. The filter improves the signal by blocking light >490nm and preventing bleed through of the light source to the detectors. The FAM, VIC and ROX signals were isolated using 10nm band pass filters (Omega Optical) centered around their respective peak emissions of 520nm, 560nm and 610nm.

The resulting excitation, emission and detection spectrum can be seen in figure 4.14. Observing this figure, it is clear that there will be some signal bleed through of emitted light (cross emission) from one fluorophore to the detector of another. These contributions are significant in duplex experiments and can result in false positive signals. It was necessary to correct for the FAM, VIC and ROX contributions to obtain the true reporter signals. This was achieved using a dye calibration matrix, discussed in Chapter 5. This combination of filters provided an excellent signal to noise ratio, aiding in the discrimination between negative and positive signals and reducing uncertainty in endpoint signal analysis. The endpoint detection system is discussed next.

4.7.2 Fluorescence Detection Rig

The droplets are continuously flowing on the digital PCR instrument, making it difficult to use a single detector and filter wheel configuration. Detection requires three separate detectors equipped with the appropriate optical filters. An image of the end point detection system can be seen in figure 4.15. Excitation is provided by a filtered 488nm laser diode (Laser 2000). A laser line generator (Omega Optical Inc.) is used to provide excitation across multiple lines. The tubing lines are held on a precisely positioned holder consisting of machined channels and strategically located through holes for detection. Optic fibers
Figure 4.14: The excitation and emission profiles for FAM (blue), VIC (green) and ROX (red) fluorophores. The solid cyan blue line represents the binned laser wavelength providing excitation for all three fluorophores. The detection windows used for endpoint fluorescence detection are indicated. The filled areas represent the individual detection windows for FAM, VIC and ROX.

are inserted into these through holes to carry the fluorescence signal to an optical array. Three charge-coupled devices (CCD) cameras (Allied Vision Technologies) equipped with optical filters are used to detect the fluorescence signal from the optic fiber array. This technique permits three colour detection for a multiple line system. An enclosure is placed around the optical detection rig to prevent natural light from interfering with detection. The thermally cycled droplets enter the optical detection system and are excited as they pass the detection region, emitting fluorescence that is recorded by the detectors.

Data acquisition is controlled using Matlab®. The cameras simultaneously capture frames at a defined frame rate, extract the intensity values from a selected region within the fibers and write the acquired data to a text file. The written text file contained time and intensity values to be used in the endpoint signal analysis algorithm, discussed in Chapter 5.
4.8 Chapter Close

This chapter outlined the design criteria for a digital PCR instrument and discussed the design of the continuous flow digital PCR instrument used in this thesis. The limitations of continuous flow technology have been presented. The fluidic network design has been discussed and the fluidic control techniques were described.

Two droplet production techniques suitable for continuous flow PCR have been examined. Microfluidic dipping is a flexible droplet production technique but cannot dispense small, repeatable droplet trains. Liquid bridge segmenters can generate very small, precise droplets at a high throughput and are therefore well suited to droplet generation for continuous flow digital PCR. The design, operation and characteristics of the continuous flow
thermal cycler have been discussed. The endpoint detection system has also been described. The integration of each of these core components yields a fully automated continuous flow platform for digital PCR, illustrated in figure 4.16. Droplets are generated, thermal cycled and fluorescently interrogated at endpoint. The performance of this platform is examined later in chapter 6 and chapter 7.
Figure 4.16: Schematic and image illustrating operation of the continuous flow digital PCR instrument.
Chapter 5

Experimental Methods

5.1 Introduction

The primary objective of this thesis was to use the continuous flow platform described in chapter 4 to perform digital PCR. This chapter details the constituents of the PCR mixes used in each section of experimentation. The sample preparation techniques are outlined and the sample loading protocol is examined. A commercial qPCR platform that was used to run benchmark experiments is also introduced in this chapter. Following this, the algorithm used to convert endpoint fluorescence data to a digital readout is described. The endpoint signal analysis steps are discussed in the order they are performed. The techniques used to count droplets, extract peak values, dye calibrate data and select thresholds are described.

5.2 Biological Sample Preparation

Time consuming sample preparation procedures are required prior to genetic analysis regardless of the PCR methodology used. The biological sample preparation protocol and reaction components are discussed here.
5.2.1 Biological Sample Preparation Protocol

Sample preparation was carried out using standard biological protocols in a UV sterilisation cabinet (Kisker). Since PCR is a highly sensitive process capable of amplifying small amounts of DNA, all necessary precautions were undertaken to reduce the risk of contamination. The UV air circulator was turned on for 15 minutes prior to use to eliminate airborne microbes within the workstation. The UV light was also switched on for 30 minutes to denature any nucleic acids in the hood, making them unsuitable for amplification and therefore eliminating contamination. Modified 384 well plates and microfuge tubes were also subjected to UV light sterilisation. The surfaces of the workstation and all items introduced to the hood were sprayed with 70% ethanol, again to reduce the risk of introducing contamination.

The reagents were removed from storage in the -20°C freezer and the 4°C fridge. The gene assay and template DNA were vortexed to ensure homogeneity, while the mastermix was gently mixed. The aliquots were then centrifuged to spin down the reaction volume to the bottom of the microfuge tube. The reaction reagents were prepared in a reagent lab and then moved to DNA lab for the addition of template DNA, once again to reduce the risk of contamination. Following the addition of template DNA in the DNA lab, the reaction aliquot was once again vortexed, followed by a final centrifuge step. The reaction contained in the microfuge tube was loaded onto the instrument or transferred to a sterile, modified 384 well plate. An oil overlay was added to the microfuge tube or well plate. All reactions were freshly prepared and immediately loaded onto the instrument for testing.

5.2.2 Reaction Components

The PCR reaction mixtures tested on the continuous flow PCR instrument used TaqMan® assays. TaqMan® assays are probe based assays, permitting sequence specific fluorescence detection. Initial tests targeted a sequence on the GAPDH gene, using cDNA as the template and TaqMan® chemistry for detection. GAPDH is commonly used as a standard endogenous control gene since it has a similar level of expression across all cells. Subsequent experiments targeted the RNase P and SRY genes, using gDNA as the template and
TaqMan® chemistry for detection. The advantages of using gDNA as the DNA template for digital PCR experiments were discussed in chapter 2.

The main components of the TaqMan® PCR reactions are the TaqMan® Gene Expression Master Mix and the TaqMan® Gene Expression Assay. The master mix contains dNTP’s, magnesium chloride, ROX passive reference dye and buffer. The gene assay contained the forward and reverse primers and the sequence specific probes. Genomic DNA dilutions were prepared from stock as required and added to the PCR mix to give the desired final gDNA concentration. Nuclease-free water was added as required. The Applied Biosystems protocol specifies an enzyme activation step of 10 minutes at 95°C, and 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, yielding a total thermal cycling time of 3600 seconds.

The experiments performed on the continuous flow instrument were undertaken to meet the thesis objectives outlined in chapter 1. The TaqMan® Gene Expression Assay, DNA template (cDNA or gDNA), template concentrations and starting reaction volumes varied for each set of experiments. The specific reaction details for each experiment are discussed in detail in chapter 6 and chapter 7.

5.3 Applied Biosystems AB7900HT qPCR Platform

A qPCR platform manufactured by Applied Biosystems, the AB7900HT, was used to benchmark the digital results and to interrogate biological samples. The qPCR system was used to interrogate samples and test the performance of a duplex assay. The AB7900HT operates using either 96-well or 384-well plates. PCR reactions are pipetted into the individual wells on the PCR plate and sealed with an adhesive cover. The prepared plates are loaded onto the arm of qPCR instrument. The arm retracts and the plate is positioned into the thermal block for thermal cycling. The thermal block operates using a Peltier based system, cycling the plate through the temperature zones required for PCR. The cycling details can be specified using the AB7900HT software. The AB7900HT uses a 488nm argon laser to excite the samples in the plate. A CCD camera and spectrograph are used to detect the fluorescence signal after each cycle. The Applied Biosystems proprietary software
normalises the fluorescence data, compiles s-curves and returns $C_q$ values for analysis.

## 5.4 Sample Loading Protocol

The reaction aliquots were either loaded directly onto the device or transferred from the reaction tube to a standard or modified 364 square well plate. The modified well plates, shown in figure 5.1, allows the dipping head to access multiple wells. The 364 well plate is a standard biological consumable micro-plate with a milled out section designed to hold an oil overlay. This permits continuous sampling of multiple wells without air ingress. The dipping tip can withdraw sample from one well, lift out of the well to an oil overlay and access another sample well. Reactions are overlayed with oil and the plate is loaded onto the dipping stage. The dipping tip is positioned in the plate oil overlay and the pumps are activated. Once the pumping system reached the specified set point the dipping protocol commenced. The dipping protocol was defined and controlled using LabVIEW. The dipping tip was positioned in the well for a specified dwell time that corresponded to a previously defined sample volume. This technique was used for microfluidic dipping and sample loading for liquid bridge dispensing. Once the required sample volume was loaded the tip moved back into the oil overlay. Additional wells were then accessed if required. This allowed no template controls to be run before and after positive reactions to assess whether contamination occurred on the device. It also allowed large sample volumes to be continuously run on the instrument by sampling positives from multiple wells.

![Figure 5.1: Modified 364 well plate and schematic illustrating well sampling under an oil overlay.](image-url)
The well plate/microfuge tube material was an important consideration as the material influences the wetting properties. Microfuge tubes were used in experiments requiring uptake of the entire sample volume. Aqueous sample does not wet the polypropylene walls, allowing pickup of the entire volume. However, the hydrophobicity of the walls can cause the sample volume to move in the well as the dipping dip is continuously introduced and removed from the sample, making polypropylene unsuitable for dipping. Polystyrene modified well plates were used for large experiments requiring well to well access. The hydrophillicity of polystyrene cause sample to wet the well walls, resulting in a dead volume that cannot be picked up. This was accounted for during analysis and is discussed further in chapter 7.

5.5 End-point Signal Analysis

The detection system used to monitor the end-point fluorescence signal from the flowing droplets in the digital PCR instrument was described in Chapter 4. The logged data files contained time-stamped fluorescence intensity data. For multiplex experiments, separate files were generated for the FAM, VIC and ROX camera’s. The raw data files were then imported into Matlab® for analysis. This software was used to determine the total number of droplets in an experiment, extract droplet peak values, calibrate the peak values, normalise the FAM and VIC signals and to produce a threshold, allowing for the discrimination between positive and negative droplets. The complete algorithm used to produce a digital experimental output (total number of droplets and number of positive droplets) is examined here. In addition, the dye calibration process - an important step required to correct raw fluorescence signals is discussed. The post-processing steps used to generate a copy number estimation from a digital experiment are as follows:

1. Raw data plot inspection.

2. Background noise reduction and signal clean-up.

3. Droplet peak detection and extraction.

4. Calibration of peak values and normalisation to passive reference dye.
5. Normalised peak signal thresholding and counting.


These endpoint analysis steps will now be discussed in detail.

### 5.5.1 End-point Plots and Droplet Counting

The end-point data files generated during data acquisition were imported into Matlab® and plotted. The raw fluorescence intensity data recorded for each line was plotted against time or data point number, providing a graphical representation of the experimental result. These plots were useful in quickly determining the outcome of an experiment. Observing these plots, it was easy to determine if there was significant droplet merging or splitting by examining the length of the droplet signatures. Air ingress or contamination could also be identified through examination of these plots. Observation of any of these events led to the rejection of the experimental run. A portion of a typical endpoint plot is shown in figure 5.2.

![Figure 5.2: A typical endpoint plot of the raw data generated by the endpoint fluorescence detection system. The droplet detection line (red) is shown.](image)

A droplet detection value was used to calculate the total number of droplets in an experiment. This line was selected to pass through the positive and negative portions of
each droplet and is shown as the red line in figure 5.2. The data was reduced to a logical array by setting the data greater than or equal to the droplet detection value, transforming the fluorescence data into a binary signal, as shown in figure 5.3. Differentiating the binary signal gives the slope, producing a positive and negative peak for each droplet. Summing the positive peaks yields the total number of droplets in the experiment. The location of the positive and negative peaks are saved for use as intervals in next step of signal analysis - peak extraction.

Manually selecting a threshold for counting the number of positives is also possible. This approach works for clean end-point curves where discrimination between positive and negative droplets is clear. However, for signals with peaks that lie between positive and negative ranges this technique is not valid. In this scenario, manual threshold selection is not meaningful and may be subject to user bias. To circumvent this problem, a histogram based approach to thresholding the data was used. This thresholding method is described in subsection 5.5.4.

Figure 5.3: Droplet counting: The raw data is transformed into a binary signal, plotted in green. Differentiation of this binary signal generates a positive and negative peak for each droplet as indicated by the black plot. Summing the positive peaks gives the total number of droplets above the specified droplet detection threshold.
5.5.2 Background Subtraction, Droplet Peak Extraction and Normalisation

Droplet peak extraction is a vital step in signal analysis as it is the peak values that are calibrated, normalised and thresholded. The endpoint data for each camera exhibited a background level due to the bleed through of the excitation light source. The baseline was subtracted from each data set to reduce the baseline to zero. The low level signal was cleaned up to eliminate any noise, reducing the data to droplet only data points. This was achieved by creating a binary signal using the droplet detection line. This binary array consisted of 1’s corresponding to the droplets and 0’s for all other data. Multiplying the original baseline reduced array by the binary array produced a new column of data free from low level noise.

The droplet peak values and locations were then extracted using an algorithm coded in Matlab®. This algorithm extracted the local maxima or peaks in a data set. As discussed in the previous subsection, the binary signal created using the droplet detection line was used to find the location of the peak intervals. The maximum values within these intervals were found, as were the peak locations. An example of peak detection from experimental data can be seen in figure 5.4.

Figure 5.4: Droplet peak detection from an endpoint fluorescence signal.

(a) Baseline reduced, low level filtered signal and detected peaks.  
(b) Section of the detected peaks.
Following peak detection and extraction, the peak values were corrected for bleed through using a dye calibration matrix - which is examined next. The calibrated droplet peak signals were then normalised to their corresponding passive reference peak signal. Normalisation was achieved by dividing the corrected detector peak signal by the corrected passive reference signal for each droplet.

### 5.5.3 Multiplex Experiments - Dye Calibration

The application of a dye calibration matrix was required for duplex experiments. There was significant cross emission of light from one reporter to the detector of another due to overlapping emission spectra of the FAM and VIC fluorophores. There was little or no cross emission from either of the two reporter dyes to ROX, the passive reference dye. The cross emission is influenced by the selection of optical filters and the design of the endpoint detection system, as discussed in Chapter 4. Figure 5.5 shows the VIC signal from droplets containing VIC fluorophores and illustrates significant VIC emission contribution to the FAM detector and some cross emission to the ROX detector. It was necessary to correct for the FAM, VIC and ROX contributions to obtain the true reporter signals. A dye calibration matrix was produced to correct for cross emission. The calibration matrix was generated by running FAM, VIC and ROX fluorophore trains through the endpoint detection system.

![Figure 5.5: Dye contributions to each detector for three droplets containing VIC fluorophores.](image.png)
and finding the average peaks values for each train, for each camera. The calibration matrix is given as:

\[
C = \begin{bmatrix}
FAM\ Camera,\ FAM\ dye & FAM\ Camera,\ VIC\ dye & FAM\ Camera,\ ROX\ dye \\
VIC\ Camera,\ FAM\ dye & VIC\ Camera,\ VIC\ dye & VIC\ Camera,\ ROX\ dye \\
ROX\ Camera,\ FAM\ dye & ROX\ Camera,\ VIC\ dye & ROX\ Camera,\ ROX\ dye
\end{bmatrix}
\]  

(5.1)

The inverse of the calibration matrix, \( A = C^{-1} \) is used to correct the peak signals. The corrected FAM peaks value can be obtained using:

\[
FAM_{\text{corrected}} = [A(1,1) \times FAM_{\text{peak}}] + [A(1,2) \times VIC_{\text{peak}}] + [A(1,3) \times ROX_{\text{peak}}]
\]  

(5.2)

Similarly, the corrected VIC values can be found using:

\[
VIC_{\text{corrected}} = [A(2,1) \times FAM_{\text{peak}}] + [A(2,2) \times VIC_{\text{peak}}] + [A(2,3) \times ROX_{\text{peak}}]
\]  

(5.3)

The corrected ROX peak values can be found by:

\[
ROX_{\text{corrected}} = [A(3,1) \times FAM_{\text{peak}}] + [A(3,2) \times VIC_{\text{peak}}] + [A(3,3) \times ROX_{\text{peak}}]
\]  

(5.4)

The three peak signal components from a droplet were inputted in the above equations to find the corrected signal for each droplet in an experiment. The corrected signal was then normalised to the corrected passive reference dye as described in the previous subsection. At this point, the endpoint data array contains the dye calibrated, normalised peaks and their time stamped locations. A threshold is required to generate a binary endpoint output consisting of the number of positive droplets and number of negative droplets. Thresholding, the final step in endpoint signal processing is discussed next.
CHAPTER 5 Experimental Methods

5.5.4 Signal Thresholding

A threshold is required to determine whether a droplet is positive or negative. There are two approaches to signal thresholding: determining an empirical threshold or selecting a threshold based on the distribution of peak data. It was not feasible to determine an empirical threshold on the continuous flow digital PCR instrument since the endpoint detection system was continuously altered and improved upon throughout the experimentation. Distribution based thresholding treats the endpoint data as a distribution containing two modes. The means of both modes can be computed and their standard deviations determined. It is possible to determine if a signal is positive or negative using this information. However, outliers that do not fall into either class may have to be rejected from the experiment resulting in the loss of data points. This is a good approach to employ when thresholding poor quality data where there is significant overlapping of the positive and negative fluorescence signals. The endpoint detection system used in this thesis ensured that there was minimal overlapping, ensuring confidence in the endpoint data. Consequently, a histogram based thresholding technique known as Otsu’s method was used to automatically threshold the fluorescence data.

Otsu’s method is most commonly used in image thresholding whereby a graylevel image is reduced to a binary image. It assumes that the data to be thresholded contains two classes of pixels (two modes) and calculates an optimum threshold that separates the two classes by minimising their combined spread or intra-class variance. Otsu’s method can also be applied to threshold the endpoint fluorescence data in a digital experiment. The fluorescence intensity data can be regarded as a data set with two modes - one mode based around the negative fluorescence level and the other based around the positive fluorescence level. Simulated data illustrating this concept can be seen in figure 5.6. Otsu’s involves iterating through all possible threshold values to find the minimum intra-class variance. The intra-class variance is given by:

$$\sigma^2_w(t) = w_1(t)\sigma^2_1(t) + w_2(t)\sigma^2_2(t)$$  \hspace{1cm} (5.5)

where \(w_i\) are the probabilities of the two classes, \(\sigma^2_i\) are the variances of the classes and \(t\)
Figure 5.6: Simulated data illustrating the bimodal distribution of endpoint peak values. The negative and positive modes are indicated.

is the threshold separating the two classes. A histogram of the acquired peak intensity data points is generated. The weights and variances are calculated for both classes at an initial threshold equal to zero. The intra-class variance is calculated. The intra-class variance is computed for all possible threshold values. The threshold that yields the lowest intra-class variance in selected as the optimal threshold.

It should be noted that for overlapping distributions there will always be a tradeoff between the probability of a false negative (classifying a positive droplet as negative) and the probability of a false positive (classifying a negative droplet as positive). In the context of digital PCR, both of these misclassification errors are undesirable and will effect the absolute quantification result. For noisy endpoint signals an alternative thresholding technique should be used, such as the approach discussed earlier. In cases where there is significant overlapping the experimental data should be rejected. An example of how this histogram based thresholding approach can be applied to an end-point fluorescence signal is discussed next.

The ideal digital PCR endpoint signal consists of two distinct fluorescence intensity levels - a positive and negative level. A clean signal obtained from the end-point detection rig can be seen in figure 5.7. This plot is a good example of a clean signal where it is easy
to discriminate between positive and negative droplets. Observing the corresponding histogram it is clear that threshold selection is straightforward, with two distinct modes visible with a separation between the two modes occurring at an approximate fluorescence level of 40. Implementation of Otsu’s method yields a threshold of 42, proving its effectiveness for clean signals. Although considerations were made in the design of the thermal cycler, endpoint optical detection system and the digital experiment design, noisy peak signals were occasionally observed. An experiment illustrating peak noise is also shown in figure 5.7. A number of peaks lie between the positive and negative range, which is reflected in the corresponding histogram showing two overlapping modes. It is difficult to select a threshold with any confidence in what is a true positive signal. For this particular test, Otsu’s method generated a threshold of 38 - a rational threshold when examining the endpoint signal and its histogram. This technique was tested for various data set types to ensure its effectiveness in threshold selection.

Otsu’s method was implemented in the endpoint analysis algorithm for all data sets as the technique was shown to work for both clean and noisy signals. However, since it is based on a bimodal distribution, it does not work for a single mode i.e. all droplets negative or all droplets positive. Knowledge of negative fluorescence levels is required in this case to determine whether all droplets are positive or negative.

The peak values extracted from the endpoint fluorescence data were thresholded using Otsu’s method - producing a binary endpoint readout consisting of the number of positive droplets and the number of negative droplets. The digital readout was then analysed using the statistical approach described in chapter 3, generating digital PCR copy number estimations and associated confidence intervals. The complete endpoint signal analysis algorithm can be found in Appendix B.

5.6 Chapter Closure

The PCR sample constituents and preparation techniques have been outlined in this Chapter. The qPCR instrument used to interrogate samples and benchmark results was described. The sample loading loading protocol has also been examined. The algorithm
Figure 5.7: Various endpoint signal types and their corresponding histograms.

(a) A clean endpoint signal.
(b) Histogram of peak values from a clean endpoint signal. Two distinct modes are visible.
(c) A ‘noisy’ endpoint signal.
(d) Histogram of peak values from a noisy endpoint signal. Two modes are visible but overlapping, complicating threshold selection.

used for endpoint signal analysis was discussed in detail, demonstrating an efficient and accurate method of producing a digital endpoint result from a continuous flow instrument.
Chapter 6

Device Characterisation

6.1 Introduction

A key aim of this project was to develop and test a fully functional continuous flow digital PCR platform. The proof of concept experiments undertaken prior to commencing the digital PCR quantification studies are described here.

This chapter demonstrates the instruments capability of amplifying a target DNA molecule and investigates the feasibility of each of the droplet generation techniques for biological processing. A proof of concept study demonstrating amplification on the digital instrument is presented. Following this, experiments investigating microfluidic dipping and liquid bridge dispensing as viable droplet generation methods are discussed.

A liquid bridge dispensing homogeneity study is examined, characterising the events observed from the endpoint fluorescence plots. The factors influencing the homogeneity of the dispensed droplets are presented, revealing an approach to minimise the concentration gradient present in long droplets. The possible sources of this effect are also discussed. The throughput of the instrument is then examined. Finally, a study investigating the maximum thermal cycler flow rates that can be used on the continuous flow thermal cycler is presented.
6.2 Proof of Concept: Amplification on the Continuous Flow Thermal Cycler

Four experiments were performed to investigate the instrument’s capability of amplifying a specific sequence of DNA. PCR reactions containing cDNA (final cDNA concentration 0.2ng/μL) as the template and a Taqman® assay targeting a sequence on GAPDH gene were prepared. The 20μL reactions were segmented using the microfluidic dipping stage and thermally cycled on the continuous flow thermal cycler. The samples were then collected downstream of the thermal cycler and stored in separate microfuge tubes for analysis.

The samples were analysed using gel electrophoresis. Gel electrophoresis is a molecular biology technique used to examine amplified products from a PCR reaction. Samples are loaded onto a porous agarose gel and the gel is immersed in a salt solution. An electric current is applied to the gel, causing the negatively charged DNA to move towards the positive pole of the electrophoresis chamber. Smaller fragments of DNA move through the gel matrix faster than large fragments, separating the DNA products in a sample by size. The amplified target, which is of known size, appears as a fluorescent band under UV light. The size of the product can be determined by observing the markers (ladders) of known base pair lengths in the adjacent lanes.

Gel electrophoresis was regularly performed on samples recovered from the digital instrument to verify sequence specific amplification. The gel electrophoresis result for this proof of concept study can be seen in figure 6.1(a). Examining the gel result, it is evident that the continuous flow digital PCR instrument successfully amplified DNA in each test. Fluorescence bands can be seen in lanes 1-4 in the image. These bands represent amplified DNA molecules fluorescing under UV light. The 100 base pair (bp) ladder is labeled in the adjacent lane. The product length in the experimental lanes correspond to that of the GAPDH target length of 122bp - demonstrating that the continuous flow instrument successfully amplified sequence specific product.

Non-specific product bands less than 100bp in size are also visible on this gel result. This product is likely primer dimer, a common by-product of PCR generated through the hybridisation and amplification of the forward and reverse primers. The formation of this
Figure 6.1: Gel results from the digital PCR instrument and the AB7900HT. (a) Amplification on the continuous flow instrument. Lanes 1-4 contain sample collected from the instrument. The four fluorescence bands in each of the lanes correspond to the 122bp GAPDH target sequence size. (b) Gel result from the AB7900HT. Lanes 1 and 2 contain sample collected from the instrument.

Product can be detrimental to the PCR efficiency as it competes with the target molecules for reagents during amplification. The accumulation of primer dimer can be ignored since the GAPDH target successfully amplified on the continuous flow platform and accumulation of this non-specific product does not contribute to the fluorescence signal due to the selected detection method. Primer dimer could contribute to false positive signals with non-specific chemistries such as binding dyes. This result demonstrates the importance of a sequence specific reporter for digital PCR. Primer dimer formation can also be seen in amplified products retrieved from the AB7900HT instrument (figure 6.1(b)), demonstrating that the accumulation of primer dimer is not exclusive to the digital PCR prototype.
6.3 Characterisation of the Droplet Production Techniques for Continuous Flow dPCR

The devices capability of amplifying target DNA in droplets was shown. The next step was to examine the suitability of each of the droplet production techniques for digital PCR. The homogeneity of the segmented droplets and the propensity for each of the droplet production techniques to cause carryover contamination is investigated here. Genomic DNA was selected as the template DNA for this study as it permitted calculation of the theoretical copy number, as discussed in Chapter 2. RNase P was selected as the genomic DNA target.

High target concentration experiments were run on the instrument to verify its capability of amplifying each droplet. The final concentration of gDNA in the PCR mix used in these experiments was 0.5ng/µL, equating to 152 copies/µL. This means that on average, an 100nL droplet would contain 15 copies. This concentration ensured that every droplet would contain target DNA molecules for amplification and would exhibit a positive endpoint fluorescence signal. No template control (NTC) trains were run on the instrument to investigate carryover contamination. The results for each of the droplet production techniques are examined in this section.

6.3.1 Liquid Bridge Dispenser

Samples with a high concentration of gDNA were prepared and loaded onto the device. The droplets were segmented using the liquid bridge dispenser at an outlet flow rate of 2.5µL/min and oil inlet flow rate of 2µL/min, generating droplets approximately 100nL in volume. The droplets were cycled and detected at endpoint. A typical endpoint result is shown in figure 6.2. The leading droplets in the train are negative while subsequent droplets have a positive amplified fluorescence signal. This observation was unexpected as the gDNA concentration ensured that each droplet would contain gDNA target molecules and should amplify. A gradient in the droplet signal can be seen after these lead negative droplets. The droplet signal gradually increases from a negative fluorescence level to a positive fluorescence level, indicating a concentration gradient in the droplets. The sample
was recovered and gel electrophoresis was performed to verify amplification of the RNase P target. The gel electrophoresis result shown in figure 6.3 shows fluorescence bands corresponding to the 87bp RNase P sequence, demonstrating that the device had successfully amplified the target sequence in subsequent droplets.

Since the device had successfully amplified, the problem could not be attributed to the performance of the continuous flow thermal cycler - each droplet containing a target molecule should produce a positive fluorescence signal. This leading edge effect was observed for all experiments at these test conditions. Barrett (2008) observed similar results when segmenting sample volumes in droplet trains for biological processing. These results indicated that there was a missing reaction component in the lead droplets. This is clearly unacceptable as the digital PCR principle is based upon distribution theory. A bias in distribution would yield inaccurate quantitative results. This leading edge effect is further investigated in the section 6.4. Carryover contamination on the liquid bridge dispenser is discussed next.

Figure 6.2: Endpoint signal from the continuous flow instrument showing the leading edge effect. The leading droplets are negative after thermal cycling.
CHAPTER 6 Device Characterisation

6.3.2 Liquid Bridge Dispensers - Carryover Contamination

The flow regime used to deliver sample to the liquid bridge had the potential to cause carryover contamination. Sample was continuously drawn from a well, causing the liquid film thickness to break down. Here, the flow regime changes from biphasic flow to single phase flow, allowing the aqueous slug (long droplet) to wet the wall of the sample inlet tube upon delivery to the liquid bridge. This allows reaction constituents to bind to the tubing wall, as illustrated in figure 6.4.

Figure 6.4: Schematic illustrating contamination of the sample inlet line as the reaction is delivered to the dispenser. The reaction is then segmented into wrapped droplets that do not contaminate.

Figure 6.3: Gel result from continuous flow instrument showing fluorescence bands corresponding to amplified 87bp RNase P target.
The loss of target molecules from the sample to the polypropylene aqueous inlet tube as sample flows from the well to the liquid bridge was a concern. Target DNA molecules that bind to the tubing do not take part in the digital PCR reaction, generating inaccurate copy number estimations. Furthermore, these DNA molecules may get picked up in subsequent experiments - skewing the results.

NTC’s were loaded before and after test reactions to investigate the effect of wetting on carryover contamination. The endpoint result from an experiment consisting of two NTC trains and a high concentration positive train can be seen in figure 6.5 (a). The front NTC’s are negative, demonstrating that there is no carryover contamination from previous experiments and that the reagents are also contamination free. As expected, the droplets in the high concentration positive train exhibit a positive fluorescence signal. The leading edge effect is not present in this experiment as steps were taken to negate the effect. These steps will be discussed in detail in later subsections. The back NTC’s, the NTC’s processed after the positive train contains a positive peak, indicating carryover contamination. An endpoint plot illustrating carryover contamination from a previous high concentration run can be seen in figure 6.5 (b). These results confirm that some DNA molecules bind to the polypropylene sample inlet line as the sample flows to the liquid bridge dispenser. These molecules are then picked up in subsequent trains and amplified.

Wash steps were used to clean the tubing surface between tests that used liquid bridge dispensers. Ethanol, nuclease free water and no template control trains were used to clean the sample inlet line. The loss of DNA at the liquid bridge and the resulting carryover contamination is clearly undesirable. However, the loss of molecules due to surface binding is similar to the loss of molecules associated with sample preparation. Molecules may bind to the disposable polypropylene pipette tip and microfuge tube surfaces. Since carryover contamination is contained within the sample delivery line, the remaining fluidic network is unaffected. The liquid bridge module can be treated as a consumable and disposed of after each experiment. Alternatively, the sample delivery line can be treated as a disposable tip that is discarded after each use. The design of a consumable liquid bridge dispenser is not within the scope of this work. Consequently, wash steps were employed to limit carryover contamination for the experiments conducted in this thesis.
Figure 6.5: Carryover contamination on the continuous flow platform. The NTC and positive trains are labeled.
6.3.3 Microfluidic Dipping - Sample Homogeneity

The viability of microfluidic dipping for biological processing was also investigated. Samples containing high concentrations of gDNA were once again prepared. The dipping protocol was specified and droplets were generated using the robotic dipping stage. An endpoint fluorescence plot from a microfluidic dipping experiment is shown in figure 6.6. Examining this result, it is clear that each of the droplets successfully amplified. The front and back NTC’s did not amplify, confirming that the droplets generated via microfluidic dipping are fully wrapped in an oil film that prevents cross and carryover contamination. Microfluidic dipping is suitable for droplet production in this regard. However, the previously discussed limitations of microfluidic dipping for droplet generation outweigh these favourable traits.

Sample inhomogeneity was considered a possible cause of the leading edge effect demonstrated earlier. The reaction contained in a well may not have a homogenous template or reagent distribution. As the dipping tip is introduced to the sample, the initial volume withdrawn may simply be missing one of these components, resulting in unamplified droplets at the start of the train. The microfluidic dipping results presented here verify that the observed effect was unique to the liquid bridge droplet production technique and not related to the homogeneity of the sample in the well. It also confirmed that the

![Endpoint fluorescence signal from droplet trains generated using microfluidic dipping.](image-url)
carryover contamination observed using the liquid bridge segmenter was due to wetting of
the sample inlet line on the liquid bridge dispenser. The experiments conducted to fully
characterise the leading edge effect are discussed next.

6.4 Liquid Bridge Dispensing: Droplet Homogeneity Study

6.4.1 Problem Identification

The endpoint curves obtained using liquid bridge dispensing and microfluidic dipping
proved that the leading droplet effect was unique to liquid bridge dispensing and was not
related to sample homogeneity in the well. This indicates that the leading droplet effect
arises from the distribution of a reagent or template component in the long droplet being
delivered to the inlet capillary for segmentation. The concentration gradient forms as the
sample is being delivered to the liquid bridge dispenser. Figure 6.7 illustrates the suspected
concentration gradient in the aqueous slug being delivered to the dispenser, the subsequent
distribution of the molecules in droplets and the resulting endpoint fluorescence signal.

Although each of the PCR components are necessary for amplification, the absence
of the following reaction constituents were deemed the most likely contributors to a nega-
tive signal:

- Target DNA molecules
- Sequence specific probes
- Forward and reverse primers

A concentration gradient of target DNA molecules is an obvious candidate, as the absence
of target DNA would result in a negative signal. The lack of sequence specific probes in
a droplet would not effect amplification but would yield a negative signal. If there are no
sequence probes to bind to the target during PCR, there will be no increase in fluorescence
as the target DNA is amplified. A droplet that does not contain the forward and reverse
primers would not amplify as the primers are essential for amplification. A number of
CHAPTER 6 Device Characterisation

Figure 6.7: Schematic illustrating the effect of a concentration gradient. (a) Concentration gradient in the aqueous being delivered to the liquid bridge dispenser. (b) Distribution of molecules in droplets after segmentation and the resulting endpoint fluorescence signal.

experiments were conducted to identify the missing component in the aqueous slug. These experiments will now be discussed.

The most apparent component required for amplification in a droplet is target DNA. A high concentration test was repeated and the endpoint curve was examined. The number of negative droplets at the beginning of the train were counted. The negative droplets were then collected and stored in a microfuge tube. The recovered sample volume of 3µL was not sufficient for gel electrophoresis. The collected sample was instead used in a new PCR reaction. Mastermix, gene assay and RNase free water was added to the collected sample and pipetted to a 96 well qPCR plate. The plate was then ran on the AB7900HT qPCR instrument.

A negative qPCR curve would prove that target DNA was not present in the lead droplets since all other reagents are available for PCR. Conversely, a positive curve would
confirm the presence of target DNA in the leading droplets - indicating that a missing reagent component is the cause of the negative droplet signals. Assuming that DNA was present but unamplified on the continuous flow instrument, the final concentration of gDNA in the new 20µL reaction was approximately 0.075ng/µL. Positive controls with a final gDNA concentration of 0.5ng/µL were prepared and run on the same plate. The presence of target molecules (previously unamplified on the continuous flow instrument) would generate s-curves with a $C_q$ value higher than the positive control. The resulting qPCR curve can be seen in figure 6.8. The recovered sample amplified, proving that target DNA was present in the lead droplets. This observation verifies the absence of a reagent component in the lead droplets, preventing the droplets from amplifying. Examining the amplification plot, it is clear that the recovered sample $C_q$ value is much lower than the positive control $C_q$ value. This was unexpected given the starting concentrations of both reactions. The positive control concentration was 0.5ng/µL whereas the test sample concentration was 0.075ng/µL. The positive control $C_q$ should be 3 cycles lower than the recovered sample $C_q$ value since the final concentration was approximately 6 times greater. However, the opposite was observed as the recovered sample had a $C_q$ value of 20.9 while the average

![Figure 6.8: qPCR curve from AB7900HT instrument. The recovered sample curve and positive control curves are shown.](image)
positive control value was 27.4, a $\Delta C_q$ of 6.5. This experiment confirmed that not only was target DNA present - it had also been previously amplified in the lead droplets on the continuous flow instrument.

These findings were vital in determining the exact cause of the negative signals exhibited by the leading droplets. Target DNA is present in these droplets. Furthermore, the target DNA is amplifying in the droplets, verifying that the reagents necessary for PCR are present. This proves that the negative signal is caused by the deficiency of sequence specific probes.

The concept of a Taqman® probe concentration gradient is supported by Barrett (2008) who processed a long droplet of pure FAM dye and observed a similar gradient. This result was reproduced on the digital instrument and is shown in figure 6.9. A concentration gradient of fluorophores is clearly present as the fluorescence levels runs from low to high as the slug passes the detector. As this distribution has been observed using dye containing FAM fluorophores, it can be assumed that the probe oligonucleotides and quenchers do not influence formation of the gradient. The concentration gradient is caused by the fluorophore molecules.

![Figure 6.9: Endpoint curve showing FAM concentration gradient in a long slug of pure FAM dye.](image)
The inhomogeneous distribution of sequence specific probes in the sample slug is unacceptable for digital PCR as false negative signals are produced, generating inaccurate results. The lead droplet effect is further characterised in the next subsection. Approaches to minimise the effect are also presented.

6.4.2 Lead Droplet Phenomenon Characterisation

A number of experiments were performed to determine the parameters influencing the probe concentration gradient. Firstly, the sample volume was varied to investigate the effect of slug length on the number of negative droplets at the front of the droplet train. The reaction constituents, concentrations and flow rates were kept the same for all experiments. The sample volume was varied from 20-80µL. The number of lead negative droplets was approximately equal for each of the experiments. This result proved that the concentration gradient is independent of the slug volume. Since the number of lead droplets is not influenced by slug volume, the effect on the endpoint result would be negligible for very large reaction volumes. The effect could be ignored by discounting a known number of droplets at the front of the droplet train. However, for small experiments the effect cannot be ignored.

The liquid bridge dispensing flow rates were also varied to investigate the relationship between the concentration gradient and the sample delivery flow rate. The sample slugs (20µL volumes containing a high concentration of target DNA) were dispensed at high flow rates and then slowed to the flow rates appropriate for thermal cycling. The sample inlet capillary length, the tubing length from the well to the liquid bridge was 210mm. The number of negative lead droplets were counted and used to calculate the volume fraction or ratio of negative droplets to total droplets, \( V_l/V_t \). The following volumetric flow rates were investigated:

1. Sample inlet 1.1µL/min, oil inlet 3µL/min, outlet 4.1µL/min.
2. Sample inlet 4.4µL/min, oil inlet 12µL/min, outlet 16.4µL/min.
3. Sample inlet 8.8µL/min, oil inlet 24µL/min, outlet 32.8µL/min.
The ratio of negative droplets to total droplet number for each experiment is plotted in figure 6.10. Observing this figure, it is clear that increasing the slug flow rate yields a lower lead droplet volume ratio. This trend demonstrates that the concentration gradient is greatly reduced at higher flow rates. Increasing the sample flow rate from 1.1µL/min to 8.8µL/min reduces the fraction of negative droplets from 0.55 to 0.2. This result was significant as it offered a method of reducing the concentration gradient in the sample slug.

An additional study was performed to investigate whether the concentration was a function of the capillary length. The sample inlet tubing length was reduced from 210mm to 70mm. Experiments were repeated at the 8.8µL/min sample inlet flow rate and the number of negative droplets counted. The fraction of negative droplets reduced from 0.2 to 0.01 by reducing the tubing length.

These results showed that the concentration gradient can be almost completely eliminated by minimising the sample inlet capillary length and dispensing at high flow rates. This finding was implemented in subsequent liquid bridge dispenser designs and digital PCR experiments. The possible mechanisms by which the concentration gradient is formed
are discussed in the next section.

### 6.4.3 Concentration Gradient Formation

The exact cause of the fluorophore gradient in flowing slugs is of interest. Although the findings from the homogeneity study aid in understanding the problem, the exact mechanism by which the concentration gradient forms remains unclear. The fluorophore properties and fluid flow characteristics were examined, revealing a number of possible causes. The proposed hypotheses describing the formation of the concentration gradient are discussed here.

The FAM probes are hydrophilic, potentially causing the probes to move away from the aqueous-oil interface. However, it is highly unlikely that the probe hydrophilicity would cause the probes to move the distances observed during experimentation. For example, in figure 6.2, approximately 30 droplets are negative and do not contain sequence specific probes. This corresponds to a 20mm section of the slug without any fluorophores present. Furthermore, the concentration gradient would be symmetrical, with probes moving away from the front and back of the aqueous slug. This was not observed. These arguments show that the concentration gradient is unrelated to probe hydrophilicity.

Low degrees of internal circulation within the sample slug was considered a possible cause of the leading edge effect. This was first proposed by Barrett (2008), who also observed the leading edge effect. King et al. (2007) demonstrated that not all slug flows produce internal circulations. This study used micro particle image velocimetry to measure the internal circulations within aqueous slugs traveling in an immiscible carrier fluid. It was found that slugs flowing at low velocities exhibit zero velocity gradients within the slugs, resulting in low levels of internal circulation (King et al., 2007). The magnitude of the leading edge effect has been shown to reduce with an increase in velocity. This points to a possible relationship with the degree of mixing in a slug - which is also proportional to velocity. However, this is unlikely as zero velocity gradients within a long slug should not affect the concentration of probes. It is assumed that the probes would remain suspended in solution in an aqueous slug with low levels of internal circulation. In addition, the tubing length would have no effect on a concentration gradient formed by internal circulation,
as seen earlier. Taking these observations into account, low levels of internal circulations within the sample slug was dismissed as a cause of the leading edge effect.

The binding of probe molecules to the tubing surface was also considered as a mechanism responsible for the fluorophore concentration gradient. The binding of gDNA to the polypropylene sample inlet tube was demonstrated earlier. The probe molecules may move away from the aqueous and stick to the tubing surface. This process continues until the surface of the tubing is saturated with probe molecules. The fluorophores in the front of the aqueous are removed as the leading edge 'supplies' probes to the internal surface of the tubing. This mechanism fits the relationship between tubing length and the concentration gradient. As a shorter length of tubing does not require as many fluorophores to functionalise the surface, a shorter front section of the slug loses its probes. However, the gradient has been observed in repeated experiments, indicating that either the probes bound to the surface are washed away by the oil flow or that the saturation of probe molecules does not occur. If the molecules are washed away, probes from subsequent sample slugs are required to re-functionalise the surface, resulting in repeated observation of the leading edge effect in experiments. Increasing the slug velocity may reduce probe binding, reducing probe loss and minimising the leading edge effect, as seen earlier. From this evidence and the observation of gDNA binding in the carryover contamination study, it is feasible that probe loss due surface binding creates the probe concentration gradient. The presence of bound probes in the sample delivery line is highly undesirable as these may be picked up in a subsequent experiments. The results would be skewed in subsequent experiments using a different assay with the same fluorophore reporter. Wash steps or liquid bridge dispenser disposal are possible methods of eliminating this problem.

6.4.4 Liquid Bridge Dispensers for Continuous Flow Digital PCR

The concentration gradient of sequence specific probes is clearly undesirable for digital PCR. This section has demonstrated that the concentration gradient is a function of the flow rate and the length of the delivery tubing. Consequently, dispensing should be performed at high flow rates for continuous flow digital PCR to minimise the concentration gradient. Furthermore, the length of the delivery line should be kept as short as possible to ensure
production of homogenous droplet trains. This method of eliminating the problem was employed in the digital PCR experiments discussed in the next chapter.

6.5 Droplet Throughput

The importance of droplet throughput was discussed in Chapter 4. A sufficiently high droplet throughput is required to achieve a short time to answer. Although important, droplet throughput was not a primary focus of this research. Thermal cycler (outlet) flow rates were selected to favour single molecule amplification. An outlet flow rate of 3µL/min was used in the experimental studies presented in the next chapter, giving the thermal cycling times specified by the chemistry protocol. This outlet flow rate yielded a droplet throughput ranging from 300-400 droplets per hour, depending on the droplet size and spacing.

A brief study was undertaken to determine the maximum thermal cycling flow rate for the instrument since this variable dictates the maximum droplet throughput. Reactions with a high concentration of gDNA were prepared, segmented and thermally cycled at various flow rates. The endpoint plots were then examined to determine whether the RNase P target had amplified for the defined flow rate, with each droplet expected to amplify given the high target concentration. The maximum outlet flow rate at which all of the droplets amplified was 6µL/min. Outlet flow rates of 7µL/min and 8µL/min revealed amplification in some but not all droplets. This is clearly undesirable, as each droplet should amplify given the presence of a target DNA molecule. An endpoint fluorescence plot for an experiment cycled at 6µL/min can be seen in figure 6.11.

The droplet throughput can be calculated by counting the number of droplets and measuring the time frame, as shown in the figure. The droplet throughput for this experiment was calculated to be 730 droplets/hour. The droplet size for this study was approximately 75nL and the droplet spacing (centre to centre) was approximately 3.2mm. Additional experiments with a reduced droplet spacing revealed a maximum throughput of approximately 1,000 droplets/hr.

The maximum flow rate that droplets will amplify at may be limited by the preheat
time, not the reduced cycling time. The droplet velocity is increased, resulting in a shorter dwell time in the denaturation block. For example, a droplet flowing at approximately 0.3 mm/s (3 µL/min) will spend 12.7 minutes in the 230 mm long denaturation zone. Increasing the velocity to 1 mm/s (8 µL/min), results in a denaturation time of 3.8 minutes, much shorter than the recommended denaturation time of 10 minutes. The DNA will not completely denature if the denaturation time is too short yielding poor amplification efficiency. The shortened denaturation time may not be sufficient to activate the polymerase, also reducing the amplification efficiency.

An experiment with a fixed denaturation time could be used to investigate whether the reduced amplification efficiency is caused by the shortened denaturation time or shortened cycling times. This can easily be achieved by adding an additional hot block module to maintain the 10 minute denaturation time at higher flow rates. If amplification is affected by the reduced denaturation time and not the reduced cycling times, the throughput can be further increased for the current design. Alternatively, a chemistry that does not require a hot start could be tested at various flow rates.
As discussed earlier, droplet throughput is not a primary objective of this work. Consequently, droplet throughput was not investigated further. The results from this study indicate the maximum flow rate and droplet throughput achievable for the current design. Additional experiments have been proposed to investigate whether reduced cycling times can be achieved using continuous flow thermal cyclers.

### 6.6 Chapter Close

This chapter demonstrated the instrument’s capability of amplifying target sequences from both cDNA and gDNA. The device successfully amplified and detected RNase P and SRY target sequences. These results were verified using gel electrophoresis.

The feasibility of the two droplet production techniques for use in biological processing were investigated. Microfluidic dipping was shown to possess the required characteristics for continuous flow PCR, preventing contamination and displaying an even distribution of reagents in each of the droplets. However, this droplet generation method has limitations in droplet size production and repeatability and hence not considered for digital PCR. Carryover contamination was found to occur when using liquid bridge dispensers. Carryover can be avoided by incorporating wash steps or disposal of the liquid bridge dispenser or sample inlet line after use.

A Taqman® probe concentration gradient was identified within the aqueous slug being delivered to the liquid bridge dispenser. The absence of this reagent component was confirmed through a number of experiments. The possible processes by which this concentration gradient is formed have been presented. Although the exact cause of the gradient is unclear, the loss of sequence-specific probes due to binding in the sample delivery tube was found to be the most probable mechanism in the formation of the concentration gradient. An approach to minimise the gradient was also presented, negating this undesirable effect and permitting the use of liquid bridge dispensers for continuous flow PCR. In the final section of this chapter, the maximum flow rate and throughput’s achievable using the current thermal design were examined.
Chapter 7

Experimental Results

7.1 Introduction

The primary aim of this project was to perform digital PCR on the continuous flow instrument. The design, development and characterisation of the device has been described in the previous chapters. This chapter discusses the digital PCR quantification studies performed on the continuous flow digital PCR instrument.

This chapter is divided into four sections. The first section demonstrates accurate singleplex digital PCR on the continuous flow instrument for various reaction volumes and gDNA concentrations. Possible sources of error are discussed, supporting the quantification results obtained on the continuous flow instrument. The second section uses an alternative liquid bridge configuration that prevents carryover contamination on the liquid bridge dispenser. This configuration is described and implemented in a digital PCR study. In the next section, multiplex digital PCR is investigated. The performance of the instrument is then examined, exploring the quantification capabilities of the continuous instrument and proposing design changes to maximise the performance of the platform. Finally, continuous flow technology is evaluated for digital PCR.
CHAPTER 7 Experimental Results

7.2 Absolute Quantification using dPCR

Performing singleplex digital PCR on the continuous flow instrument is a key goal for this project. Successful singleplex digital PCR demonstrates the platforms capability of addressing the singleplex biological applications described in chapter 1. This section discusses the first digital PCR study performed on the device. The experimental design is discussed, followed by a brief examination of the distribution of targets in droplets. The digital PCR results are presented and sources of error are introduced. Lastly, the findings from the section are summarised.

7.2.1 Experimental Design and Configuration

The first digital PCR experiment performed on the continuous flow digital PCR instrument investigated absolute quantification of a single genomic target in a 20μL sample. Samples were prepared to yield a final gDNA concentration of 0.02ng/μL, corresponding to approximately 6.1 copies/μL of the RNase P gene - the genomic target in this study. Following this, 40μL and 80μL reactions were prepared with a final gDNA concentration of 0.01ng/μL and 0.005ng/μL, respectively. Theoretically, each of the 20μL, 40μL and 80μL reactions contained 121 copies of the RNase P gene. This theoretical value was useful for benchmarking the digital PCR quantification results obtained on the continuous flow instrument.

The aim of this study was to demonstrate single molecule amplification and singleplex digital PCR on the continuous flow instrument. The effect of increased droplet number on the precision of the absolute quantification result is investigated by performing absolute quantification of various reaction volumes with the same input quantity of target molecules. Since unparalleled accuracy and precision are the key benefits of digital PCR, these qualities are examined closely in the results section. The experimental conditions are discussed next.

It was important that the entire sample volume was processed to ensure repeatability across experiments. Polypropylene microfuge tubes were used to prevent the sample from wetting the walls of well, permitting pickup of the entire sample volume. The loading tip was positioned at the bottom of the sample well. Aqueous was continuously delivered to
the liquid bridge for dispensing, generating a continuous train of droplets. This process is illustrated in figure 7.1. The endpoint detection system consisted of a single camera for this study. Correction for cross emission was not necessary as the reactions contained a single Taqman® assay employing a FAM reporter. Although normalisation of the signal using ROX is preferential, the consistency in droplet size demonstrated earlier indicates that normalisation to the passive reference dye would have little or no effect on the endpoint result.

The outlet flow rate was set to 32.8\(\mu\)L/min and the oil infuse flow rate was set to 24\(\mu\)L/min, giving a \(Q^*=0.73\) and a droplet size of approximately 58nL. These conditions yielded approximately 350, 700 and 1400 droplets for the 20\(\mu\)L, 40\(\mu\)L and 80\(\mu\)L experiments. A larger liquid bridge dispenser tip spacing was used for the first two 20\(\mu\)L experiments, yielding 105nL droplet volumes and total droplet number of 190 droplets per experiment. High flow rates were used to quickly withdraw the sample volume, countering the leading edge effect associated with liquid bridge dispensing. The loading tips were positioned in the bottom of the sample well and the entire sample volume was withdrawn and dispensed using the liquid bridge dispenser. The outlet flow rate was then reduced to the thermal cycling flow rate of 3\(\mu\)L/min once the entire sample had been segmented. Wash steps were run on the instrument after each test to reduce the risk of carryover contamination. NTC’s were also performed on the device to ensure there was no carryover contamination and on the AB7900HT qPCR instrument to ensure that the reagents were
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not contaminated. The conditions specified here were used in each experiment.

7.2.2 Theoretical Distribution of Target Molecules in Droplets

The Poisson distribution was used to predict the distribution of target molecules in droplets for each experiment. The distribution theory was useful in determining an appropriate gDNA concentration during experimental design. The distribution of 121 RNase P copies into 350, 700 and 1400 droplets is illustrated in the histogram plots shown in figure 7.2. For the 20 µL experiments, it is predicted that 247 of 350 droplets will contain no target molecules and hence exhibit an negative signal. It is expected that approximately 103 of the droplets (30%) will be positive. Of the 103 positives, it is predicted that 85 droplets will contain one target, 15 droplets will contain two targets and 2 droplets will contain 3 targets. Since most droplets will contain only one starting copy, it can be assumed single molecule amplification has been achieved if a similar proportion of positive droplets are observed at endpoint. Applying the distribution theory to the 40 µL experiments consisting of 700 droplets, the percentage of positive droplets reduces to 16%. The percentage of positive droplets further reduces to 8% for the 80 µL tests. The probability of a droplet containing more than 1 target molecule in this 1400 droplet experiment is approximately zero. A true digital effect is expected in this case - a positive droplet signal corresponds to a single target molecule in a droplet and the starting copy number can be calculated by summing the number of positives. The theoretical distribution of target molecules verifies single molecule amplification and aids in the understanding of the digital effect, both of

Figure 7.2: Histograms representing the distribution of 121 target molecules in (a) 350 droplets, (b) 700 droplets and (c) 1400 droplets.
which are examined in the next section.

Figure 7.3: Endpoint curves and scatter plots of peak values for 20\(\mu\)L, 40\(\mu\)L and 80\(\mu\)L experiments denoted by a, b and c. The calculated threshold for each experiment is shown in red.
7.2.3 Absolute Quantification Results

The 20µL experiment was repeated 6 times, providing confidence in the copy number estimations. The 40µL and 80µL experiments were repeated in triplicate. A selection of the resulting endpoint curves and scatter plots from these experiments can be seen in figure 7.3. The calculated threshold for each experiment is shown in red. This threshold was calculated using the methods outlined in chapter 5. Observing these baseline reduced fluorescence plots, it is evident that droplets have amplified. A histogram of the peak values from a 40µL experiment, shown in figure 7.4, clearly shows a bimodal distribution with modes at positive and negative values. The large separation between the modes aids in the discrimination between positive and negative signals.

![Histogram of peak values from a 40µL experiment. The peak value data forms a bimodal distribution around positive and negative values.](image)

The decrease in the proportion of positive droplets observed in the endpoint plots demonstrates the digital effect. The random distribution of positive droplets indicate that the reactions constituents are homogeneously distributed. Applying statistical distribution theory to the number of droplets and number of positives, most positive droplets contain...
only one target molecule. From this observation it can be concluded that the platform successfully performed single molecule amplification in flowing droplets. Figure 7.5 illustrates single molecule amplification on the continuous flow digital PCR instrument.

![Figure 7.5: Section of endpoint curve from a 40\mu L experiment demonstrating single molecule amplification in three different droplets.](image)

The endpoint data was analysed using the endpoint algorithm described in Chapter 5. Since a single camera system was used in this study, dye calibration and normalisation was not performed. The experimental outputs were used in the endpoint algorithm to compute the starting copy number and confidence intervals. The copy number estimations and 95% confidence intervals for each 20\mu L test is plotted in figure 7.6.

Observing figure 7.6, the copy number estimations are consistently higher than the theoretical starting copy number of 121 copies. The average estimated copy number from the 20\mu L experiments is 171 copies. Although the estimations appear somewhat inaccurate, the repeatability across the experiments is excellent. The coefficient of variance was calculated to be approximately 10%. The devices capability of performing singleplex digital PCR has been demonstrated. These results are supported by the copy number estimations obtained from the larger droplet number experiments. The copy number estimations and 95% confidence intervals from the 20\mu L, 40\mu L and 80\mu L experiments are plotted in figure 7.7.
The digital results from the 40\mu L and 80\mu L tests correlate well with the estimations obtained from the 20\mu L experiments, as shown in figure 7.7. The repeatability in the copy number estimations across the study further strengthens the absolute quantification results obtained from the continuous flow digital PCR instrument. The discrepancy between the digital quantification results and the theoretical copy number is consistent throughout the study. The possible sources of this discrepancy is discussed later.

A trend of increasing precision with increasing sample volume is evident, depicted by the increasing tightness of the confidence intervals. Statistically, this was expected as a reduction in the proportion of positives yields greater confidence in the copy number estimations. Increasing the number of micro-reactors also increases the estimation precision. The relationship between precision, the proportion of positives and the number of droplets has been discussed in Chapter 3. The increased precision is better illustrated on a precision plot, shown in figure 7.8.

![Figure 7.6: Plot of the estimated starting copy number and 95% confidence intervals obtained from 20\mu L tests performed on the continuous flow digital PCR instrument. The theoretical copy number is shown in red.](image-url)
Figure 7.7: Plot of the estimated starting copy number and associated confidence intervals for the $20\mu$L, $40\mu$L and $80\mu$L experiments. The theoretical copy number is shown in red. Tests 1-6: $20\mu$L reactions. Tests 7-9: $40\mu$L reactions. Tests 10-12: $80\mu$L reactions.

Figure 7.8: Precision plot for each of the tests in the dPCR absolute quantification study. Each reaction volume contained the same number of target molecules. The copy number estimation precision increases with an increased number of droplets for a fixed number of target molecules.

This precision plot was calculated using the mean confidence interval width value.
from each test. Observing this plot, the precision of the copy number estimation increases with an increase in droplet number (and reduction in the proportion of positives). For example, the average precision for the 20 µL experiments, tests 1 and 2 is ±19.7% compared to an average precision of ±11.3% for the 80 µL tests. The digital quantification results presented here confirm the devices capability of amplifying a single target in a flowing droplet and performing singleplex digital PCR. As expected, the digital PCR quantification precision is significantly increased by increasing the number of micro-reactors. Furthermore, the copy number estimations remained consistent for the same input of RNase P molecules regardless of the number of droplets in the experiment. This result is significant as the repeatable absolute quantification results verify that the droplets behave as individual micro-reactors that do not cross contaminate during amplification. However, there is a discrepancy between the estimated copy numbers and the theoretical values. The reproducibility in the digital PCR copy number estimations shown here indicate that this discrepancy may be attributed to errors arising from sample contamination, carryover contamination or inaccurate theoretical copy number calculations. These possible sources of error are discussed next.

### 7.2.4 Sources of Error

Contamination of the reagents was ruled out by running no template controls on the digital instrument and the AB7900HT qPCR platform. Contamination of the sample inlet tube, discussed previously in Chapter 6, was a suspected source of error contributing to the difference between the measured and theoretical starting copy number. As discussed earlier, wash steps were performed to reduce the risk of carryover. NTC’s were run frequently to ensure the device was free from contamination. However, the efficacy of these wash steps was difficult to quantify and some target molecules may remain bound to the sample delivery tubing. The contribution of carryover contamination is investigated in the next subsection.

Although contamination of the sample inlet line may play a role, a more likely source of error lies in the calculation of the theoretical copy number. The theoretical copy number is based on two assumptions. It is assumed that the specified gDNA stock concentration
is exactly 10ng/μL and that there are no pipetting errors associated with the preparation of
dilutions or samples. The manufacturer specifies that the gDNA concentration is between
9ng/μL and 11ng/μL. Therefore, the assumption that the stock concentration is exactly
10ng/μL is not correct, making inaccurate stock concentration a source of error. The tol-
erances specified by the manufacturer can be used to add error bars to the theoretical copy
number line. A plot of the singleplex digital PCR quantification results and the theoretical
copy number line with error bars can be seen in figure 7.9.

Pipetting inaccuracies are another possible source of error contributing to the ob-
served discrepancy between the estimated and theoretical copy number. According to
Lochner et al. (1996), mechanical air displacement pipettes often fail to deliver as accu-
rately and precisely as required. The pipetting accuracy and precision is influenced by the
operator, fluid and ambient temperatures, the fit of the consumable tip, air pressure, fluid
density, angle of pipetting, relative humidity, the pre-wetting of the tip and the pipetting
rhythm (Lochner et al., 1996).

The large number of variables influencing pipetting accuracy and precision illustrates
the high probability of errors introduced through liquid handling. Pipetting inaccuracies during the transfer of stock gDNA to the dilution aliquot would result in consistently higher or lower theoretical copy numbers. The effect of pipetting errors introduced during sample preparation is illustrated in figure 7.10.

Figure 7.10: Schematic illustrating the effect of pipetting errors on experimental results.

The preparation of the gDNA dilution is a probable contributor to the inaccuracy in the theoretical copy number. Genomic DNA stock is added to nuclease free water. The two liquid transfer steps involved in the dilution scheme may yield an inaccurate gDNA dilution. This inaccurate gDNA dilution is then carried forward to the sample preparation step, producing consistently inaccurate test reactions. The transfer step of the gDNA dilution to the reaction aliquot during sample preparation is also subject to pipetting inaccuracies, resulting in imprecision across experiments. The addition of reagent does not affect the quantity of gDNA present and hence does not influence the absolute quantification results. The theoretical copy number does not account for these pipetting errors. As a result, a difference between the theoretical starting copy number and the actual starting copy may be observed. The effect of pipetting errors on the theoretical copy number will now be examined.
Table 7.1: Maximum systemic and random errors according to ISO-8655

<table>
<thead>
<tr>
<th>Pipette Range (µL)</th>
<th>Maximum Systemic Error (µL)</th>
<th>Maximum Random Error (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 - 2</td>
<td>±0.08</td>
<td>≤0.04</td>
</tr>
<tr>
<td>1 - 10</td>
<td>±0.12</td>
<td>≤0.08</td>
</tr>
<tr>
<td>2 - 20</td>
<td>±0.20</td>
<td>≤0.10</td>
</tr>
<tr>
<td>10 - 100</td>
<td>±0.80</td>
<td>≤0.30</td>
</tr>
<tr>
<td>20 - 200</td>
<td>±1.60</td>
<td>≤0.60</td>
</tr>
<tr>
<td>100 - 1000</td>
<td>±8.00</td>
<td>≤3.00</td>
</tr>
</tbody>
</table>

The pipettes used for sample preparation were calibrated according to the maximum errors tolerated by ISO-8655. This standard specifies the technical specifications that must be met by an air displacement pipette. The maximum systemic error (pipette accuracy) and maximum random error (pipette precision) for a range of pipettes is shown in table 7.1.

These errors can be applied to the sample preparation protocol to examine the effect of pipetting inaccuracies on the theoretical copy number. As discussed earlier, the preparation of the DNA template dilution for use in the digital PCR experiments is one source of error. For example, 1µL of the 10±1ng/µL stock gDNA was added to 200µL of nuclease free water to create the DNA dilution. Applying the errors listed in table 7.1, 1±0.08µL gDNA was added to 200±1.60µL nuclease free water. Combining these volumetric errors with the stock concentration range yields a DNA dilution concentration range of 4.1-5.9pg/µL. The reaction volumes run on the continuous flow instrument contained a total of 8µL of the DNA dilution. Applying the pipetting errors, a range of 7.88-8.12µL of DNA dilution was added to the reaction. Therefore, the total DNA mass in any given reaction ranged between 0.32-0.48ng, corresponding to an RNase P copy number range of 98-148 copies. Applying these new error bars to the theoretical copy number range further strengthens the continuous flow digital PCR results, as shown in figure 7.11. Examining this figure, it is clear that the majority of the continuous flow absolute quantification results lie within the theoretical copy number range of 98-148 copies.

### 7.2.5 Absolute Quantification Results Summary

Reviewing the results from this study, it is clear that digital PCR has been successfully performed on the continuous flow instrument. Taking pipetting errors and imprecise gDNA
Figure 7.11: Plot of the estimated starting copy number and associated confidence intervals for the 20\(\mu\)L, 40\(\mu\)L and 80\(\mu\)L experiments. The theoretical copy number is shown in red. The upper and lower error bars (broken red lines) were calculated by combining the stock concentration range with pipetting errors. Tests 1-6: 20\(\mu\)L reactions. Tests 7-9: 40\(\mu\)L reactions. Tests 10-12: 80\(\mu\)L reactions.

stock into account, it is feasible that digital absolute quantification results are correct and produce a more accurate estimation of the actual starting copy number in the reactions than the theoretical calculations. The RNase P starting copy number was successfully quantified in a 20\(\mu\)L sample in 6 separate experiments. The experiments were repeated for 40\(\mu\)L and 80\(\mu\)L reactions, demonstrating reproducibility and increased precision in the copy number estimations. The variability between experiments can attributed to pipetting imprecision during the transfer of the diluted gDNA stock to the reagents. This study demonstrated successful digital PCR on the continuous flow instrument.

The study also revealed that digital PCR is sensitive to errors introduced during pipetting. The inaccuracies during sample preparation are important as the absolute quantification result is only as accurate and precise as the sample preparation procedure. This finding is significant and requires consideration during experimental design. For example, digital experiments investigating small changes in the ratio of two genes are highly sensitive to pipetting errors. Multiplex digital PCR is a possible approach to circumvent sample preparation issues and is discussed later in this chapter. A study investigating the effects of
carryover-contamination of the liquid bridge dispenser is presented in the next section.

7.3 Absolute Quantification using dPCR: Dip-Dispensing

Carryover contamination caused by wetting of the sample inlet line was identified in Chapter 6. It was thought that carryover contamination and sample preparation errors may contribute to the difference between the estimated and theoretical starting copy number shown in the previous study. An alternative approach to continuous flow sample delivery was used to establish whether the difference between the estimated and theoretical copy numbers was greatly influenced by carryover contamination. This novel droplet production technique and results from the study are examined here.

7.3.1 Experimental Design and Configuration

Carryover contamination at the liquid bridge dispenser is caused by the break down of the liquid film. The oil film wrapping a sample droplet breaks down and the sample wets the wall of the tubing for very long sample droplets, as discussed in the theory chapter. A dip-dispensing technique was used to maintain a liquid film between the sample and sample delivery tubing at all times, preventing wetting and carryover contamination. The dipping tip is raised into the oil overlay to provide separation between the sample slugs. This process was repeated generating wrapped sample slugs that do not wet the tubing wall. The dipped sample slugs flow to the liquid bridge and are dispensed into droplets. An illustration of the dip dispensing technique can be seen in figure 7.12.
A 2-fold dilution series study was performed on the instrument. Samples were prepared with final gDNA concentrations of 2.5pg/μL, 1.25pg/μL and 0.625pg/μL corresponding to 0.75 copies/μL, 0.375 copies/μL and 0.1875 copies/μL of the RNase P target. Reactions 60μL in volume were pipetted into modified 96-well polystyrene plates. Polystyrene well plates were used as polystyrene was shown to have better wetting properties than polypropylene, permitting microfluidic dipping. The dipping tip (sample inlet line) was dipped into the sample well to create 10mm droplets (~1.25μL). The 10mm droplets were then segmented into 80nL droplets. These droplets were generated at an outlet flow rate of 10μL/min and oil infuse flow rate of 6.5μL/min, giving a Q*=0.65. On average, there were 480 droplets per experiment. High flow rates were used to counter the concentration gradient which was also present in the 10mm droplets. The flow rates were reduced to thermal cycling flow rates once the sample had been segmented.

The entire well volume could not be processed due to the dead volume. The volume of sample processed in each experiment varied. Consequently, the endpoint results from each experiment were normalised by converting the estimated starting copy number to copies per unit volume. The results from this study are discussed next.
CHAPTER 7  Experimental Results

7.3.2  Dip-Dispensing Absolute Quantification Results

Each dilution experiment was performed in triplicate, yielding a total of 9 tests. Sample endpoint curves for each of the dilutions are shown in figure 7.13. The droplet trains corresponding to the dipped aqueous droplets are clearly visible. Three NTC trains were run before and after each experiment and can also be seen in the plots. These NTC trains were negative for all tests, proving that dip-dispensing prevented carryover contamination. The endpoint data was analysed using the same approach described in the previous section.
Figure 7.13: Endpoint curves for each dilution from the dip-dispensing study. (a) 0.75 copies/μL, (b) 0.375 copies/μL and (c) 0.1875 copies/μL. NTC trains are shown before and after each experiment.
The starting copy number was estimated for each test and converted to a starting target concentration. The digital PCR dilution series results for each of the tests is plotted in figure 7.14.

![Figure 7.14: Plot of the dilution factor versus copy number concentration. The theoretical and experimental plots are shown.](image)

The relationship between the dilution factor (fraction of the concentration of the first dilution) and target concentration is linear, as shown by the theoretical line in the figure 7.14. Examining figure 7.14, it is evident that the experimental target concentration estimations are generally lower than the theoretical values. The average estimated starting concentration for the first dilution was 0.51 copies/μL, significantly lower than the theoretical value of 0.75 copies/μL. The second and third dilution have an average target concentration of 0.27 copies/μL and 0.17 copies/μL, again lower than the theoretical values. Although the experimental values do not correlate well with theory, an approximate two-fold change in target concentration is observed in the experimental results. Furthermore, the experimental data tends towards the origin. These observations provide confidence in the accuracy of the experimental target concentration estimations. The difference between the theoretical and experimental data can once again be attributed to sample preparation errors or inaccurate
7.3.3 Dip-Dispensing versus Continuous Flow Dispensing

The digital PCR quantification results were found to consistently lower than the theoretical values when using the combined microfluidic dipping, liquid bridge dispensing droplet production approach. On the other hand, the singleplex continuous sample delivery study revealed consistently higher estimations. The difference in results between the two techniques will now be examined.

Carryover contamination using continuous flow liquid bridge dispensing was demonstrated in chapter 6. Target molecules are lost to the tubing wall using continuous flow sample delivery and subsequently washed away during the wash steps performed between experiments. Intuitively, lower copy number estimations are expected when using continuous flow sample delivery due to target molecule loss. Since this was not the case, it can be assumed the inaccuracies observed in the previous study using continuous flow sample delivery can be attributed to pipetting errors or inaccurate stock gDNA, not carryover contamination.

Contamination was eliminated using the combined dip-dispensing approach, as shown in the previous subsection. The difference between the theoretical and experimental results can be also be attributed to pipetting errors and inaccurate stock gDNA. It can be concluded that there are no significant advantages in using the dip dispensing approach. The benefits of using dip-dispensing to prevent carryover contamination are outweighed by the disadvantages associated with the approach. The disadvantages of the dip dispensing approach are: reduced droplet throughput, increased dead volume and reduction in droplet size consistency. As is difficult to dip for long droplets with great accuracy, the last droplet generated from a long slug is either slightly larger or smaller than the targeted droplet size. Consequently, liquid bridge dispensing with regular wash steps was used in subsequent studies.
7.4 Absolute Quantification using dPCR - Duplex dPCR

The absolute quantification studies presented in sections 7.2 and 7.3 demonstrate successful digital PCR on the continuous flow prototype. Since singleplex digital PCR has been demonstrated on the instrument, copy number ratios can easily be quantified by performing absolute quantification on two separate reactions, each containing one of the two specific assays of interest. The copy number ratio can then be determined by comparing the two absolute quantification results. However, these experiments are subject to the sample preparation inaccuracies identified earlier.

Biological applications quantifying extremely small changes in copy number ratio are highly sensitive to these sample preparation errors. As a result, absolute quantification of two targets in the same reaction should be used to accurately quantify the copy number ratio. The copy number ratio in a duplex experiment is unaffected by sample preparation inaccuracies - the template quantity may be inaccurate and imprecise but the ratios remain the same. This section investigates duplex digital PCR on the continuous flow prototype.

7.4.1 Experimental Design and Configuration

Taqman® RNase P FAM and SRY VIC assays were used in the duplex digital PCR study. As discussed in Chapter 2, there are twice as many RNase P copies as SRY copies in gDNA. Reactions were prepared with a final gDNA concentration of 0.003ng/μL, corresponding to 0.91 RNase P copies/μL and 0.45 SRY copies/μL. Samples 100μL in volume were prepared and pipetted into modified polystyrene well plates. The sample was then continuously drawn and segmented using the liquid bridge dispenser, processing 60μL of the reaction on average.

Samples were dispensed at an outlet flow rate of 20μL/min and oil infuse flow rate of 15μL/min, yielding a Q* equal to 0.75 and a droplet size of 70nL. There was an average of 860 droplets per experiment. The outlet flow rate was then reduced to the thermal cycling flow rate of 3μL/min once the reaction had been segmented. The absolute quantification result was converted to copies/μL since the processed sample volume varied for each experiment. The results from the duplex digital PCR study are discussed next.
7.4.2 Duplex dPCR Results

The duplex digital PCR experiment was repeated four times. Dye calibration was used to correct for FAM, VIC and ROX contributions, as discussed in Chapter 5. Dye calibration and normalisation was performed for each experiment. The data was then thresholded, producing a binary output signal.

The RNase P and SRY copy number estimations were converted to copies/µL and are plotted in figure 7.15. Examining these plots, the RNase P estimations are consistently above the theoretical RNase P concentration. The SRY copy number concentrations are also inaccurate but less precise. This results in imprecise, inaccurate experimental RNase P to SRY copy number ratios. The data from the study is summarised in table 7.2.

Figure 7.15: Estimated copy number concentrations for each of the four experiments from the duplex digital PCR study. (a) RNase P FAM concentration, (b) SRY VIC concentration and (c) copy number ratio. The theoretical value for each case is shown by the solid red line.
The RNase P digital quantification data is consistent with the singleplex RNase P study, indicating that the inaccurate experimental ratios were not caused by the RNase P assay. The estimated ratio of RNase P to SRY was generally greater than the theoretical ratio of 2:1. This was not expected, as the ratio is unaffected by sample preparation errors. This indicated that there was an issue with the SRY assay. It was thought that amplification efficiency of the SRY assay was reduced in the duplex reaction. A study was undertaken on the commercial qPCR instrument to investigate the effect of running the assays in singleplex and duplex. The results from this study are examined in the next subsection.

<table>
<thead>
<tr>
<th>Test Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical RNase P Concentration (copies/μL)</td>
<td>0.91</td>
<td>0.91</td>
<td>0.91</td>
<td>0.91</td>
</tr>
<tr>
<td>Theoretical SRY Concentration (copies/μL)</td>
<td>0.45</td>
<td>0.45</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Theoretical Ratio</td>
<td>2:1</td>
<td>2:1</td>
<td>2:1</td>
<td>2:1</td>
</tr>
<tr>
<td>Experimental RNase P Concentration (copies/μL)</td>
<td>1.31</td>
<td>1.20</td>
<td>1.23</td>
<td>1.11</td>
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<tr>
<td>Experimental SRY Concentration (copies/μL)</td>
<td>0.42</td>
<td>0.73</td>
<td>0.36</td>
<td>0.27</td>
</tr>
<tr>
<td>Experimental Ratio</td>
<td>3.1:1</td>
<td>1.65:1</td>
<td>3.4:1</td>
<td>4.11:1</td>
</tr>
</tbody>
</table>

### 7.4.3 Duplex Benchmark Study on the qPCR Instrument

Given successful singleplex digital PCR, the inconsistent results from the duplex digital PCR study indicated a problem amplifying both the RNase P and SRY targets in the same droplet. A study was performed on the AB7900HT qPCR instrument to investigate whether the inaccurate digital PCR duplex results were unique to the prototype instrument.

Singleplex RNase P FAM and singleplex SRY VIC reactions were prepared with a final gDNA concentration of 1ng/μL. Duplex reactions containing the RNase P FAM and SRY VIC assays were also prepared with a final gDNA concentration of 1ng/μL. Since the ratio of RNase P to SRY is 2:1, a \( \Delta C_q \) difference (\( \Delta C_q \)) of 1 was expected between the RNase P and SRY s-curves for both the singleplex and duplex experiments. The reactions for each experiment were run in triplicate. NTC’s were also prepared and run on the same plate to eliminate contamination as a source of error.

The amplification plots for the singleplex and duplex experiments can be seen in figure 7.16. Observing figure 7.16 (a), it is clear that the singleplex reactions have amplified as
expected with an average $\Delta C_q = 0.98$ between the RNase P and SRY reactions. However, running the assays in duplex yields a $\Delta C_q = 1.85$, as shown in figure 7.16 (b). Running both assays in the same well causes a shift in $C_q$ values, with the duplex SRY VIC crossing the fluorescence threshold at a later cycle than the singleplex SRY VIC reaction.

Figure 7.16: qPCR amplification plots for the singleplex (a) and duplex (b) experiments performed on the AB7900HT qPCR instrument.

The average $C_q$ and $\Delta C_q$ values for each of the experiments are presented in table 7.3.
Examining the table, the RNase P duplex $C_q$ is slightly lower than the RNase P singleplex $C_q$. More significantly, the SRY duplex crosses the threshold half a cycle later than the SRY singleplex, indicating that the RNase P target is preferentially amplified over the SRY target. These experiments demonstrate that the inaccurate results obtained from the duplex digital PCR study performed on the continuous flow instrument were caused by a chemistry issue.

Table 7.3: Data obtained from the singleplex and duplex experiments performed on the commercial qPCR instrument.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Average $C_q$ (n=3)</th>
<th>Standard Deviation</th>
<th>$\Delta C_q$, expected</th>
<th>$\Delta C_q$, experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase P FAM</td>
<td>26.49</td>
<td>0.03</td>
<td>1</td>
<td>0.98</td>
</tr>
<tr>
<td>SRY VIC</td>
<td>27.47</td>
<td>0.03</td>
<td>1</td>
<td>0.98</td>
</tr>
<tr>
<td>Duplex FAM</td>
<td>26.19</td>
<td>0.01</td>
<td>1</td>
<td>1.85</td>
</tr>
<tr>
<td>Duplex VIC</td>
<td>28.04</td>
<td>0.02</td>
<td>1</td>
<td>1.85</td>
</tr>
</tbody>
</table>

According to Elnifro et al. (2000), multiplex PCR can pose difficulties such as preferential amplification. The shift in the $\Delta C_q$ value observed in the duplex qPCR experiments and in the duplex digital PCR study may be attributed to the problems associated with multiplex PCR. The complications in multiplexing are described briefly in the following paragraphs.

Elnifro et al. (2000) state that the presence of more than one primer pair increases the chances of obtaining non-specific amplification products due to primer-dimer formation. Non-specific products may amplify more efficiently than the target sequences, consuming reagents and yielding reduced rates of annealing and extension, resulting in reduced amplification efficiency or one or both targets. Special attention should be made in primer design to ensure similar amplification efficiency for their respective targets and to minimise non-specific amplification (Elnifro et al., 2000).

Preferential amplification of a target sequence over another is a well documented phenomenon in multiplex PCR (Polz & Cavanaugh, 1998; Multer & Boynton, 1995). According to Wagner et al. (1994), there are two processes that induce bias in amplification - PCR selection and PCR drift. PCR selection is preferential amplification of a target due to the properties of the target and choice of primers (Elnifro et al., 2000). PCR drift is due to random fluctuations in the interactions of PCR reagents at early cycles and is most likely
to arise in the presence of very low template concentrations (Multer & Boynton, 1995).

Taking the issues associated with multiplex PCR into consideration along with the results from the duplex qPCR study, the erroneous duplex digital PCR results can be attributed to a poorly optimised duplex assay. The consistent digital quantification of the RNase P target in duplex indicates that the RNase P target was preferentially amplified to the SRY gene. This produced highly inaccurate gene expression ratios in the duplex digital PCR study. Development of suitable primer pairs for duplex digital PCR was not within the scope of this project. However, this study demonstrates the duplex or multiplex assay optimisation is an important consideration for future multiplex PCR experiments.

7.5 Instrument Performance

Digital PCR has been successfully demonstrated on the continuous flow instrument. The instrument was shown to generate accurate and precise copy number estimations. The dynamic range, precision and time to answer are important performance parameters of a digital PCR instrument and are examined in this section.

Firstly, the dynamic range of the continuous flow instrument is described in this section. The theoretical derivation of digital PCR dynamic range was presented in chapter 3. A wide dynamic range is advantageous as a digital instrument can perform absolute quantification for a wide range of starting target molecules. The precision of the instrument is then briefly discussed. Following this, the time to answer of a continuous flow instrument is examined. Finally, the feasibility of continuous flow technology for digital PCR is discussed. Limitations in the current design are presented and possible design changes are proposed to improve the instruments performance.

7.5.1 Dynamic Range

The continuous flow digital PCR instrument is not constrained to a fixed number of micro-reactors like microfluidic chip based digital PCR instruments. Theoretically, an infinite number of droplets or micro-reactors can be generated using continuous flow technology yielding an infinite dynamic range. The number of droplets that can be generated in an
experiment is limited only by the volume of sample available to be processed. Additional reagent can be added to a reaction to yield a greater number of droplets and increased dynamic range.

For a fixed reaction volume, the number of droplets that can be generated is controlled by the droplet volume. Therefore, the dynamic range of a continuous flow instrument is best visualised plotted against the droplet volume. A plot illustrating the dynamic range of a continuous flow instrument for various reaction volumes is shown in figure 7.17. The smallest droplet volumes generated on the continuous flow instrument were in the order of 50nL. Using this droplet volume, the dynamic range for various reaction volumes can be determined using the continuous flow dynamic range plot.

![Dynamic Range Diagram](image)

Figure 7.17: Dynamic range of a continuous flow instrument. Dynamic range versus droplet volume for various reaction volumes.

For a 20µL reaction, the dynamic range of the prototype is approximately $10^3$ molecules. For a 80µL reaction, the largest reaction volume processed on the instrument in this study,
the dynamic range increases to $0.8 \times 10^4$. It is evident that the dynamic range of the continuous flow instrument under investigation is limited. Methods of increasing the dynamic range of the continuous flow instrument using these curves is examined next.

Observing figure 7.17, it is clear that although increasing the reaction volume increases the dynamic range, greater rewards in dynamic range are achieved by using smaller micro-reactor volumes. Droplet’s 50nL in volume give a dynamic range of approximately $1.5 \times 10^3$ molecules for a $20\mu$L reaction volume. Increasing the reaction volume to 2mL for this droplet size increases the dynamic range to $3.3 \times 10^5$. On the other hand, reducing the droplet volume to 5nL increases the dynamic range to $1.5 \times 10^5$ for a $20\mu$L reaction. Sub-nanolitre droplets would provide a dynamic range similar to qPCR from a standard 20µL reaction volume. These droplet volumes are difficult to achieve using continuous flow technology, as discussed earlier in the device design chapter. For this instrument design, increasing the reaction volume will provide the maximum dynamic range.

### 7.5.2 Precision

The maximum precision of an instrument is influenced by the total number of micro-reactors, as shown in the theory chapter. For a continuous flow instrument, the total number of droplets is limited by the reaction volume available. As a result, highly precise estimations can be produced using large sample volumes. The maximum estimation precision in this study was $\pm 11.3\%$. This was achieved by generating 1400 droplets from a 80µL reaction. Clearly, the maximum precision that can be achieved can be increased by increasing the number of droplets. This requires large reaction volumes for the current droplet sizes generated on the instrument.

### 7.5.3 Time to answer

The time to answer is an important performance characteristic of a digital PCR instrument. Biological studies typically require a number of experimental replicates or large droplet number experiments to provide confidence in the quantification results. A short run time is advantageous as many quantitative answers - the equivalent to $C_q$’s in a qPCR experiment,
can be processed within a short time frame. The time to answer of a continuous flow digital PCR instrument has been discussed in chapter 4. The time to answer is proportional to $\frac{N_d}{\phi}$, the number of droplets divided by the droplet frequency or throughput. This means that experiments requiring many droplets may take a number of hours to complete if the throughput is not sufficiently high. The relationship between time to answer, droplet number and droplet throughput is explored here. The time to answer achieved on the digital PCR instrument is discussed, revealing significant limitations in time to answer for low droplet throughput’s.

The maximum droplet throughput achieved in this body of work was approximately 1,000 droplets/hour. Using the equations described in chapter 4, the time to answer for a 1,000 droplet study is 2 hours. A 10,000 droplet experiment - an experiment which offers a medium to low dynamic range and precision requires 11 hours to generate an answer at this droplet throughput. As a result, a large study may require a number of days to complete.

A plot of the time to answer versus droplet number for various droplet throughput’s, shown in figure 7.18, demonstrates the limitations in time to answer associated with low droplet throughput’s. This plot was constructed to determine the time to answers achievable for various droplet throughput’s for a short time to answer. Observing figure 7.18, an instrument with a droplet throughput of 80,000 droplets/hour requires just over an hour to obtain an answer for a 10,000 droplet experiment. The same droplet number experiment requires 10 hours for a droplet throughput of 1,000 droplets/hour. A 10 hour time to answer is unacceptable for a digital instrument. Although high droplet throughput’s offer shorter time to answers, the rewards in time to answer are less significant for very large experiments. For example, a 1,000,000 ($10^6$) droplet experiment requires over 10 hours for a droplet throughput of 80,000 droplets/hour.
It is clear that continuous flow instruments are not suitable for very large large droplet number experiments. The technology is best placed to perform medium to low droplet number experiments, offering accurate and sensitive quantification at a low cost. The findings from these performance indicators are used to evaluate continuous flow technology for digital PCR, discussed in the next subsection.

### 7.5.4 Evaluation of Continuous Flow Technology for Digital PCR

This study has revealed that there is a trade-off between the time to answer and the dynamic range/estimation precision on a continuous flow instrument. The larger the experiment (and hence the dynamic range and maximum precision), the longer it takes to obtain an answer. Current commercial instruments target a time to answer of less than 3 hours. Micro-reactors are simultaneously cycled and detected on array based formats, resulting in a time to answer approximately equal to the thermal cycling time.

The current instrument throughput of 1,000 droplets/hour is not suitable for large
droplet number experiments. Running lines in parallel will increase the throughput but using additional lines increases the complexity and cost of the instrument. A longer thermal cycler would permit faster outlet flow rates thus increasing the throughput and reducing the time to answer. This may require switching to positive pressure pumping to avoid the undesirable effects of large pressure drops when pumping under negative pressure.

The serial nature of continuous flow PCR makes the technology unsuitable for large droplet number experiments. As a result, the technology is best placed to perform digital PCR for applications requiring quantification of rare targets in large sample volumes. The flexibility in droplet production permits processing of large sample volumes quickly and cheaply versus commercial digital PCR platforms which require numerous consumable chips or millions of unnecessary low volume droplets. Nevertheless, design changes should be implemented to increase droplet throughput and reduce the time to answer.

7.6 Chapter Close

This chapter has demonstrated successful singleplex absolute quantification on the continuous flow digital PCR instrument. Single molecule sensitivity was demonstrated. Singleplex digital PCR was performed on various reaction volumes and concentrations. The estimations were show to be accurate and repeatable. Increased estimation precision was demonstrated for larger droplet number experiments. Significant sources of error were identified and noted for future consideration.

An alternative liquid bridge configuration was used to show that the discrepancies between the theoretical and experimental copy number was not due to carryover contamination. Duplex digital PCR was performed on the continuous flow instrument, demonstrating the requirement for an optimised duplex assay when performing duplex digital PCR.

The performance of the instrument has also been examined in this chapter. The dynamic range, estimation precision and time to answer of the continuous flow platform has been discussed. Design changes have been proposed to increase the droplet throughput of the continuous flow instrument. The recommended design changes are discussed further in
the next chapter. It was concluded that continuous flow technology is suited to quantification of rare targets in large sample volumes. For these applications, the technology offers superior flexibility, speed and reduced cost compared to current commercial digital PCR platforms.
Chapter 8

Conclusions and Recommendations

The primary aim of this project was to perform digital PCR using a unique continuous flow platform that does not require any consumable components. In order to achieve this objective, a continuous flow digital PCR instrument was designed, fabricated and characterised. Using this novel technology, droplets were generated, cycled and detected in a continuous fashion compared to conventional droplet based systems which employ a work-flow that requires consumables for droplet generation, thermal cycling and droplet detection. A number of digital PCR studies were performed on the instrument, demonstrating successful digital PCR in flowing droplets. This chapter presents the conclusions from this work. Following this, recommendations for future work are proposed.

8.1 Conclusions

- The theory governing the performance of a continuous flow instrument has been examined, revealing limitations in droplet throughput due to large pressure drops in a microfluidic network when pumping under negative pressure.

- An endpoint fluorescence algorithm was developed. This algorithm was shown to be capable of generating a binary output from the endpoint fluorescence signal. The endpoint fluorescence data is imported and plotted. The signal is cleaned of low level noise and the droplet peak values are extracted. The peak values are dye calibrated, normalised and thresholded, producing a binary output signal consisting of
the number of positive droplets and the total number of droplets.

- GAPDH and RNase P targets were successfully amplified on the instrument. Sequence specific amplification was verified using gel electrophoresis.

- Liquid bridge dispensing was shown to be a suitable droplet production technique for continuous flow digital PCR. This droplet generation method is capable of producing highly consistent low volume micro-reactors. Reaction volumes were dispensed into droplets volumes as low as 45nL and processed on the instrument. This dispensing technique was shown to be advantageous as it offers flexibility in the size of the droplets generated.

- Continuous phase sample delivery to the liquid bridge dispenser resulted in carry-over contamination on the instrument. This was eliminated by implementing a dip dispensing approach or wash steps between samples.

- The digital PCR studies demonstrated the instruments capability of single molecule amplification and detection in flowing droplets.

- Singleplex digital PCR was successfully performed on the continuous flow platform. The singleplex quantification results correlated extremely well with the theoretical concentration when taking sample preparation errors and the precision of the stock gDNA into account. The instrument accurately quantified as little as 0.178 copies/μL (178 copies/mL) of the RNase P gene in gDNA. The starting target concentration quantified on the device ranged from 0.178 copies/μL to 6.1 copies/μL. Digital PCR was performed on gDNA dilutions in various sample volumes, demonstrating highly repeatable, accurate and precise quantification.

- Duplex digital PCR was performed on the instrument. Although both the RNase P and SRY targets were successfully amplified in droplets, the copy number ratio quantification results were highly inaccurate, deviating from the expected 2:1 copy number ratio. A separate study on a commercial qPCR instrument demonstrated that these inaccuracies were caused by poor duplex assay optimisation and not related to instrument performance.
This body of work has demonstrated successful digital PCR on a continuous flow platform. To the best of the authors knowledge, this is the first time absolute quantification using digital PCR has been performed using a continuous flow instrument. This research has demonstrated that continuous flow technology is suited to quantification of rare targets in large sample volumes. The technology offers flexibility in droplet production, permitting quick and low cost quantification compared to current commercial digital PCR platforms which would require numerous high cost consumable chips.

8.2 Recommendations for future work

- Add a real time detection system to allow fluorescence monitoring cycle to cycle. This would aid in the discrimination between positive and negative signals, reducing uncertainty in noisy endpoint results. This would also permit qPCR, increasing the dynamic range of the instrument.

- Design a liquid bridge dispenser with a disposable sample delivery capillary. This would eliminate carryover contamination between samples without using wash steps.

- Determine the minimum dwell times required for flowing droplets. It is thought that reduced thermal cycling times are possible using continuous flow PCR. Identification of minimum dwell times could reduce the thermal cycling time, reducing the time to answer.

- Redesign the thermal cycler to permit higher droplet throughputs. Increasing the heating zone lengths would permit faster flow rates and higher throughputs. This design change would permit short time to answers for large droplet number experiments.

- Redesign the thermal cycler to yield smaller droplet sizes. Reducing the tubing diameter would permit small droplet volumes, increasing the dynamic range of the instrument for a given reaction volume.
• Investigate digital PCR on a multi-line system. Running lines in parallel greatly increases the throughput, further reducing the time to answer.

• Employ positive pressure pumping on the continuous flow digital PCR instrument. The recommended design changes greatly increase the pressure drop in the system. Positive pressure pumping could be used to overcome the problems posed by large pressure drops. However, positive pressure pumping complicates sample loading. Methods of automating sample loading under positive pressure pumping need to be investigated.

• Perform duplex digital PCR using an optimised duplex assay to assess the instruments capability of duplex digital PCR.
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Appendix A

Engineering Drawings
Figure A.1: Liquid Bridge Dispenser Frame
Figure A.2: Liquid Bridge Dispenser Base
Figure A.3: Liquid Bridge Dispenser Cap
Figure A.4: Thermal Cycler Blocks
Appendix B

MATLAB Algorithms

B.1 Endpoint Signal Analysis

% Showing a single detector only (FAM) as an example
    % % 1. Import data, plot data and count total number of droplets
    % Import data and load to the workspace
    FAMdata = importdata(FAM_filename);
    FAM_fd = FAMdata(:,3); % FAM fluorescence data
    FAM_td = FAMdata(:,2); % FAM time data
    % Plot time versus raw FAM fluorescence intensity data
    plot(FAM_td, FAM_fd)
    % Label axis
    xlabel('Time (s)')
    ylabel('Fluorescence Intensity (a.u.)')
    % Create binary signal, differentiate and count no. droplets
    ddetection = value; % enter droplet detection value
    bsig = FAM_fd>ddetection; % create binary signal
    der_bsig = diff(bsig); % find derivative of binary signal
    Nd = sum(der_bsig==1); % count no. of droplets
    % % 2. Background subtraction and signal clean up
    % Subtract background data
FAM_fd = FAM_fd-mode(FAM_fd);
noise_value_FAM = value; % Select value above noise FAM signal
FAM_bsig_noise = FAM_fd>noise_value_FAM; % Create binary signal
FAM_clean = FAM_bsig_noise.*FAM_fd; % Multiply data by binary to give noise free signal

% Plot background substracted, noise reduced fluorescence data
figure
plot(FAM_td,FAM_clean);
xlabel('Time (s)')
ylabel('Fluorescence Intensity (a.u.)')

%% 3. Droplet peak detection and extraction
%
% Find start and end of peaks
sp = find(der_bsig==1); %Start of peaks
ep = find(der_bsig==-1); %End of peaks
nd = length(sp); %number of droplets
intv = cell(1,Nd); % create cell intv containing the intervals to search
pkrox = cell(1,Nd);
for i = 1:Nd intv{i} = [sp(i) ep(i)]; % creates droplet intervals
[pkfam{i} pkfami{i}] = max(FAM_clean(intv{i}(1):intv{i}(2))); % finds peak values on the above intervals
pkfami{i} = pkfami{i}+intv{i}(1)-1;
end
FAM_peaks = cell2mat(pkfam); % create array containing FAM peak values

%% 4. Calibrate and normalise peak values
%
% Skip this step if single detector used
% Dye calibrate peak values using the calibration matrix A
FAM_corrected=[A(1,1)*FAM_peaks]+[A(1,2)*VIC_peaks]+[A(1,3)*ROX_peaks];
VIC_corrected=[A(2,1)*FAM_peaks]+[A(2,2)*VIC_peaks]+[A(2,3)*ROX_peaks];
ROX_corrected=[A(3,1)*FAM_peaks]+[A(3,2)*VIC_peaks]+[A(3,3)*ROX_peaks];
%
% Normalise peak values
FAM_norm = FAM_corrected./ROX_corrected;
%% 5. Threshold data and droplet counting
% Create peak value histogram
hist(FAM_norm,100)
% Threshold data using Otsu’s method
thresh = graythresh(uint8(FAM_norm))*256;
Np=sum(FAM_norm>thresh); % Count number of positive droplets
% Create scatter plot of peak fluorescence intensity values
figure
plot(FAM_norm, '. ')
xlabel('Droplet Number')
ylabel('Fluorescence Intensity (a.u.)')

%% 6. Generate copy no. estimation and 95% confidence intervals
phat=Np/Nd;
sd=sqrt((phat.*(1-phat))./(nd));
zc=1.96; % Z score for 95% confidence intervals
phatlow=phat-1.96*sd;
phathigh=phat+1.96*sd;
ghat=-log(1-phat);
ghathigh=-log(1-phathigh);
ghatlow=-log(1-phatlow);
% Estimate copy number and 95% confidence intervals
Elow=ghatlow*nd;
Emean=ghat*nd;
Ehigh=ghathigh*nd;

B.2 Theoretical Precision Plot

% Plot Precision Versus No. Target Molecules for various droplet numbers
%% 1. First find dynamic range of for each droplet number instrument
% m = N.*log(N./10)
nd1=1000;
nd2=2500;
nd3=5000;
nd4=10000;
nd5=20000;

% Dynamic range
a1=nd1.*log(nd1./10);
a2=nd2.*log(nd2./10);
a3=nd3.*log(nd3./10);
a4=nd4.*log(nd4./10);
a5=nd5.*log(nd5./10);

%% 2. Determine Binomial Response using Poisson Distribution for each case
% Determine the expected number of positives
nc1=[1:2:a1];
nc2=[1:5:a2];
nc3=[1:100:a3];
nc4=[1:10:a4];
nc5=[1:10:a5];
ppos1=1-exp(-(nc1/nd1));
ppos2=1-exp(-(nc2/nd2));
ppos3=1-exp(-(nc3/nd3));
ppos4=1-exp(-(nc4/nd4));
ppos5=1-exp(-(nc5/nd5));

% Expected number of positives
np1=ppos1.*nd1;
np2=ppos2.*nd2;
np3=ppos3.*nd3;
np4=ppos4.*nd4;
np5=ppos5.*nd5;

%% 3. Estimate Starting Copy Number and confidence Intervals.
% First, calculate the point estimate.
%probability part
pe1 = np1/nd1;
pe2 = np2/nd2;
pe3 = np3/nd3;
pe4 = np4/nd4;
pe5 = np5/nd5;

%conc estimation part
ge1 = -log(1-pe1);
ge2 = -log(1-pe2);
ge3 = -log(1-pe3);
ge4 = -log(1-pe4);
ge5 = -log(1-pe5);

% convert to copy no.
cn1 = ge1.*nd1;
cn2 = ge2.*nd2;
cn3 = ge3.*nd3;
cn4 = ge4.*nd4;
cn5 = ge5.*nd5;

% low CI prob part
plow1 = pe1 - 1.96.*sqrt(pe1.*(1-pe1)./(1-pe1)./nd1);
plow2 = pe2 - 1.96.*sqrt(pe2.*(1-pe2)./(1-pe2)./nd2);
plow3 = pe3 - 1.96.*sqrt(pe3.*(1-pe3)./(1-pe3)./nd3);
plow4 = pe4 - 1.96.*sqrt(pe4.*(1-pe4)./(1-pe4)./nd4);
plow5 = pe5 - 1.96.*sqrt(pe5.*(1-pe5)./(1-pe5)./nd5);

% low CI conc part
glow1 = -log(1-plow1);
glow2 = -log(1-plow2);
glow3 = -log(1-plow3);
glow4 = -log(1-plow4);
glow5 = -log(1-plow5);
% convert to copy no
cl1 = glow1.*nd1;
cl2 = glow2.*nd2;
cl3 = glow3.*nd3;
cl4 = glow4.*nd4;
cl5 = glow5.*nd5;

% high CI prob part
phigh1 = pe1 + 1.96.*sqrt(pe1.*(1-pe1)./nd1);
phigh2 = pe2 + 1.96.*sqrt(pe2.*(1-pe2)./nd2);
phigh3 = pe3 + 1.96.*sqrt(pe3.*(1-pe3)./nd3);
phigh4 = pe4 + 1.96.*sqrt(pe4.*(1-pe4)./nd4);
phigh5 = pe5 + 1.96.*sqrt(pe5.*(1-pe5)./nd5);

% high CI conc part
ghigh1 = -log(1-phigh1);
ghigh2 = -log(1-phigh2);
ghigh3 = -log(1-phigh3);
ghigh4 = -log(1-phigh4);
ghigh5 = -log(1-phigh5);

% convert to copy no
ch1 = ghigh1.*nd1;
ch2 = ghigh2.*nd2;
ch3 = ghigh3.*nd3;
ch4 = ghigh4.*nd4;
ch5 = ghigh5.*nd5;

% % 4. Calculate the Precision
% first calculate the mean CI length
w1 = ((cn1-cl1)+(ch1-cn1))./2;
w2 = ((cn2-cl2)+(ch2-cn2))./2;
w3 = ((cn3-cl3)+(ch3-cn3))./2;
w4 = ((cn4-cl4)+(ch4-cn4))./2;
w5 = ((cn5-cl5)+(ch5-cn5))/2;

% now calculate precision
prec1 = (w1./cn1)*100;
pred2 = (w2./cn2)*100;
pred3 = (w3./cn3)*100;
pred4 = (w4./cn4)*100;
pred5 = (w5./cn5)*100;

% Plot Precision versus Copy Number
plot(nc1,prec1)
hold all
plot(nc2,prec2,'r')
plot(nc3,prec3,'g')
plot(nc4,prec4,'y')
plot(nc5,prec5,'k')
set(gca,'YDir','reverse');
xlabel('Number of Target Molecules')
ylabel('Precision pm%')
legend('1,000','2,500','5,000','10,000','20,000')
Appendix C

Droplet Boiling

C.1 Droplet Boiling

C.1.1 Description

Droplet boiling was occasionally observed during experimental testing of the continuous flow digital PCR instrument. Gas formation due to droplet boiling was observed as the droplets were pumped through the thermal cycler. The formation and subsequent expansion and contraction of gas disrupted the outlet flow rate, resulting in varying dwell times and inconsistent droplet sizes leading to experimental failure. This highly undesirable effect was exacerbated by negative pressure pumping and the large pressure drop associated with large droplet number experiments, as discussed in chapter 3. The low pressures in the thermal cycler induce boiling at lower temperatures, resulting in increased frequency of gas formation and test failure. The droplet boiling frequency was found to be extremely inconsistent for fixed experimental conditions, making it difficult to understand and solve. The problem slowed the generation of good quality digital PCR data on the continuous flow digital PCR instrument. Consequently, a number of approaches aimed at characterising droplet boiling were investigated during the course of this research. These studies are briefly discussed in this section. Firstly, the effect of changing the pumping mode from negative pressure pumping is examined. The frequency of droplet boiling is then examined for various droplet and carrier liquids. Finally, the effect of temperature on droplet boiling...
is discussed.

C.1.2 Positive Pressure Pumping

Droplets were dispensed under negative pressure pumping in a long length of tubing, preventing the droplets from entering the thermal cycler. The pumps were stopped and the downstream capillary was then disconnected from the pump. The pump was connected to the upstream capillary, allowing the droplets to be pumped under positive pressure. In this experimental setup, the pressure gradient ran from high at the pump to atmospheric at the outlet, circumventing any issues induced by the low pressures when pumping under negative pressure. Although droplet boiling frequency appeared reduced using this approach, droplet boiling was observed. This indicated that boiling was affected by large pressure drops in the system. However, since boiling was observed when pumping under positive pressure the issue could not be entirely attributed to large pressure drops.

C.1.3 Droplet and Carrier Oil Liquids

The droplet liquid was changed to investigate whether droplet boiling was influence by the liquid type. The following droplet liquids were tested:

- Distilled water
- RNase free water
- RNase free water + template + gene assay
- TaqMan GeneExpression MasterMix
- TaqMan GeneExpression MasterMix + RNase free water
- Full reactions (MasterMix, water, assay + template)

Droplet boiling was observed for each of these droplet liquids using PD5 silicone oil as the carrier fluid. The carrier oil was also changed from PD5 to (a) AS-100 silicone oil and (b) AS-4 silicone oil to ensure that droplet boiling was not caused by an interaction between
the droplet and carrier oil. Droplet boiling was observed for both oil types, ruling out any influence of the oils on boiling.

C.1.4 Sample Preparation

The introduction of dissolved gases during sample preparation was proposed as a possible factor influencing droplet boiling and gas production. Samples were prepared and then subjected to degasification in a vacuum chamber. The degassed samples were then processed on the continuous flow digital PCR instrument. This was found to somewhat reduce the frequency of droplet boiling events, indicating that lowering the amount of dissolved gases aids in reducing boiling events and subsequent experiment failure rate.

C.1.5 Removal of Preheat

It was found that droplet boiling only occurred within the thermal cycler blocks as the flowing droplets were heated. The enzyme activation block was used to heat the droplets to 95°C for 10 minutes. The majority of observed droplet boiling cases occurred within this heating zone, with some droplet boiling occurring within the thermal cycling stage of PCR. The prolonged heating of droplets at 95°C for 10 minutes was a cause for concern as it is close to the boiling point of the droplet. The preheat stage was removed, resulting in a more favourable thermal cycling profile of 95°C for 15 seconds, 60°C for 60 seconds for a total of 40 cycles. This was shown to dramatically reduce the frequency of droplet boiling to a point at which the instrument could be reliably run. Although droplet boiling was effectively eliminated when operating under these conditions, removing the preheat is not a desirable solution. Removing the preheat step limits the instrument to ‘fast’ chemistries that do not require enzyme activation, limiting the flexibility of the instrument. An alternative solution that prevents droplet boiling is desirable and should be sought out. This requires significant research effort and was not within the scope of this project.