Cardiff University

School of Biosciences

Low Copy Number Quantification of DNA Utilising Loop-mediated Amplification (LAMP) with Bioluminescent Assay in Real-Time (BART) Reporter

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Preface

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To my family

Acknowledgements

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Abstract

Low Copy Number Quantification of DNA Utilising Loop-mediated Amplification (LAMP) with Bioluminescent Assay in Real-Time (BART) Reporter

Real time quantitative PCR is the benchmark technology of molecular diagnostics in a wide range of fields including forensic science, clinical diagnosis and the detection of genetically modified (GM) crops. There is a requirement for rapid, cheap and simple portable quantitative and specific diagnostics. Quantitative PCR is limited by a number of factors in this regard: the complex hardware is often expensive and largely laboratory limited.

Bioluminescent Assay in Real Time (BART) is a nucleic acid amplification detection system that converts inorganic pyrophosphate (PPi), a by-product of DNA synthesis, into light output. The pyrophosphate is converted into ATP which is utilised by a thermostable luciferase to convert luciferin to oxyluciferin with the emission of light. The development of isothermal amplification techniques that use the strand displacement properties of certain DNA polymerases enables the BART detection to be utilised in simple and cheap hardware at a single temperature. Loop-mediated amplification (LAMP) is an isothermal amplification method which is highly specific to the DNA target sequence and produces high concentrations of PPi.

Factors influencing the LAMP-BART detection of transgenic elements in genomic and linearised plasmid DNA have been investigated. At low target copy number, carrier DNA in the reaction mix had a positive influence on assay kinetics. Other components of the LAMP-BART mix and the assay parameters were optimised for the 35Sp LAMP-BART assay of linearised pART7 plasmid DNA targeting the 35S promoter. This assay was used to achieve single copy detection and methods were developed to determine individual copy numbers by ultra-quantification. Single copy detection with this assay enabled investigations using digital PCR tools into absolute quantification. The combination of quantitation using average time-to-peak to approximately 20 copies, with ultra-quantification to single copies and digital BART for sub-single copies per partition, shows the potential for full dynamic range quantification using LAMP-BART.

Abbreviations

ADH1	Alcohol dehydrogenase 1 gene
APS	Adenosine 5’ phosphosulphate
BART	Bioluminescent Assay in Real Time
CaMV 35Sp	Cauliflower mosaic virus 35S promoter
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dNTP	Deoxyribonucleotide
ELIDA	Enzymatic luminometric inorganic pyrophosphate detection assay
FWHM	Full width half maximum
GMO	Genetically modified organism
HPLC	High performance liquid chromatography
LAMP	Loop-mediated amplification
LH_2	Luciferin
Luc	Luciferase
MGW	Molecular grade water
NOSt	Nopaline synthase terminator
NTC	No template control
PCR	Polymerase chain reaction
PP_i	Inorganic pyrophosphate
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RLU	relative luminescent units
T_{max}	Time to peak

Further abbreviations follow the guideline described in the Nomenclature, Style and Conventions section in Biochemical Journal Instructions to Authors, The Biochemical Society, London © 2014

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Chapter 1
Introduction

1.1 Molecular diagnostics

The molecular diagnostics industry has been well established for over thirty years and continues to grow with the advent of novel approaches to the detection of biological markers. These markers include proteins, antigens and nucleic acids. In recent years, molecular diagnostics have become increasingly important for the identification of specific nucleic acid targets in a wide range of fields including infectious disease testing, identification of genetically modified (GM) crops and short tandem repeats (STRs) and single nucleotide polymorphisms (SNPs) in forensic science. The markets for the development of these products have been driven by a requirement for low cost, rapid testing, high specificity and portability. There is also a need for quantitative molecular diagnostics for calculating, for example, viral load determination and assessing the concentration of genetically modified material in a food source. For a number of years, quantitative real time PCR has been the technique of choice for this, but there are a number of emerging technologies to challenge this dominance (Morisset D et al. 2008); (Fakruddin et al. 2013); (Asiello and Baeumner 2011).

1.1.1 Molecular diagnostics of GM crops

Quantitative molecular diagnostics are required to assess the concentration of genetically modified material in food sources. Within the European Community this is due to current regulations that necessitate the labelling of food products where the concentration of genetically modified material is above 0.9% (regulation 1830/2003[4]). In the United States towards the end of 2012, California's Proposition 37 was voted upon (Carter et al. 2012). This proposed legislation, which was rejected, aimed to make mandatory the labelling of food containing GM material. This zero-tolerance would have set extraordinary demands on quantitative molecular diagnostics, which would be required to achieve single copy sensitivity and to overcome the complex problems in genomic DNA testing.

The labelling of food products containing GM is a response to political and public resistance to GM crops within the European Community which, towards the end of the 1990s, resulted in a reduction in field trials by member states with only Spain

maintaining their program with approximately 100000 hectares. This area compares to the 66.8 million hectares of transgenic crops grown in the United States in 2010 (Peng 2011) and 175.2 million hectares globally in 2013 (Marshall 2014). The only maize GM variety grown in the European Union is Monsanto's YieldGard® MON810 which is, however, banned in France, Germany and Greece, yet grown within other member states. This political uncertainty regarding the hazards of GM crops led to the European Commission in 2010 proposing to give member states the freedom to veto GM crop cultivation in their territory without any scientific explanation (Sabalza et al. 2011). Resistance to GM crops has not been aided by the scientific press with the publication of maize event NK603 reported to cause tumour formation (Séralini et al. 2012), although this paper has been scientifically discredited and the statistical analysis shown to be flawed so that the article was eventually retracted (Nature doi:10.1038/nature.2013.14268). Furthermore the accidental distribution of un-regulated maize in the United States (Macilwain 2005) whereby transgenic Bt-11 was mixed with non-approved Bt-10 have raised questions about the control systems put in place by regulators, distributors and the biotechnology companies. With such regulation comes the need for molecular diagnostics to quantify and identify transgenic contamination.

1.2 Molecular diagnostic technologies

There are many different molecular diagnostic techniques ranging from microscopy to mass spectrometry used to detect specific phenotypes and marker molecules. But it is the targeting of nucleic acids that is the focus of this project. Nucleic acids can be directly detected by several techniques such as using labelled oligonucleotide probes to hybridise to the target sequence. In dot-blot quantification, the template is bound to a membrane and a biotinylated probe hybridises. A streptavidin-peroxidase complex attaches to a molecule of biotin and catalyses the oxidation of luminal to detectable light. The amount of light generated is proportional to the concentration of the target nucleic acid. Another labelled probe method is fluorescent in situ hybridisation (FISH) (Langer-Safer et al. 1982) which uses fluorescence microscopy to observe fluorescently labelled probes hybridised to the localised RNA or DNA in cells and tissues. This approach has been used to study gene expression. However such techniques lack speed, sensitivity and convenience compared with approaches that amplify a specific target DNA such as real time quantitative PCR (qPCR; see below) which has become the benchmark technology for the detection and quantitation of genetically modified organisms. Although other methods have achieved comparable

limits of detection, including capillary electrophoresis with laser-induced fluorescence (Obeid et al. 2004) and a DNA biosensor with oligonucleotide conjugated gold nanoparticles (Kalogianni et al. 2006) which can both detect the 35S promoter at 0.1%, they have not been more widely adopted.

1.3 PCR-based molecular diagnostics

The discovery of the polymerase chain reaction (PCR) by Kary Mullis in 1983 (Saiki et al. 1985) has revolutionised molecular biology and molecular diagnostics. For the first time, low concentrations of nucleic acids could be amplified to a detectable level (Figure 1.1). Not only is this technique sensitive, but the specificity of the primers to the target sequence ensured identification of the target from other nucleic acids. The labelling of oligonucleotide primers with fluorescent markers enabled forensic scientists to genetically fingerprint crime samples by designing primers to flank short tandem repeats (STRs). PCR could be multiplexed and therefore many STRs could be analysed in the same reaction tube resulting in extraordinary discriminatory power to eliminate suspects from a crime scene.

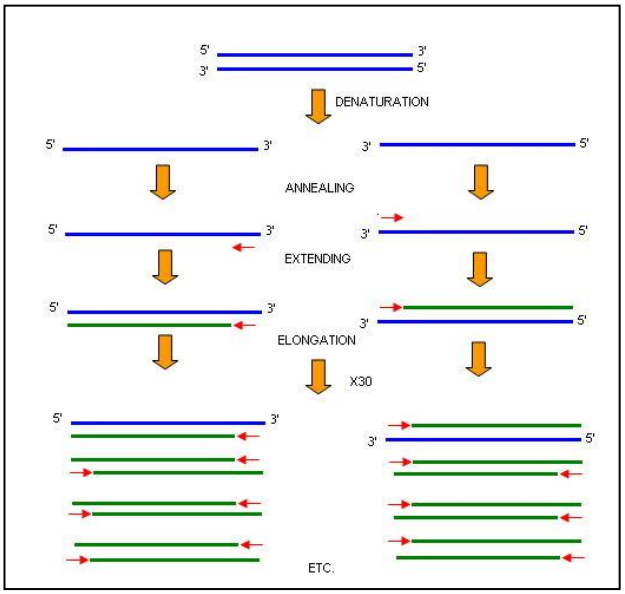


Figure 1.1: Schematic of the polymerase chain reaction (PCR). The double stranded target DNA is initially denatured by heating to 95°C. Oligonucleotide primers anneal at approximately 60°C to complementary sequences of the target strand flanking the region of interest. At 72°C Taq polymerase extends from these primers to make double stranded DNA. The cycling of denaturing, annealing and extension continues to amplify the target DNA (reproduced from www2.warwick.ac.uk).

Real time qPCR became possible using ethidium bromide (Higuchi et al. 1993) to bind to the accumulating double-stranded DNA, hence increasing the fluorescence during

amplification. The increasing fluorescence was captured with a video camera and the number of amplification cycles to achieve a threshold level of fluorescence related to the concentration of the template: the fewer the cycles, the greater the template concentration (Figure 1.2). This amplification and detection system is very sensitive, highly specific and has a wide dynamic range of quantification. The hazardous ethidium bromide has been largely replaced by other more sensitive intercalating dyes, for example SYBR green, to show the increase in fluorescence associated with duplex DNA. Different approaches to detection of the amplifying DNA include TaqMan probes (Holland et al. 1991); (Heid et al. 1996). In this method, a probe is designed to anneal between a set of specific primers. The TaqMan probe has a fluorophore at the 5' end and a quenching molecule at the 3' end. While the fluorophore and the quencher are in close proximity the fluorescence is quenched due to the principle of fluorescence resonance energy transfer (FRET). During amplification the 5' to 3' exonuclease activity of the Taq polymerase degrades the probe releasing the fluorophore which can now fluoresce via excitation from the instrumentation. The fluorescence is directly proportional to the amount of DNA template present in the qPCR.

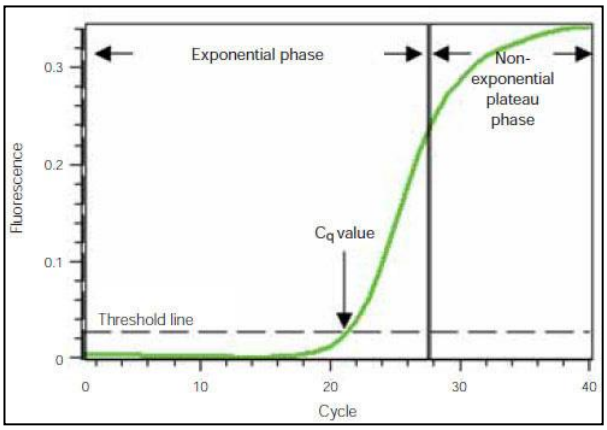


Figure 1.2: Schematic of increasing fluorescence tracking the amplification of target DNA in quantitative PCR. The C_q value in the exponential phase is proportional to the template concentration (reproduced from www.bio-rad.com).

There have been developments with these techniques for the screening and quantification of genetically modified organisms but many challenges exist for this PCR based technology (Holst-Jensen et al. 2003) including the high costs involved and the accuracy of GMO quantitation (Barbati et al. 2002).

The most common diagnostic method for GM quantification is the benchmark qPCR reaction. For this technique, primers have been designed to specifically target a universal sequence in all maize; for which the alcohol dehydrogenase 1 (ADH1) gene from *Zea mays* is often used. To detect transgenic DNA, primers designed to

specifically target generic transgenic elements such as the Cauliflower Mosaic Virus (CaMV) 35S promoter (35Sp) and the *Agrobacterium tumefaciens* nopaline synthase gene terminator (NOSt) which are commonly used to control expression of the desired trait. To discriminate CaMV 35Sp originating naturally from the virus present in *Brassica* plants primers that target the reverse transcriptase gene of the virus has been developed (Broeders et al. 2012). Event specific detection of maize has been employed for numerous maize GM crops including MON810 (Holck et al. 2002); (Hernández et al. 2003) and Bt-11 (Charles Delobel et al. 2008); (Ronning et al. 2003); (Zimmermann et al. 2000) by targeting specific sequences unique to these inserts and their position of insertion in the genome. Strategies have also been developed to target simultaneously multiple GM sequences in multiplex PCR reactions (Hernández et al. 2003); (Akiyama et al. 2005).

The polymerase chain reaction has revolutionised molecular diagnostics. It has given rise to quantitative PCR and more recently digital PCR (Vogelstein and Kinzler 1999) with the potential for absolute quantitation and other manifestations with PCR at the core. Recent additions to this family include immuno-PCR (Adler et al. 2008) and PCR-ELISA (St-Louis 2009) which have combined immunoassay detection with the polymerase chain reaction. Improvements to the kinetics of thermostable polymerases and thermocycling has achieved 30 cycles of amplification in less than 3 minutes (Wheeler et al. 2011). This rapid testing would be a highly desirable characteristic for point-of-care (POC) diagnostics (Holland and Kiechle 2005).

However, at the heart of PCR is the requirement for thermocycling and for fluorescent detection, which itself requires an excitation light source. The cost and complexity of the hardware required especially in low-resource settings, have guided nucleic acid amplification research in the direction of isothermal amplification.

1.4 Isothermal Amplification

Isothermal amplification operates at a single temperature to amplify DNA without the requirement for a thermocycler. The discovery of DNA polymerases that have strand displacement activity have led to the development of a number of nucleic acid isothermal amplification methods. PCR-based diagnostics require the denaturing of the double stranded DNA target to initiate the amplification process. However, isothermal methods rely on strand invasion by enzymes or modified oligonucleotides to start the reaction by producing single stranded template. To achieve exponential amplification, a method to promote repeated re-initiation through new strand invasion is required.

Without the need for a denaturation step in the protocol and subsequent thermocycling, the cost of the hardware needed can be greatly reduced.
The simplest form of isothermal amplification is rolling circle amplification (RCA). This amplifies circular DNA or RNA templates using strand displacement polymerases to produce concatemers of tandem repeats (Lizardi et al. 1998) resulting from initiation events that then run continuously around a circular template (Figure 1.3).

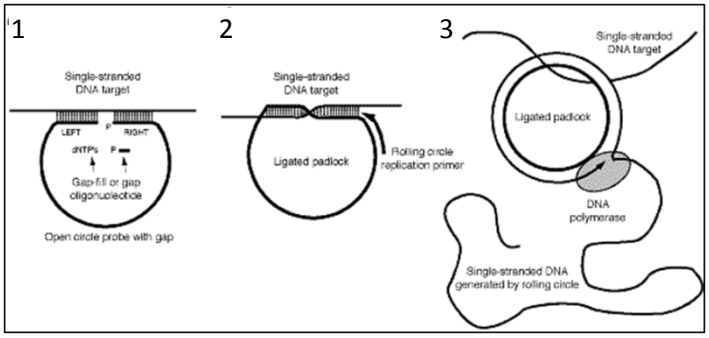


Figure 1.3: Rolling circle amplification (1) open circle probe binds to the single stranded DNA target leaving a small gap which is filled by ligation (2) the rolling circle replication primer binds to the ligated padlock probe (3) amplification of the padlock probe using a strand displacing DNA polymerase (reproduced from Lizardi et al. 1998).

For single stranded templates, formation of a single-stranded circular DNA molecule can be achieved with padlock probes annealing to the ssDNA target with a gap between the ends that can be ligated together (Banér et al. 1998). A primer binding to the circular molecule can then be extended continuously by the polymerase. The simplicity of RCA continues to be attractive to molecular diagnostics and more recently to nanobiotechnology (Ali et al. 2014), but is inherently a slow amplification and is only applicable to circular templates. To overcome these limitations of RCA and improve the efficiency of isothermal amplification, two basic approaches have been used. The first is to design primers that promote amplification through the inclusion of sequences that promote loop formation, or through the modification of their composing nucleotides.

1.4.1 Isothermal amplification with redesigned or modified oligonucleotides

Loop-mediated amplification (LAMP) (Notomi et al. 2000) has found numerous applications with both DNA and RNA targets. The simplicity and specificity of using turn-back primers (Kimura et al. 2011) for strand invasion and a displacement polymerase has been complemented with a range of real time and endpoint detection systems. There are further details for this method in section 1.5.

A similar technique to LAMP is SMART-Amp2 (Hall et al. 2002); “smart amplification process version 2”). This has asymmetrically designed primers to reduce the formation of non-specific amplification products. The specificity of this method is enhanced by using *Thermus aquaticus* MutS to irreversibly bind to mismatched dsDNA preventing further polymerisation and reducing non-specific amplification. SMART-AMP2 has become a useful tool for single nucleotide polymorphism (SNP) identification (Mitani et al. 2007); (Lezhava and Hayashizaki 2009); (Aw et al. 2012).

Multiple displacement amplification (MDA) is an isothermal amplification method that uses the strand displacement activity of phi29 DNA polymerase and multiple sequence-independent primers (Dean et al. 2002). The technique has been successful with genomic DNA templates to low copy number but the sensitivity is limited by background amplification compromising the results. Recent improvements to the method and the reagents used, has resulted in the development of a digital MDA system (Blainey and Quake 2011).

The first technique to use chimeric DNA/RNA primers and displacement primers to form the single stranded template with a strand displacing polymerase is the reaction déplacement chimeric (RDC) developed by Bio Mérieux (Craponne, France) (Cleuziat and Mandrand 1998).The polymerase initiates amplification after the RNase H degrades the RNA region between the hybridised primer and the DNA template. There are no applications of this isothermal amplification in the primary scientific literature although patents and an assessment report exist (http://www.patentstorm.us/patents/5824517.html; http://www.coextra.eu/pdf/report1449.pdf).

Isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) (Shimada et al. 2002) uses a pair of chimeric 5’-DNA-RNA-3’ primers and a thermostable RNase H enzyme with the activity of a strand displacement polymerase. The method requires the heat denaturation of the template to initiate amplification which adds complexity to the hardware design and the cost of the assay. A similar method to ICAN but with differently designed chimeric primers is the single primer isothermal amplification (SPIA) (Williams et al. 2010).

The isothermal target and signalling probe amplification (iTPA) (Jung et al. 2010) approach brings together some of the features of LAMP and ICAN and has been coupled with fluorescence resonance energy transfer cycling probe technology (FRET CPT) for amplification detection. Although early indications of assay sensitivity are promising and there are simple hardware requirements, the complexity of the primer design could limit the applications of this method.

1.4.2 Isothermal amplification with enzymic strand displacement

There is a group of isothermal amplifications that are characterised by enzymic strand displacement in amplification initiation, cycling or both. Helicase-dependent amplification (HDA) is one of these methods that separate the double-stranded DNA template at amplification initiation (Vincent et al. 2004). DNA helicase unwinds the double-stranded DNA and single-stranded DNA binding proteins and target specific primers bind to the strands. Amplification of the target follows with a DNA polymerase to produce double-stranded copies of the template. The simplicity of the method is beneficial; however the reaction time is slow in comparison to other isothermal amplification technologies. Continued research with HDA has seen advancements with assay speed (Chow et al. 2008); (Jeong et al. 2009).

Another method that uses single-stranded DNA binding proteins is the recombinase polymerase amplification (RPA) technique (Piepenburg et al. 2006). The recombinase enzyme and target specific primer complex insert the primer into the double-stranded target which is stabilised by ssDNA binding proteins and the primer extended by strand displacing DNA polymerase. This method is fast, sensitive and does not require dsDNA template pre-treatment. Using reverse transcriptase, the method has been modified for RNA detection.

Some isothermal amplification methods require restriction endonucleases to nick the primer template duplex for strand displacement by the polymerase. Strand displacement amplification (SDA) (Walker et al. 1992), isothermal exponential amplification reaction (EXPAR) (Van Ness et al. 2003) and the nicking and extension amplification reaction (NEAR) (Maples et al. 2009) all use primers designed with restriction sites, strand displacement polymerases and the requirement of template denaturation to initiate the reaction.

The cyclic enzymatic amplification method (CEAM) uses exonuclease III to release fluorescence from quenched fluorescent probes upon hybridisation to the template (Cui et al. 2010). The initiation of the cyclic reaction requires denaturation.

1.4.3 RNA producing isothermal amplifications

Three techniques that produce RNA amplicons from RNA template are nucleic acid sequence-based amplification (NASBA) (Compton 1991), transcription mediated amplification (TMA) (GEN-PROBE, San Diego USA) and self-sustained sequence replication (3SR) (Fahy et al. 1991). In the NASBA approach the target RNA provides the template to produce cDNA using reverse transcriptase from a primer with the T7

promoter sequence. RNase H removes the template RNA and a further primer produces dsDNA with the T7 promoter, which synthesises multiple RNA copies using T7 DNA-dependent RNA polymerase. The newly generated RNA acts as a template for isothermal amplification cycling. However, there is a requirement for heat pre-treatment of the RNA or dsDNA template. The majority of applications of isothermal amplification have targeted infectious disease; there have been developments with isothermal amplification in the area of GM detection with the invention of NASBA amplification implemented microarray analysis (NAIMA) (Dobnik et al. 2010).
Many of these and other isothermal amplification techniques are owned by commercial organisations, for example Inchworm by Enzo Biochem (New York, US), and mechanistic details are only available in the filed patents and not in the scientific literature, such that their robustness and practicality is not always clear.

1.5 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a simple, rapid, specific and cost-effective isothermal nucleic acid amplification method (Notomi et al. 2000); (Tomita et al. 2008). LAMP has also shown robustness to inhibitory substances to a greater extent than PCR (Kaneko et al. 2007); (Kiddle et al. 2012). Two distinct types of primer are required: LAMP primers which are responsible for hairpin loop formation and amplification initiation, and displacement primers which displace the initial loop structure with the strand displacement activity of the DNA polymerase. The primers are designed for six distinct sequences of the target resulting in a high degree of specificity. The complexity of primer design is greater than for PCR and a number of publications (Torres et al. 2011); (Kimura et al. 2011); (Parida et al. 2008) as well as the Eiken Chemical Company Ltd website (http://loopamp.eiken.co.jp/e/) provide support for this. The LAMP reaction can be accelerated with the further addition of LOOP primers (Nagamine et al. 2002) which bind to the single stranded hairpin loop formed by the LAMP primers to form additional amplification products and, as a by-product, inorganic pyrophosphate. The LAMP reaction can also be accelerated by STEM primers (Gandelman et al. 2011) which bind in the region between the hairpin loops, although loops do not need to form, creating more products of amplification. A recently improved recombinant *Bst* polymerase has been reported to reduce assay time by 50% when compared to polymerisation by a wild-type Bst DNA polymerase (Tanner et al. 2012).

1.5.1 LAMP mechanism

The LAMP amplification mechanism is complex: The amplification is initiated by strand invasion by the forward and backward inner primers (FIP and BIP) which are composed of sense and anti-sense sequences for the target molecule. DNA polymerisation proceeds from these primers and the newly synthesised strands are displaced by primers designated F3 and B3. The displaced single stranded DNA originating from either FIP or BIP can now form a hair pin loop due to the anti-sense sequence of that LAMP primer. Towards the 3' end of this structure, the alternative inner primer anneals and extends, which in turn is displaced by the appropriate displacement primer. The displaced 'dumbbell' structure has hairpin loops at both ends and is the target structure for cyclic amplification. Further inner primers enable the cycling between the two forms of this 'dumbbell' structure which also produces intermediates that are elongated into concatemers of the original target sequence. The amplification is conducted at temperatures of between 60 and 65°C without the need to denature the target to initiate the amplification. However there is evidence that pre-denaturing the template increases the LAMP assay sensitivity (Aryan et al. 2010). Figure 1.4 shows the annealing of the FIP and F3 displacement primer to start the LAMP amplification from single stranded template. Without denaturation, the double stranded DNA requires strand invasion by the LAMP primers of the "breathing" DNA, which could be a limiting step. By denaturing the template, this potentially limiting step is removed and sensitivity is increased.

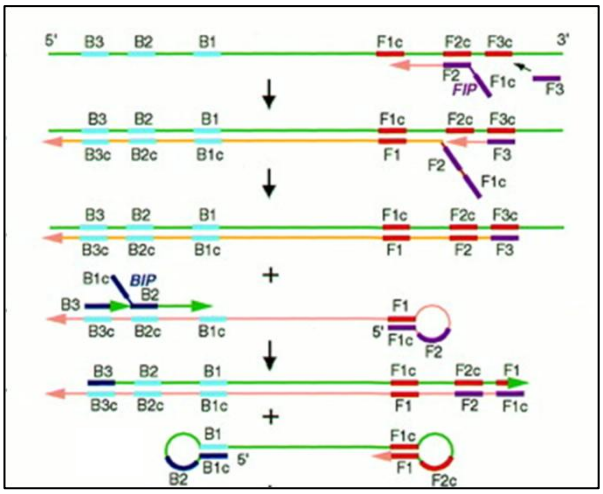


Figure 1.4: Loop-mediated amplification (LAMP) initiation of amplification to the formation of the "dumbbell" structure required for the cycling and elongation steps. Loop primers and STEM primers anneal to the single stranded loops and inter-loop regions and produce further amplicons. The elongation phase of the amplification produces concatemers with cauliflower-like structures (reproduced from Nagamine et al. 2002).

1.5.2 Nucleic acid detection using LAMP

The LAMP process can be detected in a number of ways. Firstly the inorganic pyrophosphate by-product can be analysed using the turbidity of magnesium pyrophosphate (Mori et al. 2001) or real-time turbidimetry (Mori et al. 2004). Visual determination of fluorescence using calcein and manganese in the LAMP assay has been described (Tomita et al. 2008) and has been used quantitatively for the detection of *Brucella* spp. (Soleimani et al. 2013). Calcein fluorescence is quenched by manganese ions binding to pyrophosphate and therefore a positive amplification is characterised by bright fluorescence. Intercalating dyes such as SYBR green I, ethidium bromide and Pico Green, have been used for end point detection of LAMP amplification. Recently the detection of SYBR green fluorescence has been enhanced into a real time format for the quantitative detection of a fungus (Zhang et al. 2013). LAMP amplification has been used in the molecular diagnosis of GM contamination in crops. The detection of 35Sp and NOSt sequences was shown with samples containing 10 copies of transgenic soya (Lee et al. 2009) by visual examination of the multimeric LAMP products on a gel. Visual examination of positive LAMP results has also been described (Randhawa et al. 2013) for maize event T25 and for the rapid detection of cry1ab gene in rice (Li et al. 2013). The detection of GM using LAMP coupled to bioluminescence (see section 1.6) has shown sensitivity of 0.1% or 50 copies of GM target per 20µl volume (Kiddle et al. 2012).
A limitation to LAMP detection has been the inability to multiplex a number of target sequences simultaneously, but steps to overcome this with modified LAMP primers incorporating a quencher-fluorophore duplex region (Tanner et al. 2012) has shown that up to 4 target sequences can be detected in one LAMP reaction. The sensitivity of LAMP to amplify single copies of template has seen some early developments in absolute quantitation using digital LAMP (Gansen et al. 2012); (Sun et al. 2013); (Zhu et al. 2012) and microfluidic devices.

1.6 Bioluminescent Assay in Real Time (BART)

To detect and quantify nucleic acid amplifications without complex equipment and associated costs, alternative methods have been developed based on inorganic pyrophosphate detection. During DNA amplification, a molecule of inorganic pyrophosphate is produced with each addition of a nucleotide. The amount of this low molecular weight by-product synthesized is proportional to the concentration of the template. Therefore the inorganic pyrophosphate concentration can be used to

estimate the concentration of the nucleic acid template in a given sample. As discussed above with LAMP amplification, there are a number of methods to detect inorganic pyrophosphate, but a further alternative is the enzymatic luminometric inorganic pyrophosphate detection assay (ELIDA; (Nyrén and Edwin 1994), which converts inorganic pyrophosphate with the substrate adenosine-5'-*O*-phosphosulphate (APS), catalysed by the enzyme ATP-sulphurylase, to ATP. Using firefly luciferase, the ATP produced in the previous reversible reaction is required to oxidise the substrate luciferin to oxyluciferin, with the emission of light (Figure 1.5).

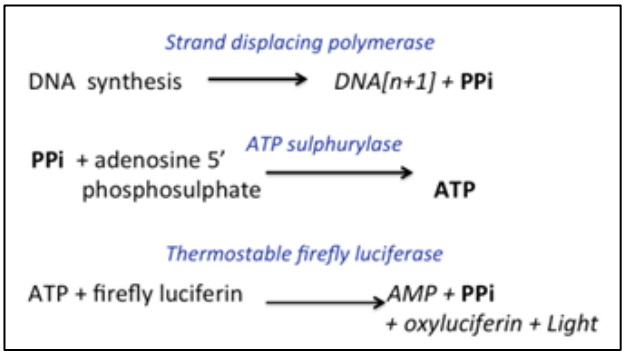


Figure 1.5: The chemistry of the isothermal DNA amplification reaction coupled with BART detection.

In Pyrosequencing® these coupled reactions are used to detect the liberation of inorganic pyrophosphate from the addition of each correct dNTP (Ronaghi et al. 1996). The development of recombinant firefly luciferases with increased temperature stability to tolerate the 60 to 65°C temperature range of some isothermal amplification technologies, led to the invention of BART (Bioluminescent Assay in Real Time) by Prof. Jim Murray and Dr. Laurence Tisi (Murray and Tisi 2004).

1.6.1 BART detection of nucleic acid amplification

BART operates in a closed tube format reducing the risk of contamination from highly amplified template DNA. The production of inorganic pyrophosphate from DNA synthesis is rapidly converted to light which is recorded continuously by a simple CCD camera or light diode detection system connected to a computer. The temperature of the assay is regulated by a heat block without the need for thermocycling in the case of isothermal amplification.

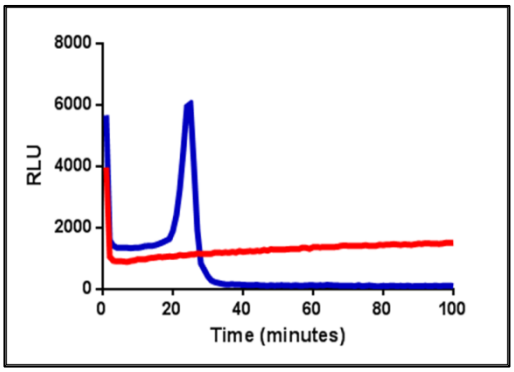


Figure 1.6: Bioluminescent light output from nucleic acid amplification with BART detection. The figure shows the light intensity from a negative sample (red trace) and a positive sample (blue trace) against the assay time. The positive sample is characterised by a BART peak and the time of maximum light intensity defined as time-to-peak (T_{max}).

The light output for a sample with or without DNA template is characterised by an initial flash of light which decays to a baseline level of luminescence. The initial relatively high light output at the start of the assay is due to the concentration of dATP present (required for DNA synthesis) which is an alternative, but less efficiently utilised, substrate for luciferase. A sample without a DNA template specific for the primers in the assay will approximately maintain this baseline level of light output. However a positive sample will be characterised by a sharp peak of light which decays rapidly to an almost undetectable level (Figure 1.6). This BART peak is created by the exponential increase in inorganic pyrophosphate production during DNA synthesis, being converted into ATP and into increasing light output. The peak occurs when APS is depleted and the luciferase is inhibited by the increasing concentration of unprocessed inorganic pyrophosphate. The time from the start of the assay to the time at which the light intensity peaks is referred to as time-to-peak or T_{max}. The value of T_{max} is inversely proportional to the concentration of the original sample DNA template. The selection of LAMP isothermal amplification for high specificity and high inorganic pyrophosphate production, coupled with BART, has been used to quantitatively detect DNA and RNA targets (Gandelman et al. 2010) and transgenic elements in genetically modified crops (Kiddle et al. 2012). Validation of a LAMP-BART molecular detection system has also been used to rapidly and reliably detect *Listeria* in food samples (Fortes et al. 2013). The combination of a nucleic acid amplification technology with BART has considerable potential to provide a simple and low-cost HIV viral load monitoring strategy in a resource limited environment (Tisi et al. 2012). Quantitation using LAMP-BART has been limited by the high variability between replicates at very low copy number. A linear relationship between template concentration and time-to-peak was observed between 5.5×10^8 and 550 copies of the *Chlamydia* Artificial plasmid Template (ChAT) by Gandelman et al. (2012). However,

the variance for 55 and 5.5 copies per assay was high and a non-linear relationship was observed. The LOQ is therefore limited in this assay to a value between 55 and 550 copies per assay. The LAMP-BART assays of genomic DNA template using 35Sp, NOSt and ADH1 primers produced highly variable results with all extraction techniques assessed (Kiddle et al. 2012). The LAMP-BART assay with the lowest variance over the range of template concentrations was the 35Sp assay with Promega Wizard extracted genomic DNA. The data becomes non-linear for this assay at approximately 10^4 copies per assay.

1.7 Aims and objectives

In this project, the aim was to study the quantitation of DNA by LAMP-BART at low copy numbers and to develop approaches with improved quantitative capability. The objective was to use simple artificial DNA templates to observe the parameters that affect the LAMP-BART assay kinetics and to use these observations to devise improvements, and then test these for genomic DNA quantification.

The project also aimed to detect single copies of a DNA target and to develop quantitation methods to differentiate individual copy numbers at ultra-low copy numbers. The objective of single copy detection was to develop a digital BART assay for an artificial DNA template with the further aim of using digital BART for genomic DNA quantification.

The ultimate aim of the project was to develop a LAMP-BART assay to quantify DNA over a full dynamic range using the various techniques of average Tmax, ultra-quantification and digital BART.

Chapter 2
Materials and methods

2.1 Reagents and chemicals

2.1.1 Chemicals for LAMP-BART assays

General laboratory chemicals were purchased from Sigma (Poole, UK) and these included trehalose, potassium chloride (KCl), polyvinylpyrrolidone (PVP), dithiothreitol (DTT), molecular grade water and mineral oil. ThermoPol 10X buffer, Isothermal amplification 10X buffer, Diluent D buffer, VENT buffer, ATP sulphurylase, *Bst* DNA polymerase large fragment, magnesium sulphate ($MgSO_4$) were all supplied by New England Biolabs (MA, USA). BARTMaster and Lumopol buffer were supplied by Lumora (Ely, UK). Salmon sperm carrier DNA and the four deoxyribonucleotides (dNTPs) were supplied by Invitrogen (CA, USA). Luciferin potassium salt (LH_2) was supplied by Europa Biotech (Ely, UK). Adenosine-5'-*O*-phosphosulphate (APS) was supplied by Biolog Life Science (Bremen, Germany). Ultra-Glo™ Luciferase was supplied by Promega (WI, USA).

2.1.2 Reagent preparation for LAMP-BART assays

Salmon sperm carrier DNA was supplied at a labelled concentration of 10mg/ml and quantified using the NanoDrop 1000 spectrophotometer for a more accurate concentration (typically 14.2mg/ml). Diluted 1/10 and 1/100 for use in LAMP-BART assays at 100ng per partition, used undiluted for digital LAMP-BART assays and stored at -20°C.

For LAMP-BART assays and for PCR formats, molecular grade water from Sigma was used. Individual aliquots of 1.5ml in 2ml screw-top tubes were prepared and stored at -20°C.

ThermoPol was superseded by Isothermal Amplification buffer for the digital BART isothermal amplifications (Chapter 5), supplied at 10X concentration and used directly in the LAMP-BART assay at 1X concentration, stored at -20°C.

A 25% trehalose solution was prepared by adding 12.5g to 50ml molecular grade water to produce aliquots of 420µl in 2ml screw-top tubes for storage at -20°C.

A 1M DTT solution was prepared by adding 1.54g to 10ml molecular grade water to produce aliquots of 100µl in 2ml screw-top tubes for storage at -20°C.
A 40mg/ml PVP solution was prepared by adding 0.4g to 10ml molecular grade water to produce aliquots of 100µl in 2ml screw-top tubes for storage at -20°C.
A 1.2M potassium chloride solution was prepared by adding 0.895g to 10ml molecular grade water to produce aliquots of 100µl in 2ml screw-top tubes for storage at -20°C.
Deoxyribonucleotides were supplied at 100mM for each of dATP, dCTP, dGTP and dTTP and prepared by adding 10µl of each dNTP to 160µl molecular grade water to produce aliquots of 65µl at 5µM with respect to each dNTP, in 2ml screw-top tubes for storage at -20°C.
A 10mg/ml D-luciferin K^+ salt solution was prepared by adding 10mg to 1ml molecular grade water to produce aliquots of 25µl in 2ml screw-top tubes for storage at -20°C.
10mM adenosine-5'-*O*-phosphosulphate (APS) was supplied at the desired concentration (determined by the manufacturer by UV absorbance at λ_{max}) and each vial produced 16 aliquots of 60µl in 2ml screw-top tubes for storage at -20°C.
0.55mg/ml Ultra-Glo™ luciferase was produced by adding 10µl 1M DTT, 100µl 10X ThermoPol and 790µl molecular grade water to 100µl Ultra-Glo™ luciferase, supplied at 5.5mg/ml, to produce aliquots of 25µl in 2ml screw-top tubes for storage at -20°C.
25U/ml ATP sulphurylase was supplied at 300U/ml and subsequently diluted when required by adding 4µl ATP sulphurylase to either 44µl Diluent D buffer or VENT buffer and stored at -20°C.
Bst DNA polymerase large fragment was supplied at 8000U/ml and used directly in the LAMP-BART assay to give an assay concentration of 8U/ml, stored at -20°C.
Individual mineral oil aliquots of 1.5ml in 2ml screw-top tubes were prepared and stored at room temperature.

2.1.3 Reconstitution of primers

Oligonucleotides for LAMP and PCR DNA amplification were supplied initially by Eurofins MWG Operon (Ebersberg, Germany) and later Sigma (Poole, UK). The primer pellets were hydrated with the required volume of molecular grade water, as indicated on the company's technical datasheet, to 100µM. The primers were labelled and stored at -20°C. The details of each primer were entered into the Lab Collector database.

2.1.4 Consumables for LAMP-BART

Axygene® Maxymum Recovery® pipette tips 10µl, 100µl and 1000µl from Corning Life Sciences (MA, USA) were used for all LAMP-BART and PCR procedures.
PCR microplate Thermo-Fast 96 well non-skirted white plates from Fisher Scientific (Loughborough, UK) were used for LAMP-BART assays.
X-clear Advanced Polyolefin Starseal was used to seal the microplate before amplification and non-stick 1.5ml tubes were supplied by STARLAB (Milton Keynes, UK).
Ultra non-stick 2ml tubes with screwcap for DNA template storage were supplied by Alpha Laboratories (Eastleigh, UK).

2.2 Genomic template

2.2.1 Maize event Bt11 certified reference material

The Certified Reference Materials for maize event Bt-11 (Figure 2.1) have been developed and produced by the Institute for Reference Materials (Geel, Belgium) for Fluka GmbH (Buchs, Switzerland). They contain defined mass fractions of maize kernel powder derived from the genetically modified maize Bt-11 and wild type variety 'Pelican', both supplied by Syngenta Seeds S.A.S. (Nérac, FR). The percentage GMO of the reference maize samples are certified at 0, 0.1, 0.5, 1, 2 and 5% w/w. The maize event Bt-11, developed by Syngenta Seeds, contains the transgene *cry1Ab* from *Bacillus thuringiensis* subspecies *kurstaki* for resistance to the European corn borer (*Ostrinia nubilalis*) and the phosphinothricin N-acetyltransferase encoding gene (*pat*) from *Streptomyces viridochromogenes* for tolerance to glufosinate herbicide (ISAAA GM approval database; www.isaaa.org/gmapprovaldatabase/). The expression of the *cry1Ab* and *pat* transgenes is regulated by the constitutive promoter 35S from the cauliflower mosaic virus and terminator from the nopaline synthase gene (NOSt) from *Agrobacterium tumefaciens*.

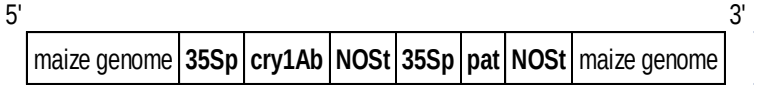


Figure 2.1: Graphical representation of the Bt-11 maize event transgenic cassette with two copies of the CaMV 35S promoter and the NOS terminator (Yang et al. 2007).

2.2.2 Maize event Mon810

Maize kernels from Monsanto of the maize event MON810 (Figure 2.2) were cleaned with molecular grade water to remove the anti-fungal coating before grinding to a powder for extraction. MON810 contains the CaMV sequence, but the insertion of the cassette into the maize genome failed to insert the NOS terminator sequence.

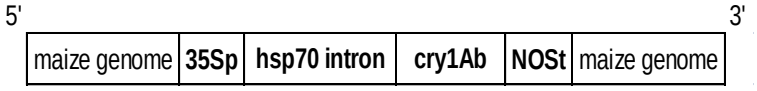


Figure 2.2: Graphical representation of the Mon810 maize event transgenic cassette with one copy of the CaMV 35S promoter and the intended position of the NOS terminator which failed to insert into the maize genome.

Sequencing of the CaMV 35S promoter in Mon810 showed 100% alignment to the reference data on GenBank, attempts to sequence the NOSt element in Mon810 were unsuccessful due to a truncation event at the 3' end of the *cry1Ab* gene causing the complete loss of the NOSt sequence (Rosati et al. 2008). Attempts to germinate the Mon810 seeds were also unsuccessful.

2.2.3 Maize event SMARTSTAX™

The maize event SMARTSTAX™ developed by Dow AgroSciences is a stacked trait result of conventional cross breeding of four parental lines with herbicide tolerance and resistance to numerous lepidopterans and coleopterans. The maize genome contains a truncated duplication of the 35S promoter sequence (Figure 2.3).

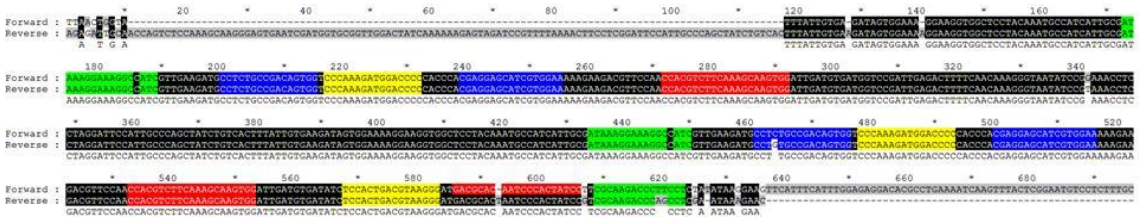


Figure 2.3: Graphical representation of the CaMV 35S sequence in maize event Smartstax™ which shows the truncated duplication of the promoter. Sequences for the displacement primers are indicated in green, loop primers in yellow and LAMP primer sequences in red and blue. The set of LAMP primers complement the sequence between positions 430 and 630. There is also complementation of the B3 displacement, BIP LAMP primer, B-loop and part of the FIP primer upstream of the complete primer set, which suggests that part of the 35S promoter sequence is partially duplicated. This enhancement of the 35S promoter is used to target gene expression in a particular part of the plant.

2.2.4 Growing GM maize from seeds to maturity

Maize seeds from the commercial variety SMARTSTAX™ were grown in a soil sand mix (approximately 10% sand) with fungicide and insecticide added. Each pot containing a seed just below the surface was watered periodically within the growing room. Details of the plants were entered onto the Phytotracker database (Nieuwland et al. 2012). The young plants were transferred to a GM glasshouse when 50cm tall to grow to maturity. Six months from germination the maize cobs were harvested and the plants and soil autoclaved before disposal.

2.2.5 Germinating GM maize seeds on agar

The maize seeds were initialled cleaned with 60% ethanol before sterilisation with 25ml bleach solution (5ml bleach sanitizer, 20ml Millipore Elix water and 50µl Silwet) and repeated washing with autoclaved MGW. The dried seeds were planted with sterile tweezers into GM micro agar and transferred to the growth room under the lights for germination.

2.2.6 Germinating GM maize seeds on filter paper

The maize seeds were cleaned and sterilised and positioned between wetted filter paper in a large petri-dish. The dish was foil wrapped and transferred to the growth room to promote germinating and the growth of roots and etiolated leaf tissue.

2.3 Plasmid template

The plasmid pART7 (Gleave 1992) containing the CaMV 35S promoter sequence in linearised and circular forms was supplied by Lumora (Ely, UK).
A plasmid containing the CaMV 35S promoter sequence and the NOSt sequence from *Agrobacterium tumefaciens* was selected from the Murray Laboratory plasmid database on Lab Collector. The stock archive of this plasmid pUC35S GUS (Murray Lab plasmid no. 374) is stored at -20°C and fresh plasmid stocks are generated by bulking up through *E. coli* transformation. This plasmid was used in both linear and circular forms (Figure 2.4) and for the construction of a new plasmid containing the maize alcohol dehydrogenase gene to replace the GUS gene.

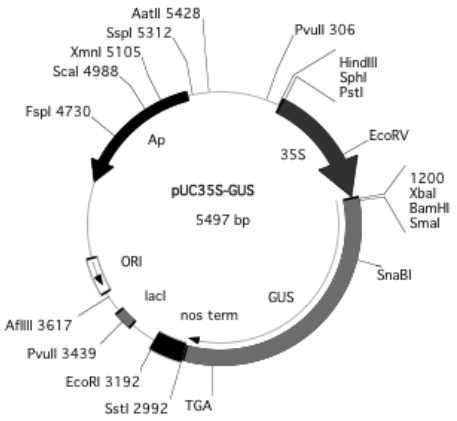


Figure 2.4: Plasmid map of pUC35S GUS (plasmid number 374)

2.3.1 Bulking up plasmid pUC35S GUS from lab stock

The stock plasmid pUC35S GUS was used to transform DG1 chemically competent *Escherichia coli* cells from Eurogentec (Seraing, Belgium). To each tube of cells thawed on ice, 2µl of the plasmid was added. The cells were gently mixed and incubated on ice for 30 minutes before heat-shocking at 42°C for 35 seconds. Each tube was immediately transferred to ice for 2 minutes. To each tube was added 250µl of Lysogeny Broth (LB) (Bertani 2004) from laboratory stock, and the tubes were incubated with shaking at 37°C for 30 minutes. Under sterile conditions, 100µl of the transformed cells were spread on a LB agar plate containing 50µg/ml of the antibiotic ampicillin to select for transformed cells with resistance. The plates were incubated at 37°C inverted overnight.

The following morning a single colony of transformed *E. coli* was selected and transferred to 20ml of LB medium containing ampicillin (50µg/ml) in a 40ml screw-top tube under sterile conditions. The tubes were incubated at 37°C overnight in a shaking incubator.

2.3.2 Purification of the pUC35S GUS plasmid DNA

To obtain high quality and good quantities of the plasmid from the transformed overnight cell culture, the alkaline lysis mini-prep method was used (Sambrook et al. 1989). The tubes were removed from the incubator and kept on ice for 30 minutes to stop growth. After centrifugation at 2000rpm for 10 minutes, the supernatants were carefully discarded. To each pellet was added 200µl of ice cold Solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0) and vortexed vigorously. Each re-suspended pellet was transferred to a 1.5ml tube containing 400µl of freshly prepared Solution II (0.2M NaOH, 1% (w/v) SDS). The tubes were rapidly inverted five

times and kept on ice and 300µl of ice cold Solution III (60ml of 5M potassium acetate, 11.5ml of glacial acetic acid, 28.5ml of water was added). To disperse the Solution III throughout the lysate, each tube was inverted and vortexed for 10 seconds. The tubes were stored on ice for 5 minutes and then centrifuged at 12000g for 5 minutes at 4°C. To a fresh tube, 600µl of the supernatant was added to 600µl phenol:chloroform (25ml phenol, 24ml chloroform, 1ml isoamyl alcohol) and vortexed. The mixture was centrifuged at 12000g for 2 minutes at 4°C, and 550µl of the supernatant transferred to a fresh tube. The double-stranded DNA was precipitated by mixing with 550µl propan-2-ol and allowed to stand for 2 minutes at room temperature before centrifuging at 12000g for 5 minutes at 4°C. The supernatant was discarded and the pellet washed with 1ml of 70% ethanol at 4°C. The supernatant was discarded and the pellet allowed to dry before it was re-dissolved in 50µl TE buffer (10mM Tris-HCl pH8.0), 1mM EDTA pH8.0) containing 20µg/ml RNase and stored at -20°C.

2.3.3 Restriction digest of pUC35S GUS

Analysis of the purified pUC35S GUS was by cleavage with two restriction enzymes and agarose gel electrophoresis of the undigested and digested DNA (Figure 2.5). The restriction endonucleases Bam HI and Sac I from New England Biolabs (MA, USA) cut the plasmid on either side of the GUS gene. Restriction followed NEB guidelines; briefly 1µl of each of the endonucleases was incubated at 37°C for 1 hour with 2µl pUC35S GUS, 2µl NEB buffer 4 and 14µl molecular grade water. Conformation of the expected fragment size was achieved by loading the digested plasmid, undigested plasmid and 10kb SmartLadder from Eurogentec (Seraing, Belgium) to a 1% agarose gel for 30 minutes at 100V. Band visualisation was by UV fluorescence of the nucleic acid stain SafeView™ from Applied Biological Materials (Vancouver, Canada).

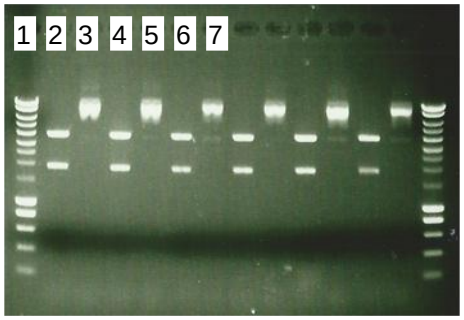


Figure 2.5: 1% Agarose gel with 10kb ladder. Lanes 1 and 14 contain the 10kb ladder, lanes 2, 4, 6, 8, 10 and 12 contain repeat the digested pUC35S GUS plasmid and lanes 3, 5, 7, 9, 11 and 13 contain the undigested plasmid.

2.3.4 Linearised pART7 aliquots from Lumora

The preparation of the linearised pART7 dried aliquots was performed in Lumora by Guy Kiddle; 100ng/µl pART7 linear plasmid was diluted ten times to give a concentration of $2x10^9$ copies/µl. 20µl of this stock was diluted in 10ml of molecular grade water containing 100µl of 10mg/ml salmon sperm DNA and gel loading buffer to give a final concentration of $4x10^6$ copies/µl. This was thoroughly mixed, aliquoted into 100µl replicates before freezing at -20°C. The aliquots were dried down in a centrifugal evaporator without heating until the target was present as a blue bead of glycerol. The dried aliquots were then stored at -20°C and should contain $4x10^8$ copies of the CaMV 35Sp target.

To re-suspend an aliquot, 400µl of molecular grade water was added and the template left to stand for 10 minutes before vigorous vortexing. The starting concentration for use in a titration series is therefore 10^6 copies/µl.

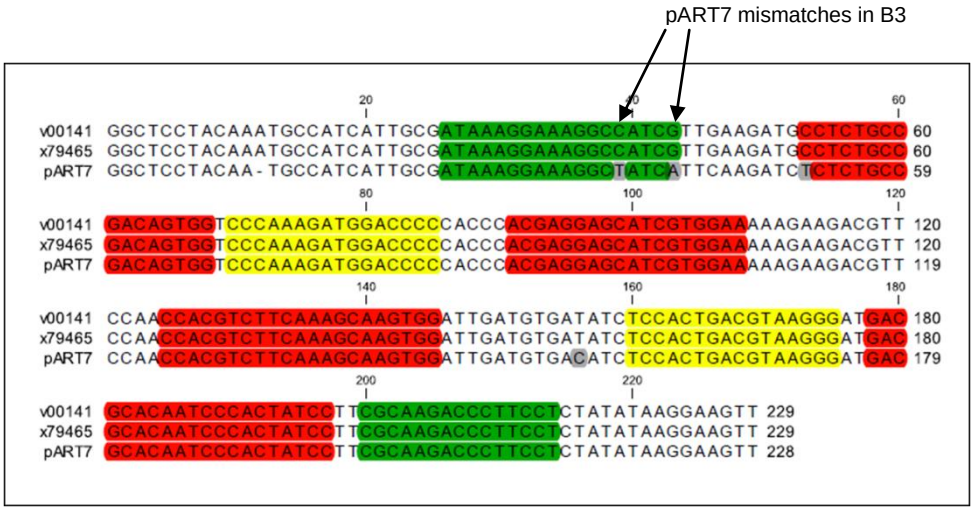


Figure 2.6: Graphical representation of the CaMV 35S sequence in pART7 showing the mismatches in the displacement primer B3. Sequences for the displacement primers are indicated in green, loop primers in yellow and LAMP primer sequences in red and blue.

The sequencing data from the pART7 plasmid indicated two mismatches in the CaMV 35Sp LAMP B3 displacement and one in the B2 section of the LAMP BIP primer (Figure 2.6). Both primers were redesigned specifically for this target.

2.3.5 Construction of pUC35S ADH1 plasmid

In section 2.3.3 the GUS gene was removed successfully by cutting with Bam HI and Sac I in a double digest (Figure 2.7). Both fragments had sticky ends.

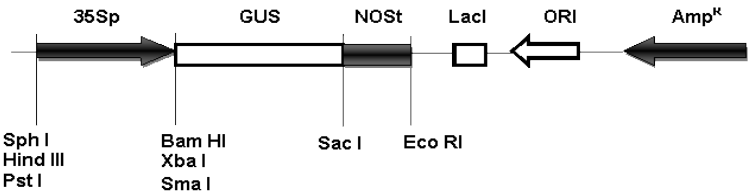


Figure 2.7: Graphical representation of the restriction sites around the GUS gene in the plasmid pUC35S GUS

2.3.5.1 PCR amplicon of ADH1 gene from maize

The primers for the maize ADH1 gene were designed to complement sequences outside the target area for both LAMP primers and PCR primers. The 20mers were designed by Primer3 software to have a melting temperature of approximately 60°C http://frodo.wi.mit.edu/primer3 (Rozen and Skaletsky 1998). The recognition sequences for the restriction enzymes were then added to the 5' end with an additional four base pairs. The primers (Figure 2.8) were designed with restriction sequences for Sph I, Bam HI, Sac I and Eco RI to facilitate the additional removal of the 35Sp and NOSt sequences if required. Both primers have weak secondary structure, no predicted primer-dimer formation and melting temperature of approximately 82°C.

Target, Type, Notation, Version	Length	T_{MELT}	GC Content	Dimer	Secondary	Primer Sequence (5' to 3')
ADH1, Insert, Forward	36	82.6°C	47%	No	Weak	TATTGCATGCGGATCCAATCAGGGCTCATTTTCTCG
ADH1, insert, Reverse	36	82.2°C	52.7%	No	Weak	GTCCGAATTCGAGCTCAGAAAGAAACGCCTCCTTGC

5′ TATTGCATGCGGATCCAATCAGGGCTCATTTTCTCG 3′
5′ GTCCGAATTCGAGCTCAGAAAGAAACGCCTCCTTGC 3′

Figure 2.8: PCR primers to produce an amplicon of maize ADH1 with flanking restriction sites in blue. Values for TMELT, primer dimer and secondary structure formation derived from Sigma technical datasheets accompanying the primers.

The PCR reaction was run on an Eppendorf Mastercycler® pro vapo.protect™ thermal cycler using Qiagen PCR mastermix with an initial 3 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C.The PCR product was visualised and assessed by agarose gel electrophoresis and purified using the PCR clean-up protocol of the Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit.

2.3.5.2 Ligation of ADH1 insert

The maize ADH1 amplicon was digested with the restriction endonucleases Bam HI and Sac I following NEB guidelines to produce overhanging single stranded termini

compatible with the digested large fragment of pUC35S GUS. The ratio of insert to vector was calculated from the following equation:

$$X \text{ ng insert} = \frac{(Y \text{ bp insert}) \times (\text{ng vector})}{(\text{total bp in vector})} \quad \times \text{ ratio (1:1 to 1:3 vector:insert)}$$

6µl of vector and 2µl of the ADH1 insert were combined with 1µl of NEB ligase and 1µl NEB ligase buffer for incubation for 1 hour at 16°C. *E. coli* DG1 chemically competent cells were transformed with the new construct and bulked up using the method described in section 2.3.2.

2.3.5.3 Sequencing of pUC35S ADH1

15µl of the newly constructed pUC35S ADH1 at a concentration of 80ng/µl, was submitted to Cardiff University DNA Sequencing Core to confirm the ligation of the maize ADH1 sequence (Figure 2.9).The results were checked against reference sequences for CaMV 35S promoter, NOS terminator and the maize ADH1 gene using the CLC Sequence Viewer v6 workbench and were shown to be correct.

GAATCAAAGGCCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCGCCGTA
AAGACTGGCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATAT
TCGTCAACATGGTGGAGCACGACACACTTGTCTACTCCAAAAATATCAAAGATACAGT
CTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCT
CCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAA
GGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCC
TCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAA
AGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGA
CGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGG
AAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTAGAGGATCCAATCAGGGCTCA
TTTTCTCGCTCCTCACAGGCTCATCTCGCTTTGGATCGATTGGTTTCGTAACTGGTGA
GGGACTGAGGGTCTCGGAGTGGATTGATTTGGGATTCTGTTCGAAGATTTGCGGAG
GGGGGCAATGGCGACCGCGGGGAAGGTGAATCAAGTGCAAAGGTCCGCCTTGTTT
CTCCTCTGTCTCTTGATCTGACTAATCTTGGTTTATGATTCGTTGAGTAATTTTGGGG
AAAGCTTCGTCCACAGTTTTTTTTTCGATGAACAGTGCCGCAGTGGCGCTGATCTTGT
ATGCTATCCTGCAATCGTGGTGAACTTATTTCTTTTATATCCTTTACTCCCATGAAAAG
GCTAGTAATCTTTCTCGATGTAACATCGTCCAGCACTGCTATTACCGTGTGGTCCATC
CGACAGTCTGGCTGAACACATCATACGATCTATGGAGCAAAAATCTATCTTCCCTGTT
CTTTAATGAAGGACGTCATTTTCATTAGTATGATCTAGGAATGTTGCAACTTGCAAGG
AGGCGTTTCTTTCTGAGCTCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTT
TCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAAT
TACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTT
TATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGC
GCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAATTC
GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACAC
AACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTA
ACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGC
GCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGA
GCGG

Figure 2.9: Part sequence of the successfully constructed plasmid pUC35S ADH1 to show the LAMP target sequences for CaMV 35Sp (in blue), maize ADH1 reference gene (in yellow) and NOSt (in green) with positions of displacement primers (in grey).

2.3.5.4 Linearization of plasmid pUC35S ADH1

The plasmid pUC35S ADH1 containing the three LAMP primer target sequences 35Sp, ADH1 and NOSt, was linearised by cutting with Bam HI to NEB guidelines. The single

cut in the plasmid occurs in a flanking region between the 35S and the ADH1 LAMP target sequences. Briefly, 24µl of the plasmid was restricted with 4µl of Bam HI in 4µl of each of NEB buffer 3, NEB BSA and MGW overnight at 37°C. The linearised plasmid was purified according the guidelines from a Macherey-Nagel kit.

2.4 Extraction protocols

2.4.1 Preparing plant tissue for extraction using liquid nitrogen

Leaf and root tissue and maize kernels were ground to a fine powder with mortars and pestles using liquid nitrogen to lyse the cells by cryopulverisation. The resulting powder was kept at -20°C until required for genomic DNA extraction.

2.4.2 Promega Wizard® kit extraction

The Promega Wizard® Genomic DNA Purification Kit was used for the extraction of genomic DNA from the various maize events according to the manufacturer's instructions for plant tissue. To summarise the protocol; 600µl of Nucleic Lysis Solution was added to 40mg of maize powder and vortexed to reduce conglomeration. The sample was then incubated for 30 minutes at 65°C with periodic vortexing to expose all the material to the solution. To remove RNA, 3µl of RNase solution was added and the sample inverted to mix. This was followed by incubation for 15 minutes at 37°C and cooling at room temperature. 200µl of Protein Precipitation Solution was added and the sample was vortexed vigorously for 20 seconds. The pellet formed after centrifugation for 3 minutes at 13000rpm was discarded, and the supernatant was carefully transferred to another tube. 600µl isopropanol at room temperature was added and the tube was inverted several times before centrifugation for 1 minute at 13000rpm. The supernatant was decanted and the DNA pellet was washed by inversion with 600µl 70% ethanol at room temperature. After centrifugation for 1 minute at 13000rpm the supernatant was aspirated carefully and the pellet left to air dry for at least 15 minutes. Finally 100µl DNA rehydration solution was added and the extract was incubated for 1 hour at 65°C with periodic mixing. Some extracts were rehydrated with the reduced volume of 50µl and with molecular grade water to replace TE buffer due to the chelating nature of EDTA, depending on the experiment. Genomic DNA extracts were stored at 4°C until required.

2.4.3 CTAB extraction

50mg of powdered plant material was weighed into a 1.5ml tube and 500µl CTAB buffer (2% CTAB (hexadecyltrimethylammonium bromide), 100mM Tris-HCl pH 8.0, 20mM EDTA, 1.4M NaCl) was added and mixed. 5µl β-mercaptoethanol was added and incubated at 65°C for 30 minutes with shaking. To this was added 500µl CIA (24ml chloroform, 1ml isoamyl alcohol) and mixed for 10 minutes on a shaker before centrifuging at maximum speed for 5 minutes at 4°C. The aqueous phase was transferred and the CIA extraction was repeated. The aqueous phase was combined with an equal volume of propan-2-ol and incubated on ice for 5 minutes before centrifuging at maximum speed for 5 minutes. The supernatant was discarded and the pellet washed with 1ml of 70% ethanol at 4°C. The supernatant was discarded and the pellet allowed to dry before it was re-dissolved in 50µl TE buffer (10mM Tris-HCl pH8.0), 1mM EDTA pH8.0) containing 20µg/ml RNase and stored at -20°C.

2.4.4 Phenol:chloroform with ethanol precipitation

Phenol:chloroform:isoamyl alcohol in the ratio 25:24:1 was used to purify DNA extracts. Briefly, the DNA extract and phenol:chloroform reagent were thoroughly mixed then centrifuged to separate the two phases. The salt balance of the removed top phase was adjusted to 0.3M with sodium acetate, before the addition of 100% ethanol, mixed and kept at -20°C for 1 hour. After centrifugation and discarding the supernatant, the pellet was washed with 70% ethanol and dried before it was re-dissolved in 50µl TE buffer, MGW or rehydration buffer.

2.4.5 DNA purification using a Macherey Nagel kit

PCR amplicons were purified using the protocol for PCR clean-up from the Nucleospin® guide of the Macherey-Nagel (Düren, Germany) DNA purification kit. Briefly the amplicon volume was adjusted and a buffer added before transfer to a Nucleospin® column to bind the DNA. The silica membrane was washed and dried and the DNA eluted with 50µl of elution buffer into a final 1.5ml tube.

2.4.6 Chelex extraction

15mg of ground plant material was mixed with 300µl of 5% Chelex® 100 resin from Bio-Rad (CA, USA) for 1 minute followed by vortexing for 10 seconds. Incubation in a

boiling water bath for 5 minutes was followed by vortexing for 10 seconds. The supernatant was removed after centrifuging at 13000rpm for 1 minute (HwangBo et al. 2009).

2.4.7 Lumora fast extraction technique

Fast extraction columns from Lumora (Ely, UK) were used to extract DNA from maize powder. The method follows the procedure for stool samples (McElgunn et al. 2014). Briefly the maize powder was added directly to the column which was sealed before gently shaking to mix. The twist-off tab at the bottom of the column was then snapped off and the column placed into a collection tube, put in a heat block at 100°C and incubated for 10 minutes. The pressure inside the column increases and heat elution gently directs the material through the resins. Inhibitors are bound, cells lysis occurs and liberated DNA is eluted into the collection tube.

2.4.8 Assessing genomic DNA quality

Genomic DNA samples were analysed on a 0.8% TAE agarose gel containing SafeView™ and visualised using UV fluorescence in a Syngene light box. The absorbance of the samples was measured between 220 and 350 nm on a NanoDrop 100 Spectrophotometer. The ratios of 260:230 and 260:280 were recorded as an indication of contamination.

2.4.9 Sequencing template DNA

DNA for sequencing was submitted to Cardiff University DNA Sequencing Core at appropriate concentrations depending on sample type and size. Plasmid DNA was submitted at 50 to 100ng/µl. Genomic DNA was sequenced from PCR amplicons using primers designed to flank the 35Sp, NOSt and ADH1 sequences. The PCR primers were designed to produce amplicons of approximately 200bp which were submitted at a concentration of 2ng/µl (Figure 2.10).

Target, Type, Notation, Version	Length	T_{MELT}	GC Content	Dimer	Secondary	Primer Sequence (5' to 3')
35Sp, Sequencing, Forward	20	63.5°C	45.0%	No	None	AAACCTCCTCGGATTCCATT
35Sp, Sequencing, Reverse	20	63.8°C	55.0%	No	None	GATTTCAGCGTGTCCTCTCC
NOSt, Sequencing, Forward	20	64.1°C	45.0%	No	Weak	TCATCCAGCGTGATTGGTAA
NOSt, Sequencing, Reverse	20	63.7°C	55.0%	No	Weak	AGTCAGCCTCTCGATTGCTC
NOSt, Sequencing, Forward, v2	20	62.5°C	45.0%	No	None	TAAAGAAGGAGTGCGTCGAA
NOSt, Sequencing, Reverse, v2	21	62.1°C	47.6%	No	Weak	CACAGTACCGAAGTTTGATCG
ADH1, Sequencing, Forward	18	66.1°C	55.5%	No	Weak	TGGTTTGCTTGCCCACAG
ADH1, Sequencing, Reverse	20	64.1°C	45.0%	No	None	TTCACCACGATTGCAGGATA

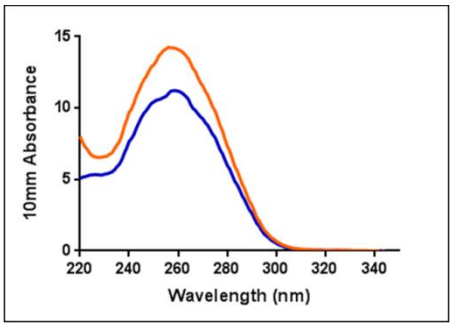
Figure 2.10: PCR primers designed to produce an amplicon of the 35S promoter, NOS terminator and maize ADH1 sequences for sequencing. Values for TMELT, primer dimer and secondary structure formation derived from Sigma technical datasheets accompanying the primers. All primers were designed using Primer3 software.

Sequencing results were aligned to reference sequences using CLC Sequence Viewer v6 workbench.

2.5 Quantification techniques

2.5.1 NanoDrop 1000 spectrophotometer quantification

The NanoDrop 1000 spectrophotometer (Thermo Scientific) was used to quantify double stranded DNA. The absorbance of the sample at 260nm is used to calculate the DNA concentration which is expressed in ng/µl. A small volume of 1 µl of the test sample is required for loading to the device. The absorbance of the sample is shown graphically for the wavelength range from 220 to 350nm (Figure 2.11).The ratio 260:280 gives an indication of the purity of the DNA, a value of approximately 1.8 suggests a sample largely free from contaminants such as protein and phenol which absorb strongly at or near 280nm. The ratio 260:230 should be in the range 2.0 to 2.2 but this may be reduced if there is contamination with carbohydrates, EDTA and other contaminants that absorb at 230nm.



Sample ID	ng/ul	260/280	260/230
1	704.54	1.92	2.14
2	559.10	1.87	2.08

Figure 2.11: Graphical trace of the absorbance of two double stranded genomic DNA samples to show the peak at 260nm (sample 1 in orange and sample 2 in blue). Table of results with DNA concentration in ng/µl and ratios 260:280 greater than 1.8 and 260:230 between 2.0 and 2.2 for both samples.

2.5.2 Agarose gel quantification

Agarose gel quantification followed the method from Eurogentec with SmartLadder. To a 0.8% TAE agarose gel with SafeView nucleic acid stain was added 5µl of the 10kb SmartLadder and to the other lanes 10µl containing 2µl test sample, 1µl loading dye and 7µl MGW. Electrophoresis was run for 1 hour at 100V and the DNA bands visualised with a UV transilluminator. The gel image was analysed using ImageJ software to calculate the intensity of the DNA bands for the ladder and the test samples and to quantify in the range 15 to 100ng.

2.5.3 Agilent Bioanalyzer

The plasmids pART7, pUC35S GUS and pUC35S ADH1 and an amplicon of maize ADH1 were quantified with the Agilent 2100 Bioanalyzer from Agilent Technologies (Waldbronn, Germany) for DNA concentrations between 0.5 and 50 ng/µl and DNA sized between 50 and 10380 base pairs. This method is therefore not suitable for the quantitation of large genomes. This lab-on-a-chip technology uses electrophoresis to separate nucleic acid fragments by size within a DNA chip containing interconnected microchannels. The sample and ladder results are analysed to give sample size and quantification (Figure 2.12).

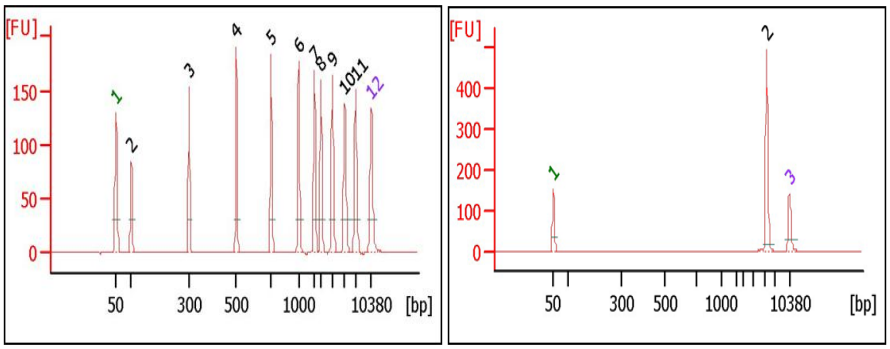


Figure 2.12: Electropherogram results for the ladder peaks between 50 and 10380 base pairs and the upper and lower markers and the DNA sample indicated by the number 2.

2.5.4 Quantitative polymerase chain reaction (qPCR)

Genomic and plasmid DNA samples were assayed by real time quantitative polymerase chain reaction using a Corbett Rotor-Gene thermal cycler with SYBR® Green JumpStart™ Taq ReadyMix™ from Sigma (Poole, UK). The reaction volume of 20µl consisted of 5µl of template DNA or negative control, 10µl of JumpStart, 1µl of each primer (100µM) and 3µl MGW. The parameters were set at 94°C for 2 minutes for activation of the hot-start Taq polymerase, followed by 40 cycles of 94°C (30 seconds), 50°C (30 seconds) and 72°C (30 seconds). Melt analysis followed the thermal cycling. Data was analysed with the Rotor-Gene 6000 software and with Microsoft Excel.

2.5.4.1 CaMV 35S promoter primer design

Forward and reverse primers for PCR and qPCR were synthesised by Sigma (Poole, UK) and are listed in figure 2.13.

Target, Type, Notation, Version	Length	T_{MELT}	GC Content	Dimer	Secondary	Primer Sequence (5' to 3')
35Sp, qPCR, Forward, P3	20	63.0°C	50.0%	No	Very Weak	GAAGGGTCTTGCGAAGGATA
35Sp, qPCR, Reverse, P3	20	64.4°C	50.0%	No	Weak	ACAGTGGTCCCAAAGATGGA
35Sp, qPCR, Forward, GK	20	63.3°C	50.0%	No	Very Weak	GATTCCATTGCCCAGCTATC
35Sp, qPCR, Reverse, GK	20	63.7°C	45.0%	No	None	CAACGATGGCCTTTCCTTTA
35Sp, qPCR, Forward, M1*	25	64.5°C	40.0%	Yes	Weak	ATTGATGTGATATCTCCACTGACGT
35Sp, qPCR, Reverse, M1*	25	66.2°C	40.0%	No	Very Weak	CCTCTCCAAATGAAATGAACTTCCT
35Sp, qPCR, Forward, M3‡	21	63.1°C	42.9%	No	Very Weak	CGTCTTCAAAGCAAGTGGATT
35Sp, qPCR, Reverse, M3‡	22	65.6°C	45.5%	No	Very Weak	TCTTGCGAAGGATAGTGGGATT

Figure 2.13: qPCR primers for the 35S promoter sequence. Values for TMELT, primer dimer and secondary structure formation derived from Sigma technical datasheets accompanying the primers. Primers marked with 'GK' are from (Kiddle et al. 2012), primers marked M1* are from (Kuribara et al. 2002) and those marked M3‡ are from (Fernandez et al. 2005). All others were designed using Primer3 software. Evaluation of 35Sp primers in qPCR from (Holden et al. 2009).

2.5.4.2 Optimisation of qPCR for 35Sp assays

The primers for the 35S promoter target denoted M3 (Fernandez et al. 2005) were selected at a concentration for each primer of 5µM in the total assay volume. Melt curve analysis was used to analyse the results for primer interactions and contamination in negative controls. Cycle threshold (C_t) values were used against cycle copy number to calculate amplification efficiency of the qPCR by the following equation:

$$\text{Efficiency} = -1+10^{(-1/\text{slope})}$$

Amplification efficiency between 90% and 110% are acceptable.

2.5.5 Estimating copy number

The estimations of the copy number of double stranded DNA can be calculated using the following equation once the mass and length is known:

$$\text{Copies of target} = \frac{\text{ng of double stranded DNA x Avogadro's constant } (6.022 \times 10^{23})}{\text{Length in base pairs} \times 10^{9} \times 650 \text{ Daltons}}$$

For the maize genome the length is assumed to be 2.4×10^9 base pairs and the genome diploid. Calculations of copy number for the plasmid pART7 assumed the length is 4900bp and 5497bp for pUC35S GUS (5497bp) and 4330bp for pUC35S ADH1. The online copy number calculator was used at http://www.uri.edu/research/gsc/resources/cndna.html.

2.6 LAMP-BART assays

2.6.1 BARTmaster assays

Freeze-dried BARTMaster is made by Lumora (Ely, UK) and contains all the components for 25 LAMP-BART reactions at 5µl with the exception of an amplification buffer, water and the primers and template. Initial work with maize genomic DNA and CaMV 35Sp and maize ADH1 primers used the following method: To each BARTMaster was added 188µl of Lumopol from Lumora, 7.5µl of additional *Bst* polymerase large fragment, 130µl molecular grade water and 50µl of LAMP primer mix. To each of the plate partitions 15µl of this master mix was added and 5µl of the test sample or controls.

For some of the digital LAMP-BART assays a version of BARTMaster called LAMP-20 was used to enable 5µl total assay volume per 384 partitions. To one pot of LAMP-20 200µl of isothermal amplification buffer (NEB) was added and 1672µl of molecular grade water. The volume was transferred to an ultra non-stick 2ml tube (Alpha Laboratories) and combined with 30µl *Bst* polymerase large fragment, 16µl of each LAMP primer, 8µl of each loop primer, 4µl of each displacement primer, 2µl of 10mg/ml salmon sperm DNA (Invitrogen) and 40µl of template. The master mix plus template was mixed by inversion and centrifuged at 14000rpm for 1 minute. The tube was inserted into the Qiagen robot for 384 partition plate loading.

2.6.2 Non-BARTmaster assays

The individual aliquots of the reagents were combined to give final concentrations in the LAMP-BART assays of: 87 mM trehalose, 10 mM DTT, 0.4 mg/ml polyvinylpyrrolidone, 60 mM KCl, 300 µM each dNTP, 100 µg/ml luciferin, 250 µM adenosine-5'-O-phophosulphate, 5.5 µg/ml Ultra-Glo™ recombinant luciferase, 375 mU/ml ATP sulphurylase, 0.32 U/µl Bst polymerase large fragment, 1.6 µM each LAMP primer, 0.8 µM each loop primer and 0.4 µM each displacement primer diluted in Thermopol buffer (NEB). For a final reaction volume of 20µl, 5µl of DNA template or negative sample was added. The final volume was covered with two drops of mineral oil to prevent evaporation during the assay. The plate was sealed with a clear plastic adhesive cover and assayed on the Cardiff 'Lucy' or the Lumora 'Bison' platform normally at 60°C for 92 scans. The light output from 1 minute time integrals was collected for each analysed plate partition and saved as a Microsoft Excel .csv file.

2.6.3 Primer set 1: CaMV 35S promoter (35Sp)

Displacement primers F3 and B3 (Eiken notation), LAMP primers FIP and BIP and loop primers F-loop and B-loop (Figure 2.14), were designed to target the cauliflower mosaic virus 35S promoter sequence (GenBank accession number X79465). The promoter is present in the majority of transgenic maize events. The primers were stored at -20°C until required at a concentration of 100pmoles/µl.

Type, Name, Version	Length	T_{MELT}	GC	Dimer	Secondary	Primer Sequence (5' to 3')
Disp., F3, v1*	15	57.0°C	60.0%	No	Very Weak	AGGAAGGAAAGGCCATCG
Disp., F3, v3	20	50.7°C	40.0%	No	Very Weak	CTTATATAGAGGAAGGGTCT
Disp., B3, v1*	18	59.1°C	44.4%	No	None	ATAAAGGAAAGGCCATCG
Disp., B3, v2‡	18	51.2°C	33.3%	No	None	ATAAAGGAAAGGCTATCA
Disp., B3, v3	21	55.6°C	33.3%	No	Weak	GATAAAGGAAAGGCTATCATT
Disp., B3, v4	20	61.2°C	40.0%	No	None	ATAAAGGAAAGGCCATCGTT
LAMP, FIP, v1*	39	81.0°C	46.1%	No	Weak	GTCTTCAAAGCAAGTGG-TTTT-GGATAGTGGGATTGTGCG
LAMP, FIP, v2a‡	41	85.4°C	53.6%	No	Strong	CCACGTCTTCAAAGCAAGTGGGGATAGTGGGATTGTGCGTC
LAMP, FIP, v2‡	45	85.3°C	48.8%	No	Strong	CCACGTCTTCAAAGCAAGTGG-TTTT-GGATAGTGGGATTGTGCGTC
LAMP, BIP, v1*	37	86.2°C	56.7%	No	Strong	TTCCACGATGCTCCTCG-TTTT-CCTCTGCCGACAGTGG
LAMP, BIP, v2	38	84.9°C	52.6%	No	Strong	TTCCACGATGCTCCTCG-TTTT-TCTCTGCCGACAGTGGT
Loop, F-Loop, v1*	16	55.0°C	56.2%	No	None	TCCACTGACGTAAGGG
Loop, B-Loop, v1*	16	61.4°C	62.5%	No	Weak	GGGGTCCATCTTTGGG

Figure 2.14: LAMP primers for 35S promoter sequence. Values for TMELT, primer dimer and secondary structure formation derived from Sigma technical datasheets accompanying the primers. Primers marked with * were designed by (Lee et al. 2009) and those with ‡ were designed by Guy Kiddle (Lumora)

2.6.4 Primer set 2: *Zea mays* alcohol dehydrogenase 1 (ADH1)

Displacement primers, lamp primers and LAMP primers for loop-mediated amplification were designed to target the *Zea mays* alcohol dehydrogenase reference gene ADH1 sequence (Figure 2.15; GenBank accession number NM_001111939). The primers were stored at -20°C until required at a concentration of 100pmoles/µl.

Type, Name, Version	Length	T_{MELT}	GC Content	Dimer	Secondary	Primer Sequence (5' to 3')
Disp., F3, v1‡	19	59.5°C	42.1%	No	None	CTTTGGATCGATTGGTTTC
Disp., F3, v2	20	65.9°C	45.0%	No	None	CGCTTTGGATCGATTGGTTT
Disp., B3, v1‡	17	53.7°C	41.1%	No	None	CCCAAAATTACTCAACG
Disp., B3, v2	20	61.3°C	40.0%	No	None	CCCCAAAATTACTCAACGAA
LAMP, FIP, v1‡	47	84.6°C	49.0%	No	Moderate	CCCCTCCGCAAATCTTCGAACAG-TTTT-GTAACTGGTGAAGGACTGAG
LAMP, FIP, v2	47	85.5°C	51.0%	No	Strong	CCCCTCCGCAAATCTTCGAACAG-TTTT-GTAACTGGTGAGGGACTGAG
LAMP, FIP, v3	44	83.6°C	50.0%	No	Strong	CCCTCCGCAAATCTTCGAAC-TTTT-GTAACTGGTGAGGGACTGAG
LAMP, BIP, v1‡	50	79.6°C	38.0%	No	Moderate	GGTGATCAAGTGCAAAGGTC-TTTT-CATAAACCAAGATTAGTCAGATCAAG
LAMP, BIP, v2	46	79.6°C	41.4%	No	Moderate	GGTGATCAAGTGCAAAGGTC-TTTT-CCAAGATTAGTCAGATCAAGTC
Loop, F-Loop, v1‡	20	63.9°C	55.0%	No	None	CGCCTTGTTTCTCCTCTGTC
Loop, F-Loop, v2	16	50.4°C	50.0%	No	None	CCTTGTTTCTCCTCTG
Loop, B-Loop, v1‡	21	65.7°C	52.4%	No	Weak	CCAAATCATCCACTCCGAGAC
Loop, B-Loop, v2	22	66.7°C	50.0%	No	None	CCAAATCAATCCACTCCGAGAC
Loop, B-Loop, v3	16	54.2°C	56.2%	No	None	CAATCCACTCCGAGAC

Figure 2.15: LAMP primers for maize ADH1 reference gene sequence. Values for TMELT, primer dimer and secondary structure formation derived from Sigma technical datasheets accompanying the primers. Primers marked with ‡ were designed by Guy Kiddle (2012).

2.6.5 Primer set 3: Nopaline synthetase terminator (NOSt)

A final set of primers for loop-mediated amplification were designed to target the *Agrobacterium tumefaciens* nopaline synthetase terminator NOSt sequence (Figure 2.16; GenBank accession number V00087;41). The primers were stored at -20°C until required at a concentration of 100pmoles/µl.

Type, Name, Version	Length	T_{MELT}	GC Content	Dimer	Secondary	Primer Sequence (5' to 3')
Disp., F3, v1*	22	59.2°C	36.3%	No	Weak	CGCGATAATTTATCCTAGTTTG
Disp., F3, v2	20	57.5°C	40.0%	No	Weak	GCGCGATAATTTATCCTAGT
Disp., B3, v1*	19	61.0°C	36.8%	No	None	CGTTCAAACATTTGGCAAT
Disp., B3, v2	20	63.5°C	40.0%	No	None	GATCGTTCAAACATTTGGCA
LAMP, FIP, v1*	52	80.6°C	36.5%	Yes	Moderate	GCATGACGTTATTTATGAGATGGG-TTTT-CGCTATATTTTGTTTTCTATCGCG
LAMP, FIP, v2	46	76.0°C	30.4%	No	Very Weak	GCATGACGTTATTTATGAGA-TTTT-TCGCGCTATATTTTGTTTTCTA
LAMP, BIP, v1*	44	81.0°C	40.9%	No	Moderate	CATGCTTAACGTAATTCAACAG-TTTT-TGAATCCTGTTGCCGGTC
LAMP, BIP, v2	43	80.6°C	39.5%	No	Moderate	CATGCTTAACGTAATTCAACA-TTTT-TGAATCCTGTTGCCGGTC
Loop, F-Loop, v1*	22	58.2°C	40.9%	No	None	GATTAGAGTCCCGCAATTATAC
Loop, F-Loop, v2	20	57.2°C	40.0%	No	None	GATTAGAGTCCCGCAATTAT
Loop, B-Loop, v1*	23	56.8°C	21.7%	No	Weak	AAATTATATGATAATCATCGCAA
Loop, B-Loop, v2	22	54.5°C	27.2%	No	Weak	GAAATTATATGATAATCATCGC

Figure 2.16: LAMP primers for NOS terminator sequence. Values for TMELT, primer dimer and secondary structure formation derived from Sigma technical datasheets accompanying the primers. Primers marked with * were designed by (Lee et al. 2009).

2.6.6 Hardware for LAMP-BART

There are a number of light detection devices with controlled temperature wells that have been developed for BART. These are the 'LUCY', the 'PDQ', the 'BISON' and the 3M™ Molecular Detection System from 3M Food Safety (MN, USA). Further details of the 3M™ Detection System can be found in the validation of the system for the detection of *Listeria* (Fortes et al. 2013).

2.6.6.1 LUCY for 96 well plates

The 'LUCY' (Figure 2.17) is composed of a programmable TRobot thermal cycler from Biometra (Göttingen, Germany) within a Syngene Chemi Genius Bio Imaging System from Synoptics (Cambridge, UK). A CCD camera above the thermal cycler is used to record the light output from each well, using specially designed React IVD software developed by Synopics. The software converts the output of light into numerical values

in a Microsoft Excel spreadsheet. The 'LUCY' was used for a maximum of 96 BART assays over a range of temperatures and time integrals.

Figure 2.17: Photograph of a LUCY detection system for BART to show the position of the TRobot thermal cycler within Syngene Bio Imaging System.

2.6.6.2 Qiagen robotics

For digital LAMP-BART assays on 384 well plates, a QIAgility robot from Qiagen (Limburg, Netherlands) was used to set-up the assay before manual loading of mineral oil. The robot was programmed to deliver 5µl per partition of the reaction mix via pipette tips to the microtitre plate positioned in a pre-cooled manifold. The remaining volume of reaction mix was confirmed with a visual inspection for correct loading.

2.6.6.3 BISON for 384 well plates

For digital LAMP-BART assays in Lumora, the 'BISON' platform (Figure 2.18) was used for the applicability to 384 well plates. The device developed by Lumora couples assay heating control to a CCD camera and an algorithm to interrogate the BART signals to generate positive and negative decisions for each sample.

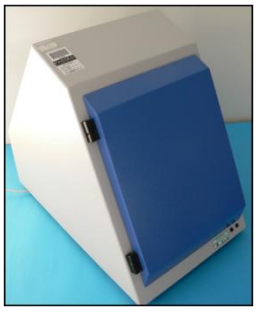


Figure 2.18: Photograph of a BISON detection system for BART. Within the blue door is a heating block which can be modified for 96, 384 and 1536 well plates and a CCD camera.

2.6.7 Lumora inhibitor test strips

To test samples for inhibition, single shot inhibitor controls supplied by Lumora were used. Each inhibitor control tube contained LAMP-BART reagents, a set of LAMP primers and the specific supertemplate associated with them. Each assay required the addition of 15µl of reconstitution buffer allowing 10 minutes for reconstitution. 5µl of the sample was added before a few drops of mineral oil and 60°C LAMP-BART run conditions. Deviation from the control average T_{max} value of 17 minutes was indicative of inhibition by the sample.

2.7 Amplification visualisation

2.7.1 Agarose gel for LAMP products

For LAMP amplicon resolution, a 2% agarose gel was prepared with 2g agarose and 100ml of 1x TAE buffer, melted and cooled to approximately 60°C. To visualise the LAMP ladder pattern from positive results without the large amount of fluorescence from luciferin, SafeView nucleic acid stain was replaced with GelRed Biotium from Cambridge Bioscience (Cambridge, UK). 10µl of 10000x GelRed was added to 100ml of agarose gel. To the outer gel lanes was added 5µl 10kb SmartLadder from Eurogentec (Seraing, Belgium) to approximate the size of LAMP products. The lanes in between were loaded with 10µl of sample, MGW and loading buffer in a 5:5:1 ratio. Electrophoresis of the DNA was for 40 minutes at 100V. The DNA bands were visualised either with a UV or blue-light transilluminator and photographed.

2.7.2 Excising gel bands and purifying the DNA

The gel bands visualised on a blue-light transilluminator with an amber filter were cut out with a fresh razor blade and transferred to a 1.5ml tube. The DNA was purified from the agarose using the protocol for DNA extraction from agarose gels from the Nucleospin® guide of the Macherey-Nagel (Düren, Germany) DNA purification kit. Briefly the gel slice is solubilised and transferred to a Nucleospin® column to bind the DNA. The silica membrane was washed and dried and the DNA eluted with an elution buffer into a final 1.5ml tube.

2.8 Data analysis and bioinformatics

2.8.1 Software for analysis of results

LAMP-BART assay results were saved as raw data files in Microsoft Excel .csv format. Statistical analysis of the results was with Microsoft Excel 2007 and GraphPad Prism version 6 was used for all graphical output. Analysis of data from the Corbett Rotor-Gene thermal cycler used the accompanying Rotor-Gene 6000 series software version 1.7 for quantitation and melt curve analysis. The data was also analysed with Microsoft Excel 2007 and graphs produced with GraphPad Prism version 6. The Agilent Bioanalyser produced results in a .pdf format which were viewed with Adobe Reader XI. Sequencing data was analysed using CLC Sequence Viewer v6 workbench or MultAlin multiple sequence alignment (Corpet 1988).

2.8.2 Random number generation

To randomly pick a result from the single copy number LAMP-BART assays, the random integer set generator was used at www.random.org operated by Randomness and Integrity Services Ltd.

2.8.3 Digital PCR statistics

For digital PCR Poisson correction the web-based application uCount^{SM} was used to calculate copy numbers per partition within confidence levels. The application is found at www.dna.utah.edu/ucount/uc.html with guidelines for digital PCR (Huggett et al. 2013).
Without confidence levels, the mean copy number per partition can be calculated by using the equation below (Dube et al. 2008):

Copies per partition = –ln ((total partition - positives) / total partitions)

Amplification efficiency for digital PCR and BART reactions was assumed to be 100%. The calculator of amplification efficiency on the Agilent website uses the gradient of the slope from the PCR calibration curve to calculate this.
http://www.genomics.agilent.com/biocalculators/calcSlopeEfficiency.jsp

2.8.4 Bioinformatics

A number of useful informatic tools were used from the MaizeGDB website focused on *Zea mays* including Genome Browser and BLAST. The Basic Local Alignment Search Tool was also used at the National Library of Medicine website.

The European Nucleotide Archive (ENA) at http://www.ebi.ac.uk/ena/home was used to find reference nucleotide sequences with GenBank accession numbers from sequence data.

LAMP-BART primer sequences were provided by Lumora or were previously published. The later versions of these primers used the calculations of melt temperature, GC content, primer length, secondary structure and potential primer-dimerisation from Sigma's website.

Chapter 3
LAMP-BART Quantification

3.1 Introduction

Quantitation using BART coupled with loop-mediated amplification (LAMP) was previously described by Gandelman (2010) and shows that the time-to-peak of light output is proportional to the target concentration. The light profile is characterised by an initial flash of light from the luciferase followed by a baseline level of luminescence which is maintained for assays without template. For positive results the light output increases exponentially from the baseline until the concentration of inorganic pyrophosphate reaches a critical level for the inhibition of luciferase. The result is a peak of light corresponding to a particular time interval; the time-to-peak or T_{max}. Replicate assays are used to determine average T_{max} and they also indicate the variance between time-to-peak values for copy numbers per partition. The variation between replicates observed with qPCR assays can be extremely low which results in quantification to low copy numbers. Assays using LAMP are characterised by increasing variance with decreasing copy number (which is described in more detail in Chapter 4) and this results in high limits of quantification (LOQ) when compared to the benchmark technology of qPCR.

The limit of detection (LOD) and limit of quantitation (LOQ) for qPCR have yet to achieve a generic definition (Nutz et al. 2011). LOD for qPCR is commonly defined as the lowest target concentration at which 95% of the replicate assays are detected. Stochastic variation at very low target copy number limits the sensitivity of qPCR to 3 copies per partition (Bustin et al. 2009). However digital PCR can be used to assay lower copy numbers. The LOQ of a qPCR quantification assay can be defined by the lowest concentration of the linear dynamic range of cycle threshold values against log copy number; which is normally 5 to 6 orders of magnitude. These definitions for LOD and LOQ have been used for LAMP-BART assays.

From the International Service for the Acquisition of Agri-biotech Applications (ISAAA) website (www.isaaa.org/gmapprovaldatabase/cropevents/) as of 2012 there were 65 approved maize events. Three of these were available as kernels or powdered seeds in the lab: Bt-11 from Syngenta Seeds, MON810 from Monsanto and SmartStax from

Dow AgroSciences. MON810 is the simplest of the events containing one trait for resistance to corn borers from the gene cry1Ab. The event was designed to contain the transgenic cassette with the NOS terminator but this failed to insert (Rosati et al. 2008). Bt-11 contains two traits; in addition to the cry1Ab gene is a further gene to confer tolerance to glufosinate herbicide. SmartStax is an example of a stacked trait (Que et al. 2010) and is the result of conventional cross-breeding of events MON88017, MON89034, TC1507 and DAS59122-7.

For any detection strategy there is a requirement for extracting the genomic DNA. For GM crop material this may be from leaf tissue in a field environment or from a food product in a laboratory. Extraction methods have been compared for both qPCR testing (Elsanhoty et al. 2010) and for LAMP amplification (Kiddle et al. 2012) for which the Promega Wizard Genomic DNA purification kit produced improved results. Rapid extraction methods for plant genomic DNA have been developed using phenol:chloroform (Kang and Yang 2004) and can be completed within 30 minutes. Faster still is the extraction method based on boiling chelating resins (McElgunn et al. 2014) which may be viable for GM testing.

This project used three GM assays to target CaMV 35S promoter, NOS terminator and the maize ADH1 reference gene. The main focus was on the 35S promoter which is common to the majority of maize events and is frequently targeted in GM screening with qPCR (Holden et al. 2009). The promoter is well characterised and strongly and constitutively expresses the trait of interest. Increased expression has been shown by duplication of the 35S promoter in the insert (Kay et al. 1987) and enhanced 35S promoters have been developed which have a truncated duplication of the promoter upstream of the complete sequence. Transcriptional enhancement has also been achieved with chimeras of the 35S promoter (Patro et al. 2012). These adaptations have not changed target sequences for the PCR primers, but the discovery of a single nucleotide polymorphism (SNP) within the 35S promoter sequence of maize event TC1507 (Morisset D. et al. 2009) illustrated how the sensitivity of an assay can be greatly reduced by such a sequence alteration leading to inaccurate results.

In this chapter the aim was to improve the sensitivity and variation between replicates in LAMP-BART assays of various templates to quantify over a wide linear dynamic range of template concentrations using average T_{max}. Using primers designed for GM detection in maize, the objective was to improve quantification of maize genomic DNA

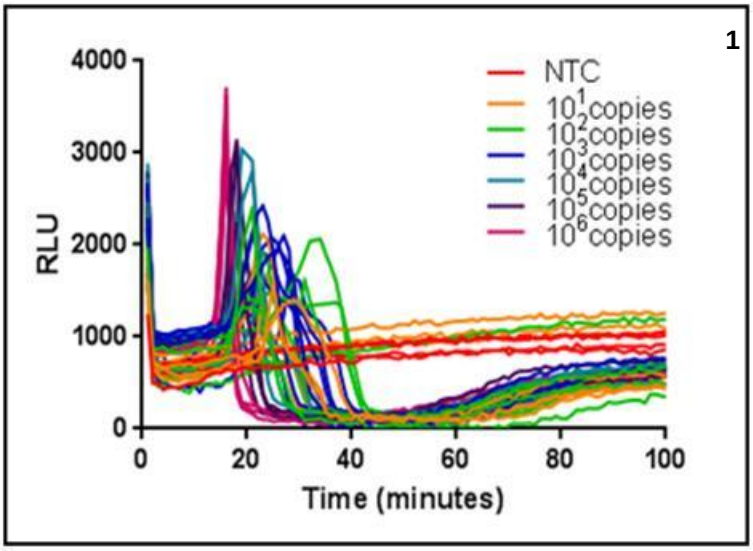
by understanding in greater detail, the parameters that affect LAMP-BART quantification of artificial template.

3.2 LAMP-BART assays of artificial template

In this section two plasmid linearised DNA templates and an amplicon from a low molecular weight LAMP ladder band were assessed for sensitivity and reproducibility over a wide range of copy numbers. As well as detection of template copies, the aim was to investigate the limit of quantitation of these templates using time-to-peak calculations.

3.2.1 LAMP-BART assay of linearised plasmid DNA (pUC35S GUS)

The first template investigated was the plasmid pUC35S GUS from lab stocks, which contains the 35Sp sequence and was linearised and purified before titration. The initial quantification was by NanoDrop and calculations of copy number were based on those values. Using the original version of 35Sp LAMP primers, the serially diluted template was assayed with LAMP-BART (Figure 3.1).



copies	mean	SD	cv	fastest	s/rate
10^1	24.69	2.69	11%	22.74	63%
10^2	24.75	5.46	22%	20.58	88%
10^3	24.36	2.58	11%	20.58	100%
10^4	20.58	0.88	4%	19.5	100%
10^5	18.41	0.00	0%	18.41	100%
10^6	16.25	0.00	0%	16.25	100%

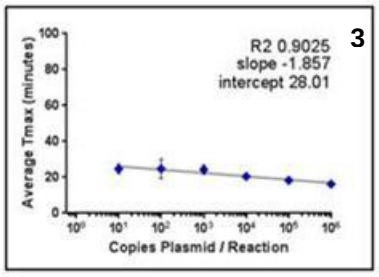
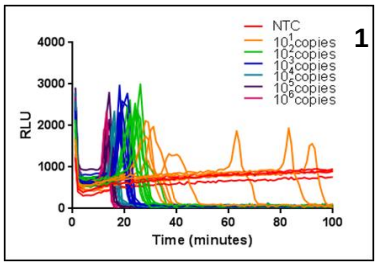


Figure 3.1: LAMP-BART 35Sp assay of linearised pUC35S GUS plasmid (1) The chart shows the light output against assay time for plasmid template concentrations of 10^6 copies per partition (in purple) to 10 copies per partition in orange, no template control (NTC) samples in red. The numbers of replicates are: 4 for 10^6, 10^5 and 10^4, NTC and 8 for 10^3, 100 and 10 copies per partition. (2) The summary results table shows the average $T_{max,}$ standard deviation, coefficient of variation, fastest T_{max} value and the success rate for each copy number (3) The graph shows the average T_{max} plotted against the target copy number from 10 copies to 10^6 copies per partition.

The serial dilution from 1 million to 10 copies per partition showed 100% amplification frequency and low variation between replicates for 10^6 to 10^3 copies per partition. At copy numbers below 1000 the variation increases and the amplification frequency reduces. This also suggests that the limit of quantitation is between 10^4 and 10^3 copies per partition. Although the correlation to the semi-log line for average T_{max} against copy number is greater than 0.9, the slope is shallow and quantitation could be limited. The LAMP-BART assay of this template successfully detected 10 copies of the template per partition with 63% amplification frequency, but the variability between replicates is too high at this level for quantification.

3.2.2 Linearised plasmid DNA (pART7)

An alternative to the linearised plasmid target pUC35S GUS is the linearised pART7 plasmid which is described in more detail in Chapter 4. Initial quantification of this plasmid was based on results from the laboratories in Cardiff and Ely using both the Agilent Bioanalyzer and NanoDrop. Individual homologous dried frozen aliquots were prepared for single use of defined template copy number per µl. As with the previous plasmid template, the original version of 35Sp LAMP primers was used and the serially diluted template was assayed with LAMP-BART (Figure 3.2).



copies	mean	SD	cv	fastest	s/rate
10^1	48.82	26.66	55%	28.14	100%
10^2	23.55	1.71	7%	21.66	100%
10^3	18.95	0.82	4%	18.41	100%
10^4	15.71	0.62	4%	15.17	100%
10^5	14.09	0.00	0%	14.09	100%
10^6	13.01	0.00	0%	13.01	100%

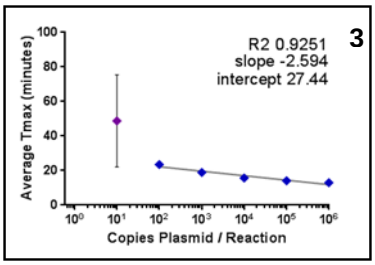


Figure 3.2: LAMP-BART 35Sp assay of linearised pART7 plasmid (1) The chart shows the light output against assay time for plasmid template concentrations of 10^6 copies per partition (in purple) to 10 copies per partition in orange, no template control (NTC) samples in red. The numbers of replicates are: 4 for 10^6, 10^5 and 10^4, NTC and 8 for 10^3, 100 and 10 copies per partition. (2) The summary results table shows the average $T_{max,}$ standard deviation, coefficient of variation, fastest T_{max} value and the success rate for each copy number (3) The graph shows the average T_{max} plotted against the target copy number from 10 copies to 10^6 copies per partition.

The LAMP-BART 35Sp assays of the serial dilution of the pART7 template from 1 million to 10 copies per partition showed 100% amplification frequency. However the variation between replicates was high for the 10 copies per partition assay. Between 10^6 and 10^2 copies per partition the correlation to the semi-log line was strong and the slope is steeper than observed with the other plasmid template allowing for improved differentiation between copy numbers. The limit of quantitation appears to be at

approximately 100 copies per partition which is an improvement on the LOQ suggested by the pUC35S GUS data.

The linearised pART7 plasmid was selected as the model template for improvements to limit of detection and quantification, reproducibility and LAMP-BART kinetics.

3.2.3 Low molecular weight LAMP amplicons

A feature of LAMP isothermal amplification is the formation of amplicons of increasing size which can be visualised on an agarose gel as a ladder pattern. This distinctive pattern was used to confirm LAMP amplification from non-specific primer interactions. The formation of low molecular weight products will form after amplification initiation which is potentially a time limiting step giving rise to variation in time-to-peak values. Sizing these products and relating them to specific hairpin structures is problematic due to the combination of single and double stranded DNA and secondary structure, but a low molecular weight band should show low variation between replicates because the amplification initiation step has been overcome. In this example a band corresponding to approximately 250bp on the ladder was carefully excised from an agarose gel using a blue light transilluminator, purified and NanoDrop quantified. The conversion of the DNA concentration to copy numbers of the template was a guideline only due to the uncertain DNA size, separation from other amplicons and initial quantification value. Therefore the results are plotted against the dilution factor (Figure 3.3).

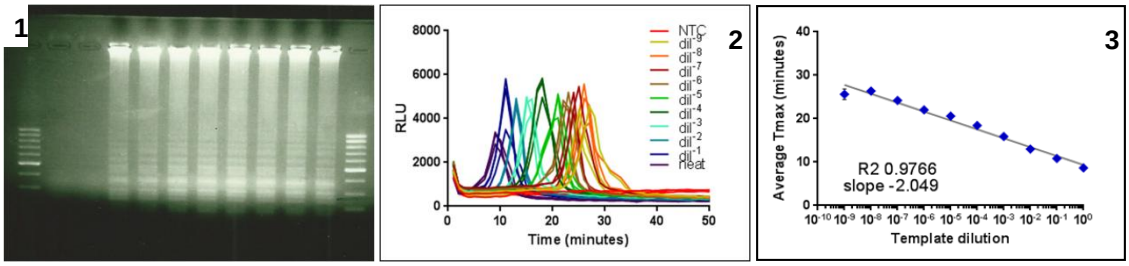


Figure 3.3: Agarose gel of 35Sp LAMP amplicons with 1kb SmartLadder, band at approximately 250bp excised, purified and quantified (2) LAMP-BART 35Sp assay of diluted purified LAMP amplicon (3) average T_{max} against serial dilution of purified LAMP amplicon

For each dilution there were three replicates and the serial dilution went as low as 10^{-9} which as a guideline represented only a few copies. The variation between replicates that had been previously observed with very dilute samples was not evident with these dilutions. However, the uncertain template copy number and identity of the LAMP amplicon are not appropriate for a model template for further study. Also the high concentration of this template produced by LAMP amplification reinforces the

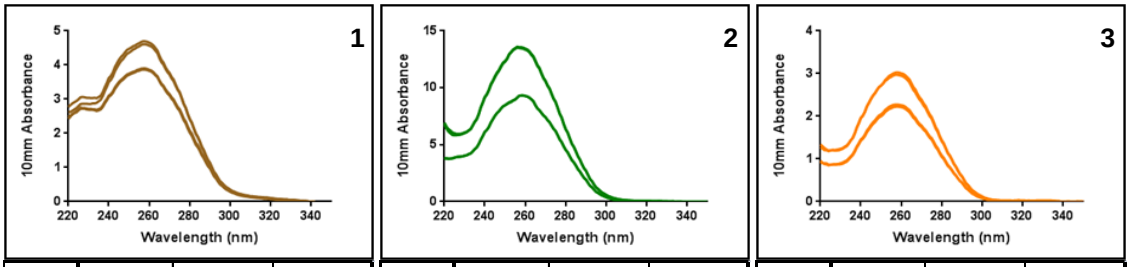
requirement to keep amplified product areas away from LAMP-BART assay preparation and therefore to use this type of template could be very contaminating.

3.3 LAMP-BART assays of genomic template

In this section, the purity and the quality of the maize genomic DNA from different sources was assessed. The effect of genomic DNA purity was investigated further in section 5.6.1.4 of Chapter 5. The aim was to select a source of maize genomic template to investigate the parameters that affect limit of detection, amplification frequency, variability between replicates and quantitation.

3.3.1 Source of plant DNA

Attempts to germinate the seeds from maize event MON810 were unsuccessful possibly due to the age and storage conditions that preceded planting in soil, in agar or on damp filter paper. The multiple trait maize event SmartStax® did however germinate and was grown for two weeks in the dark to provide etiolated leaf and root tissue. Approximately 40mg of either SmartStax® maize seeds, leaf or root tissue were extracted using the Promega Wizard genomic DNA kit and the resulting genomic DNA assessed with the NanoDrop spectrophotometer (Figure 3.4).



seed	ng/ul	260:230	260:280
1	228.47	1.60	1.88
2	232.92	1.53	1.93
3	192.93	1.42	1.85
4	191.56	1.42	1.89
mean		1.49	1.89
SD		0.09	0.03

leaf	ng/ul	260:230	260:280
1	464.31	2.05	2.3
2	463.90	2.04	2.31
3	667.62	2.10	2.22
4	672.11	2.11	2.21
mean		2.08	2.26
SD		0.04	0.05

root	ng/ul	260:230	260:280
1	110.68	2.11	2.53
2	112.98	2.09	2.52
3	150.27	2.09	2.47
4	147.54	2.08	2.38
mean		2.09	2.48
SD		0.01	0.07

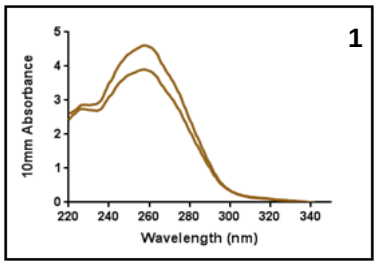
Figure 3.4: NanoDrop results for DNA extracts from (1) maize seeds (2) maize leaf material (3) maize root tissue

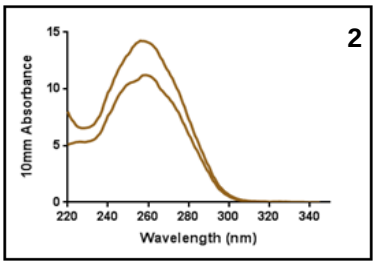
The ratios 260:280 and 260:230 which can indicate contamination of the DNA are higher for the genomic DNA originating from etiolated root and leaf tissue. The ratio 260:230 for maize seeds is low and is indicative of contamination that could influence LAMP-BART reactions. The high yield of DNA from leaf tissue may be affected by non-

template DNA derived from chloroplasts which will add to the quantification by NanoDrop.

3.3.2 Purification of genomic DNA

Maize kernels are more likely to be the origin of the genomic DNA used for GMO testing of food stuffs, than leaf and root material. The purity of the genomic DNA derived from maize kernels, extracted with a Promega Wizard genomic DNA extraction kit was shown to be improved by refinement of extracts with phenol:chloroform followed by ethanol precipitation. The NanoDrop spectrophotometer was used to assess the purity (Figure 3.5).





seed	ng/ul	260:230	260:280
1	230.70	1.57	1.91
2	192.25	1.42	1.87
mean		1.49	1.89
SD		0.10	0.02

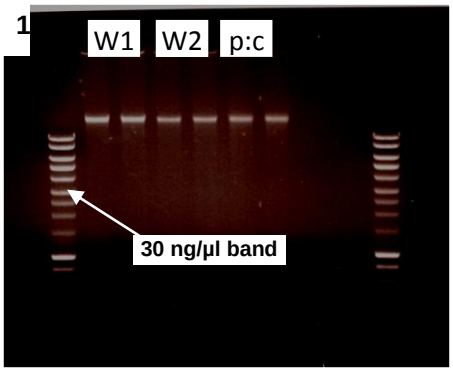
seed	ng/ul	260:230	260:280
1	704.54	2.14	1.92
2	559.10	2.08	1.87
mean		2.11	1.90
SD		0.04	0.04

Figure 3.5: NanoDrop results for DNA extracts from (1) maize seeds (2) maize seeds refined with phenol:chloroform (3) and (4) summary results tables

The ratio 260:280 indicated a slightly improved value from an already high degree of DNA purity. However, the ratio 260:230 showed a marked improvement in contamination reduction.

3.3.3 Assessing the quality of genomic DNA

As well as using the NanoDrop to assess the purity of the genomic DNA used in LAMP-BART and PCR assays, agarose gel electrophoresis was also used (Figure 3.6).



Sample	NanoDrop (ng/µl)	260:230	260:280	Gel (ng/µl)
Wizard 1	264	1.51	1.73	>30
Wizard 2	221	2.25	1.92	28
phenol:chloroform	223	2.56	1.90	26

2

Figure 3.6: Agarose gel electrophoresis of (L to R in duplicate) (W1) Promega Wizard extracted maize genomic DNA with sub-optimal 260:230 and 260:280 NanoDrop ratios, (W2) Promega Wizard extracted maize genomic DNA with optimal 260:230 and 260:280 NanoDrop ratios, (p:c) phenol:chloroform refined maize genomic DNA with optimal 260:230 and 260:280 NanoDrop ratios, negative lanes (2) summary results table

The bands of genomic DNA for the three extracts were not smeared in appearance indicating that the quality of the extracted genomic DNA is high. The DNA concentration calculation from agarose gel quantification was lower than the values from the NanoDrop. This is a result that has been previously observed in Kiddle (2012) and represents an issue with the use of the different methods of quantification for genomic DNA.

3.3.4 Certified Reference Materials Bt11 LAMP-BART assay

In this investigation, European Reference Material maize samples with percentage GM contamination of wild type maize with event Bt-11; 0.1%, 0.5%, 1%, 2% and 5% were extracted with the Promega Wizard™ Genomic DNA purification kit. Primers were used for ADH1 as the maize reference gene and for the 35Sp to target the decreasing transgenic element from 5% to 0.1%. The aim of this investigation was to investigate the detection of low percentage GM contamination in a background of non-GM genomic DNA and to assess variability and quantitation over a range of GM copy number concentrations (Figure 3.7).

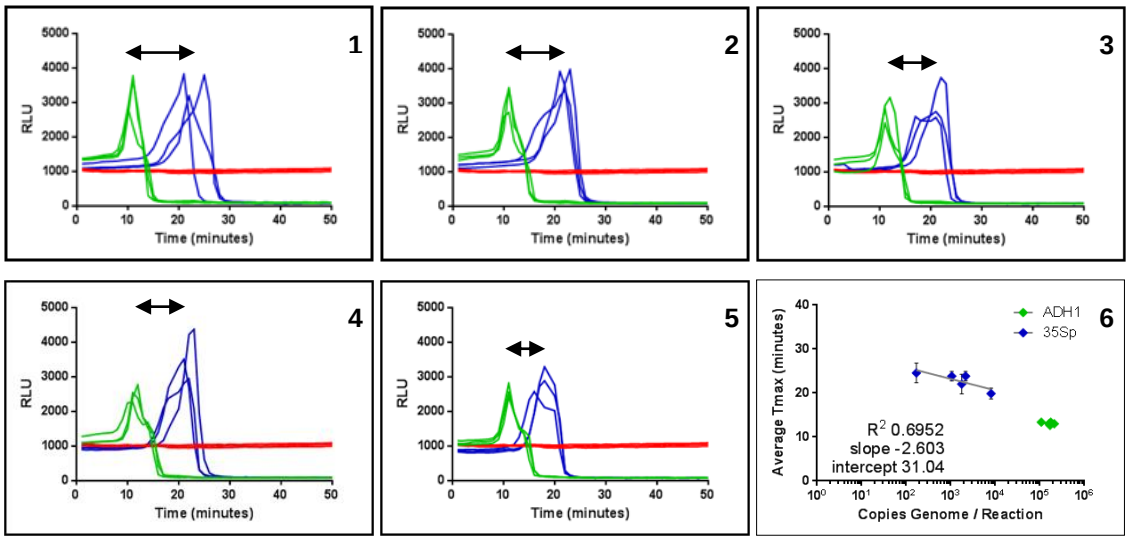


Figure 3.7: ADH1 LAMP-BART assays of maize genomic DNA in green and 35Sp LAMP-BART assay of GM maize Bt-11 genomic DNA in blue, all assays in triplicate, BART light output against assay time (truncated to 50 minutes) (1) 0.1% GM extract (2) 0.5% GM extract (3) 1.0% GM extract (4) 2.0% GM extract (5) 5.0% GM extract (6) the average Tmax results for the LAMP-BART assays of ADH1 ranged from 12.66 to 13.26 minutes representing the variation in DNA yield from the extractions. It was calculate that the 5% sample had 8107 copies of the 35Sp copies and the 0.1% had 170 copies. The 2%, 1% and 0.5% samples had 2162, 1776 and 1062 copies of the 35Sp sequence respectively, which are closely grouped on a logarithmic scale.

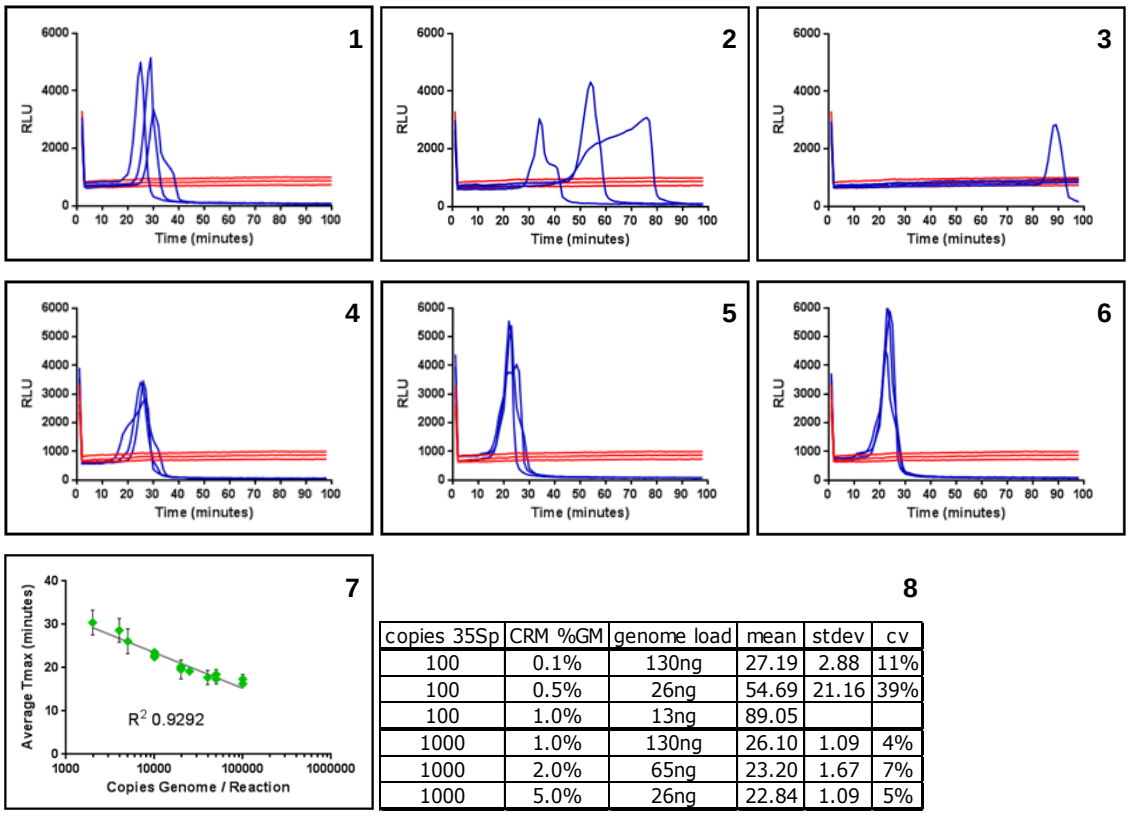
The five extracts originated from 40mg of the percentage GM maize powder and the time-to-peak values for ADH1 are consequentially closely matched. The 0.1% GM sample equated to a calculated copy number per partition of 170 copies per partition which was successfully detected in all replicates. The 5% sample equated to approximately 8100 copies per partition. NTCs were clear for all assays. Increasing average T_{max} was observed with decreasing copy numbers of the 35Sp target to a semi-logarithmic line with a correlation of 0.84 (panel 6). Variation between the triplicate peaks was observed for all the percentage GM samples and although there is a correlation between average T_{max} and copy number, quantification of genomic DNA in this assay would be uncertain.

3.4 Effect of non-template carrier DNA in amplification assays

3.4.1 Effect of genome load on the detection of 100 transgenic copies per partition using LAMP-BART

The reference maize GM samples 0.1%, 0.5%, 1%, 2% and 5%GM were extracted with the Promega Genome Wizard™ kit and further refined with phenol:chloroform. To compare the sensitivity of the five samples at low copy number a titration series was made so that the GM copy number was constant at 100 copies per assay, but the total

genomic content varied due to the different GM proportions in the original samples. The samples were assayed with LAMP primers designed for the cauliflower mosaic virus (CaMV) 35S gene promoter (35Sp) for the GM component of the maize samples. The samples were also assayed for the maize alcohol dehydrogenase 1 (ADH1) reference gene for the genomic element of the maize samples. The 0.1% GM maize was diluted to 100 copies per partition and had a corresponding genome load in the sample of 100000 copies, which equates to 130ng of DNA. The 0.5% GM sample at 100 copies per partition has 20000 genome copies equivalent to 26ng of non-target DNA. The 1%, 2% and 5% GM samples had correspondingly lower concentrations of non-target genomic DNA. The aim of the assay was to investigate the variation between replicates and amplification frequency of the template DNA in different concentrations of non-target DNA (Figure 3.8).



copies 35Sp	CRM %GM	genome load	mean	stdev	cv
100	0.1%	130ng	27.19	2.88	11%
100	0.5%	26ng	54.69	21.16	39%
100	1.0%	13ng	89.05		
1000	1.0%	130ng	26.10	1.09	4%
1000	2.0%	65ng	23.20	1.67	7%
1000	5.0%	26ng	22.84	1.09	5%

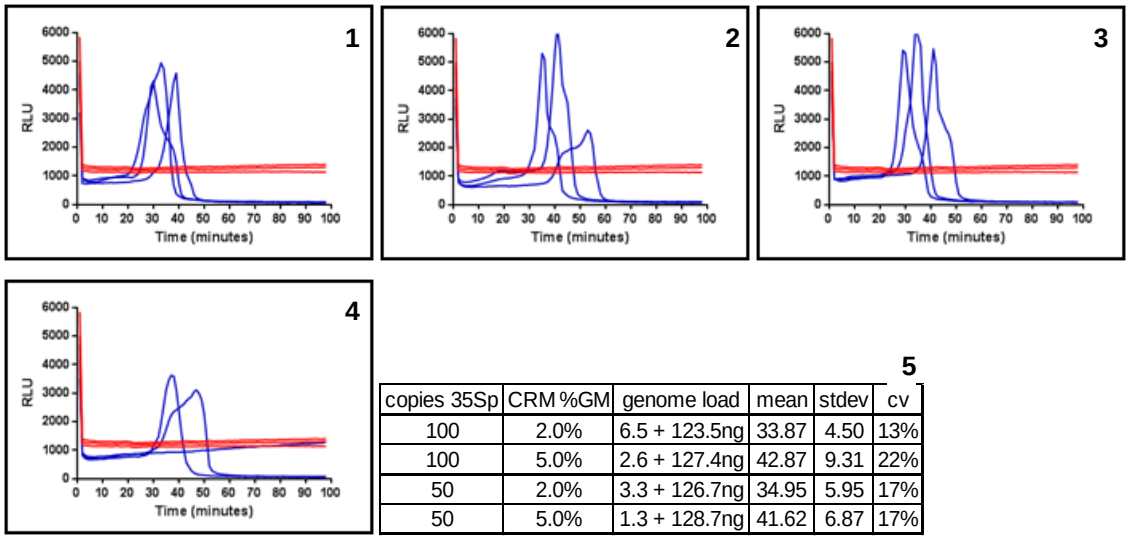
Figure 3.8: 35Sp LAMP-BART assays of maize genomic DNA with percentage GM maize genomic DNA, copies of the GM maize (35Sp sequence) in triplicate, BART light output against assay time (1) 100 copies per partition 35Sp from 0.1% GM (2) 100 copies per partition 35S from 0.5% GM (3) 100 copies per partition 35Sp from 1% GM (4) 1000 copies per partition 35Sp from 1%GM (5) 1000 copies per partition 35Sp from 2% GM (6) 1000 copies per partition 35Sp from 5% GM (7) ADH1 LAMP-BART results (8) summary of results and genome load for the assays per partition

This LAMP-BART 35Sp assay at 100 copies per partition showed an improved reproducibility associated with the 0.1% GM maize sample with standard deviation of 2.88 minutes. For the 0.5% sample at this copy number the standard deviation for the

three replicates had increased to 21.16 minutes. The amplification frequency dropped with the 1%, 2% and 5% samples (data not shown for 2% and 5%) with only two of the three replicates positive. At 1000 copies of the 35Sp target per partition the variance between the replicates was low and the average T_{max} values from the 1% GM sample was slower than the 2% and 5% (there is no data for 0.1% and 0.5% GM for 1000 copies per partition because of the low starting DNA concentration of the samples).
A linear relationship was observed for the five percentage GM samples diluted with respect to the 35Sp copy number, assayed for the ADH1 reference gene, which strongly indicates that the observed differences between the percentage GM samples were not as a result of titration errors. All NTCs were clear.
The data suggest that the total genomic DNA per partition has an impact on sensitivity, reproducibility and average T_{max} values. Approximately 100000 copies of non-target genomic DNA in an assay equating to approximately 60ng per partition, has a positive effect on the assay.

3.4.2 Effect of increased genome load on the detection of 100 transgenic copies using LAMP-BART

The 0% GM sample was used in this experiment to further increase, during the titration, the genome load for the 2% and 5% GM samples. The genome load was increased to 100000 copies per partition and the 2% and the 5% samples were assayed with the 35Sp primers at 100 copies GM per assay. In the previous assay the untreated samples had reduced amplification frequencies. The samples were also assayed at 50 copies of 35Sp per partition (Figure 3.9).



5

copies 35Sp	CRM %GM	genome load	mean	stdev	cv
100	2.0%	6.5 + 123.5ng	33.87	4.50	13%
100	5.0%	2.6 + 127.4ng	42.87	9.31	22%
50	2.0%	3.3 + 126.7ng	34.95	5.95	17%
50	5.0%	1.3 + 128.7ng	41.62	6.87	17%

Figure 3.9: 35Sp LAMP-BART assays of maize genomic DNA with percentage GM maize genomic DNA, copies of the GM maize (35Sp sequence) in triplicate, 100000 copies per partition of genome load, BART light output against assay time (1) 100 copies per partition 35Sp from 2.0% GM (2) 100 copies per partition 35Sp from 5.0% GM (3) 50 copies per partition 35Sp from 2.0% GM (4) 50 copies per partition 35Sp from 5.0% GM (5) Summary table showing the calculated genome load added to the sample dilutions with average T_{max}, standard deviation and coefficient of variation results.

The assays for the 2% and 5% GM samples at 100 copies 35Sp per partition showed an improvement on the 66% amplification frequency observed previously. The average T_{max} for the 2% GM and the 5% GM was 34 and 43 minutes respectively, in contrast the 0.1% GM in the previous experiment had an average T_{max} value of 27 minutes. Increased genome load does appear to improve the success rate of the 2% and 5% samples at 100 copies per partition. Average T_{max} increased for these assays with increasing percentage GM.

The results from this assay for 100 and 50 copies with additional genomic load were added to the results from the previous section for 200, 500 and 1000 copies per partition (data not previously shown for 200 and 500 copies per partition) and displayed in the graphs below (Figure 3.10).

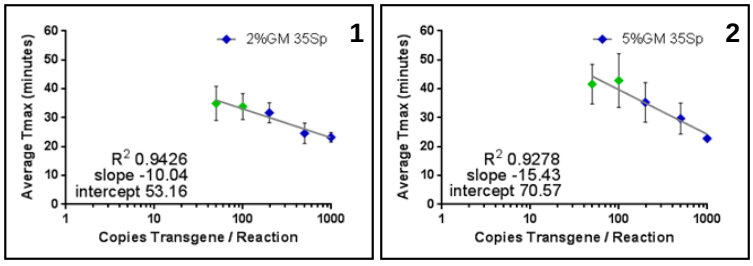


Figure 3.10: GM-LAMP-BART 35Sp assays of 2% and 5% GM contaminated maize with genome load adjusted to 100000 genome equivalents for all samples (1) 2% GM samples with additional results for 100 and 50 copies/partition (2) 5% GM sample with additional results for 100 and 50 copies/partition

The values for average T_{max} for the 2% and 5% GM samples for 100 and 50 copies per partition correlate strongly with the previous results and suggest that other factors, such as impurities, are influencing the amplification for these samples. The 5% and 2% samples are the most diluted and any inhibitors should be more concentrated in the samples with lower percentage GM. Molecular grade water was used for the dilutions and therefore the difference in average T_{max} between these simples is not from the chelation of magnesium ions by the EDTA in TE buffer. The difference between the average T_{max} for any given target concentration originated from the 2% and 5% extracts.

3.4.3 The effect of genome load on transgene detection of 50 to 300 copies per partition using LAMP-BART

To investigate the effect of genome load in a wide range from 1ng to 390ng per partition on a range of copy numbers with respect to the 35S promoter sequence, multiple extracts were prepared using the Promega Wizard™ kit. The percentage GM samples were titrated to 300, 200, 100 or 50 copies 35Sp per partition without the addition of extra genome from the 0% GM extract. Therefore for 50 copies 35Sp per partition from the 0.1%GM maize powder there was a total genomic load equating to approximately 65ng and for 50 copies 35Sp from the 5%GM maize there was approximately 1ng per partition genome load.

The experiment was also designed to compare %GM samples that had only been extracted with the Promega Wizard kit to those that had been further refined with the phenol:chloroform method. The effect of denaturation was also investigated with the aim of producing faster and more reproducible time- to- peak data by increasing the favourability of LAMP initiation. The aim of these experiments was to assess the optimal concentration of genome load in a LAMP-BART assay with different template conditions (Figure 3.11).

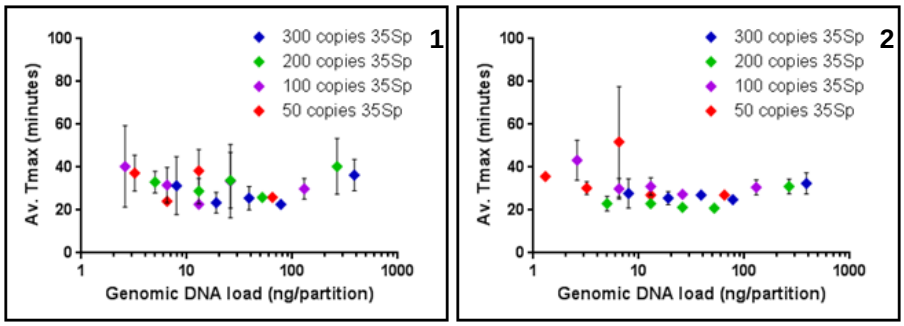


Figure 3.11: GM-LAMP-BART 35Sp assays of 0.1%, 0.5%, 1%, 2% and 5% GM contamination at 300, 200, 100 and 50 copies 35Sp per partition, Average T_{max} values according to the genomic load of the sample (1) Promega Wizard Genomic DNA kit extracts in native form (data published in Kiddle et al 2012) (2) Promega Wizard Genomic DNA kit extracts denatured. All non-template controls were clean.

The first two graphs for Promega Wizard™ extracted samples whether native or denatured show a range of genomic load within which the reproducibility, sensitivity and time-to-peak times are good. This range appears to be from about 30ng to 110ng per partition. Outside of this range the time-to-peak values appear to increase and so does the variation between replicates. There is greater amplification frequency for the denatured samples: 100% amplification frequency is achieved for all copy numbers with the exception of 2 out of 3 replicates at 50 copies with 3ng genome load and 1 out of 3 for 50 copies with 1ng genome load.

Percentage GM maize extracts prepared at the same time as the others were refined by phenol:chloroform extraction followed by ethanol precipitation. These extracts were then diluted in the same way as previously to provide 300, 200, 100 and 50 copies 35Sp per partition for each of the percentage GM samples (Figure 3.12).

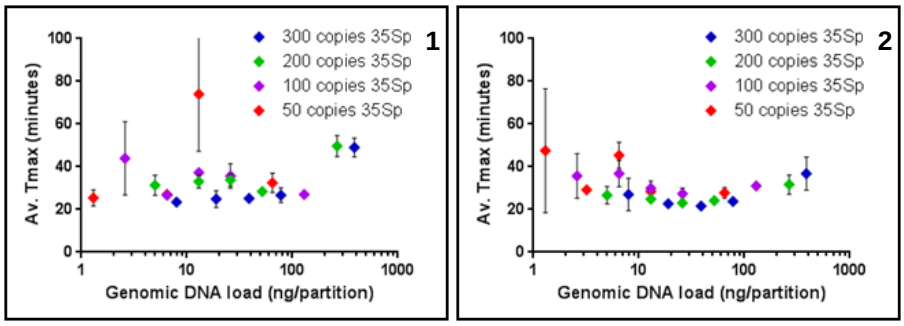


Figure 3.12: GM-LAMP-BART 35Sp assays of 0.1%, 0.5%, 1%, 2% and 5% GM contamination at 300, 200, 100 and 50 copies 35Sp per partition, Average T_{max} values according to the genomic load of the sample (1) Promega Wizard Genomic DNA kit extracts refined with phenol:chloroform in native form (2) Promega Wizard Genomic DNA kit extracts refined with phenol:chloroform denatured. All non-template controls were clean.

The results are a similar picture to the unrefined samples with a range of genome load between approximately 30ng to 110ng per partition with the faster time-to-peak values, low variation between replicates and high amplification frequency. The 300 copies per

partition results with a genome load of 390ng per partition were over 10 minutes slower than the 300 copies per partition with 78ng of total genome load. The denatured samples again had a slightly increased amplification frequency for the 50 copies per partition. It was the lowest genome load samples that had reduced amplification frequency at this level. 2 out of 3 for 50 copies at 6ng per partition, 1 out of 3 positive at 3ng per partition and 2 out of 3 at 1ng per partition.

All the sets of data for percentage GM samples show high variability and reduced sensitivity at low levels of non-target DNA when assayed with LAMP-BART for the 35S promoter. Also, above a threshold of approximately 100ng/partition the time to peak is significantly increased. The data for the phenol:chloroform refined percentage GM samples has a similar range outside of which reproducibility deteriorates which suggests that the quality of the DNA extraction is not affecting the results. The non-target DNA carrier may act in a number of ways at sufficient concentration to improve sensitivity and reproducibility; by limiting the sticking of target DNA to the sides of the plastic tubes, as an alternative target for DNase activity or to sequester primers before the assay starts and preventing mis-amplification in sub-optimal conditions. At high concentrations of carrier the time to peak values are slowed. These assays are produced by the 0.1% GM extract that has been suspended in TE buffer and would receive only the slightest dilution with molecular grade water to 300 and 200 copies GM per assay. Therefore for these samples the concentration of EDTA is highest and there is a possibility that the kinetics of the amplification is slowed by the chelation of the magnesium ions. Another possibility for the slower kinetics could be the effect of high levels of carrier reducing the interaction between primers, reactants and the target DNA.

3.4.4 The effect of increased genome load on transgene detection of 50 to 300 copies per partition using LAMP-BART

To investigate higher concentrations of genome load on the low copy number 35Sp assays with LAMP-BART (Figure 3.13), formulations of the 0% and 0.1% GM ERM maize powders were prepared to 0.075% and 0.05%. Both were extracted with the Promega Wizard kit and further refined with phenol:chloroform. Determination of DNA concentration in TE buffer was by NanoDrop spectrophotometry and agarose gel electrophoresis.

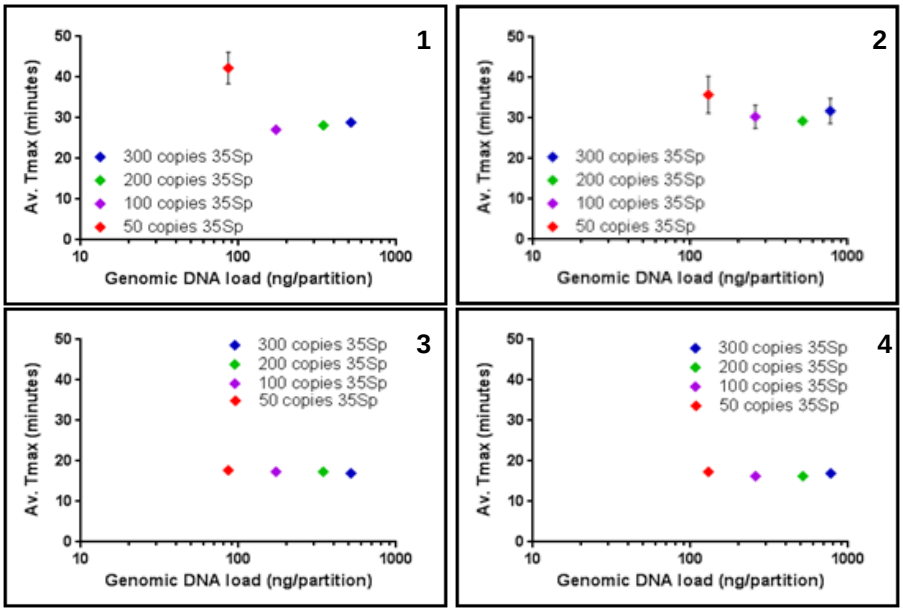


Figure 3.13: GM-LAMP-BART 35Sp assays of 0.075% and 0.050% GM contamination at 300, 200, 100 and 50 copies 35Sp per partition, Average T_{max} values according to the genomic load of the sample (1) Promega Wizard Genomic DNA kit extract of 0.075% GM refined with phenol:chloroform in native form (2) Promega Wizard Genomic DNA kit extracts of 0.050% GM refined with phenol:chloroform in native form (3) LAMP-BART Inhibitor control assay for 0.075% GM samples (4) LAMP-BART Inhibitor control assay for 0.050% GM samples. All non-template controls were clean.

The high levels of genome load have compromised the time to peak values for both of these assays. The effect is more pronounced with the 300 and 200 copies GM per assay as increasing copy number does not result in faster T_{max} values. The data suggest that quantitation breaks down at high genome load.

The slower time to peak values may be the result of inhibitors in the concentrated samples; therefore the titrations were tested with inhibitor controls supplied by Lumora. The controls were unaffected by any of the samples and therefore the LAMP-BART assays were not affected by any inhibitors that may be present in the samples. Also the presence of EDTA in the TE buffer of the extracts does not appear to be having an impact on the LAMP-BART inhibitor controls.

In summary the LAMP-BART genome load experiments suggested that the concentration of non-template genomic DNA could influence the amplification frequency, time-to-peak and variability between replicates at 100 copies of the transgene per partition. At a higher copy number of the transgene this effect was less apparent. Increasing the concentration of non-target genomic DNA for samples that had previously low levels of genome load showed improvement to the amplification frequency and variation between replicates. Low copy number experiments (between 50 and 300 copies of the transgene) with a range of sample-associated genome loads

from 1ng to 390ng per partition, indicated a range between 30ng and110ng per partition on non-target genomic DNA, in which the LAMP-BART assay results had improved amplification frequency, variation between replicates and time-to-peak times.

3.4.5 Effect of genome load on 35Sp qPCR assay

In this experiment, the ERM maize GM samples were titrated to 300, 200, 100 and 50 copies 35Sp per assay for analysis by qPCR. The aim was to investigate the sensitivity, amplification frequency and efficiency of the qPCR assay in comparison to the LAMP-BART assay of the same samples. The 35Sp primers were designed using Primer3 software and were used at a concentration of 5µM on a Corbett Rotor-Gene thermal cycler with SYBR® Green JumpStart™ Taq ReadyMix™ for 40 cycles (Figure 3.14).

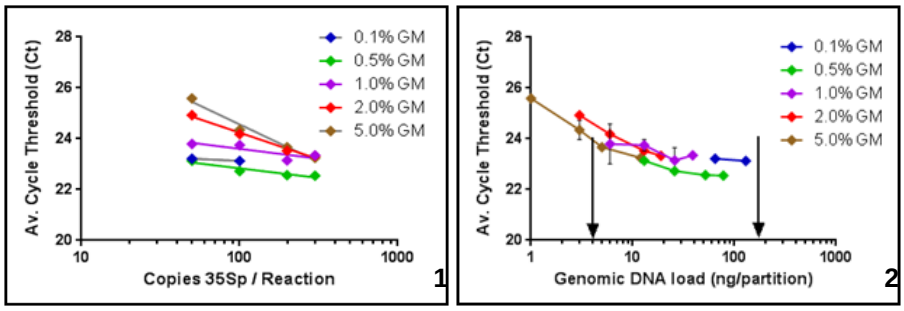


Figure 3.14: qPCR with 35Sp primers and percentage GM maize genomic DNA template for 300, 200, 100 and 50 copies 35Sp per partition (●) 5% GM samples (●) 2% GM samples (●) 1% GM samples (●) 0.5% GM samples (●) 0.1% GM samples (1) cycle threshold (Ct) against copies 35Sp per partition (L to R 50, 100, 200 and 500 copies per partition) (2) cycle threshold (Ct) against the concentration of genomic DNA in each sample

The qPCR 35Sp assay appears to be greatly affected by genome load of the samples. The 5% GM samples with the lowest range of genome load had 100% amplification frequency, excellent quantitation between copy numbers, low variation between replicates, but a sub-optimal amplification efficiency of 118%. The amplification efficiency for the other samples was sub-optimal with the gradient of the slope tending towards horizontal. The amplification frequency for all the 50 copy samples was 100% which is better than the LAMP-BART assay. At the other end of the scale the 200 and 300 copies per partition assays from the 0.1% GM sample gave no result due to the excessively high background fluorescence. The use of SYBR green with high total genome DNA content produces background fluorescence that hampers amplification detection by qPCR.

In summary, the LAMP-BART assay of low copy number template appeared to show improved results at non-target genomic DNA concentrations of 30ng to 110ng per partition. The qPCR results are not improved by genome load in this range of concentrations. The efficiency of the qPCR amplification was reduced with the increase in genome load. The amplification frequency was compromised with concentrations of genome load above approximately 100ng per partition.

3.4.6 Effect of non-specific DNA on transgene quantification using qPCR

The linearised plasmid pUC35S GUS containing the 35Sp sequence was used instead of the GM genomic DNA to investigate the effect of non-target DNA on qPCR amplification frequency, amplification efficiency and quantification. Non-template genomic DNA was replaced with salmon sperm carrier DNA of known concentration.

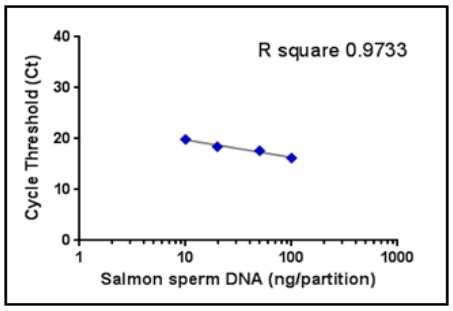


Figure 3.15: qPCR cycle threshold (Ct) values against the salmon sperm DNA concentration in the NTCs. An increase in carrier DNA concentration (10, 25, 50 and 100ng per assay) produces an increase in amplicon formation indicated by decreasing cycle threshold values.

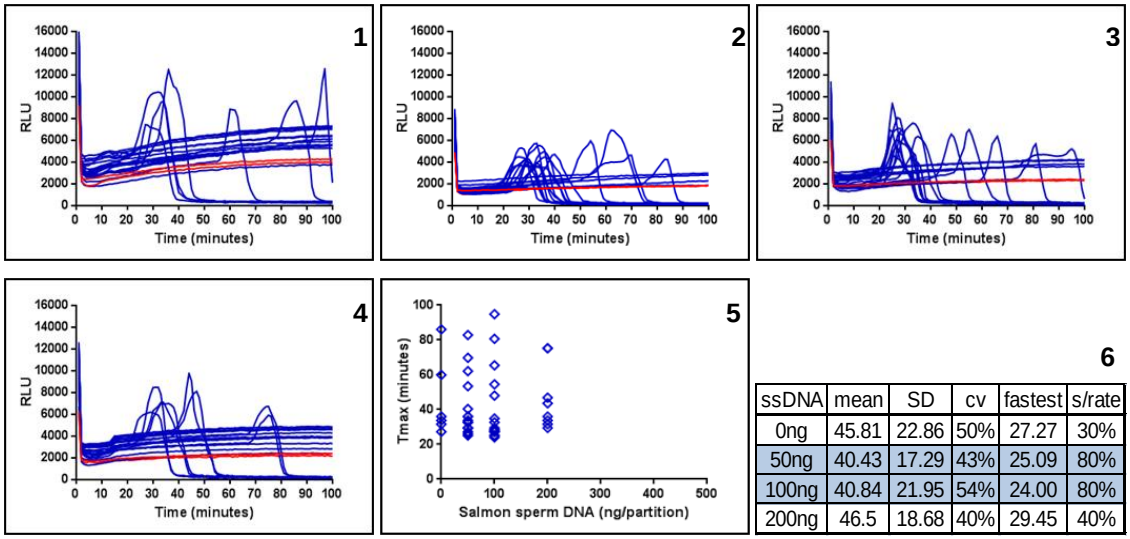
The results of the qPCR were fast cycle threshold (Ct) values for all of the samples (data not shown) and positive results for the non-template controls (Figure 3.15) which contained the same concentrations of carrier DNA. The plotted data from the NTCs indicated either a contamination with the 35Sp sequence or non-specific product formation between the primer set and the salmon sperm DNA. A review of the melt data from the Rotor-Gene software indicated a higher melt temperature for the product formed in the NTCs over that of the 35Sp amplicon. An alternative supply of salmon sperm DNA carrier produced similar results, as did a number of different PCR primer sets for the CaMV 35S promoter. Sequence data, although not conclusive, indicated a strong possibility that non-specific product formation is responsible for both the fast cycle threshold data and for the positive results in the NTCs.

3.4.7 Effect of non-specific DNA on transgene detection using LAMP-BART

The aim of these experiments was to assess the optimal concentration of carrier for an artificial template with easy to control concentrations of carrier DNA at copy numbers of the transgene approaching the limit of detection. Plasmid template was assayed with LAMP-BART, using either 35Sp or NOSt primers and salmon sperm carrier DNA to replace the non-template genomic DNA

3.4.7.1 Effect of non-specific DNA on transgene detection of 10 copies per partition using LAMP-BART with 35Sp primers

The aim of this experiment was to assess the optimal concentration of non-template DNA for LAMP-BART assays at low copy number using the 35Sp primer set. The linearised plasmid was titrated to 10 copies per partition and concentrations of carrier DNA ranged from 0ng to 50, 100 and 200ng per partition. The samples were assayed by LAMP-BART with the original set of 35Sp primers (Figure 3.16).



ssDNA	mean	SD	cv	fastest	s/rate
0ng	45.81	22.86	50%	27.27	30%
50ng	40.43	17.29	43%	25.09	80%
100ng	40.84	21.95	54%	24.00	80%
200ng	46.5	18.68	40%	29.45	40%

Figure 3.16: GM-LAMP-BART 35Sp assay of 10 copies of linearised plasmid with increasing concentration of salmon sperm DNA (1) without additional DNA (2) with 50ng salmon sperm DNA per partition (3) with 100ng salmon sperm DNA per partition (4) with 200ng salmon sperm DNA per partition (5) time-to-peak values for each assay plotted against the carrier DNA (6) summary of results (concentration of salmon sperm DNA in ng/partition)

The highest amplification frequency of 80% was observed for the assays with 50 and 100ng per partition of the carrier DNA. The average T_{max} and fastest T_{max} values for these two assays were faster than the 0ng and 200ng per partition assays. The previous results from genomic DNA with non-template genomic DNA indicated

improved LAMP-BART results from a range of approximately 30ng to 110ng per partition genome load. The results from this assay with the highest amplification frequencies are within this range.

3.4.7.2 Effect of high concentrations of non-specific DNA on transgene detection of 10 copies per partition using LAMP-BART with 35Sp primers

In a second experiment, the range of concentrations of salmon sperm carrier DNA was increased from 0ng per partition to 50ng, 500ng, 5µg and 10µg to investigate the effect of high concentrations of non-target DNA on the LAMP-BART assay. The linearised plasmid pART7 was used at 10 copies per partition with the range of carrier DNA concentrations (Figure 3.17). The assay used the new set of LAMP 35Sp primers designed specifically for the pART7 plasmid.

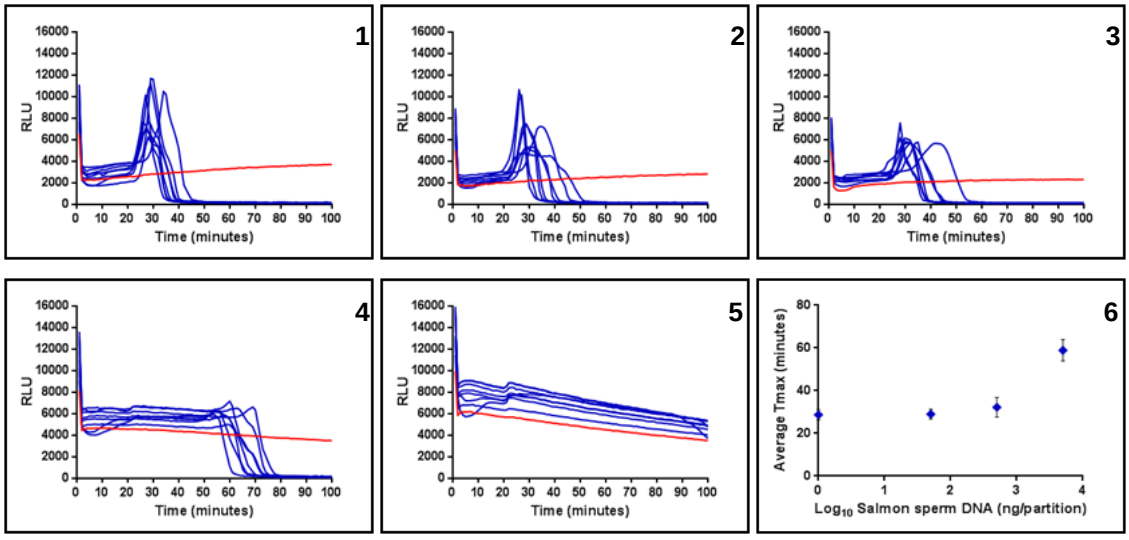


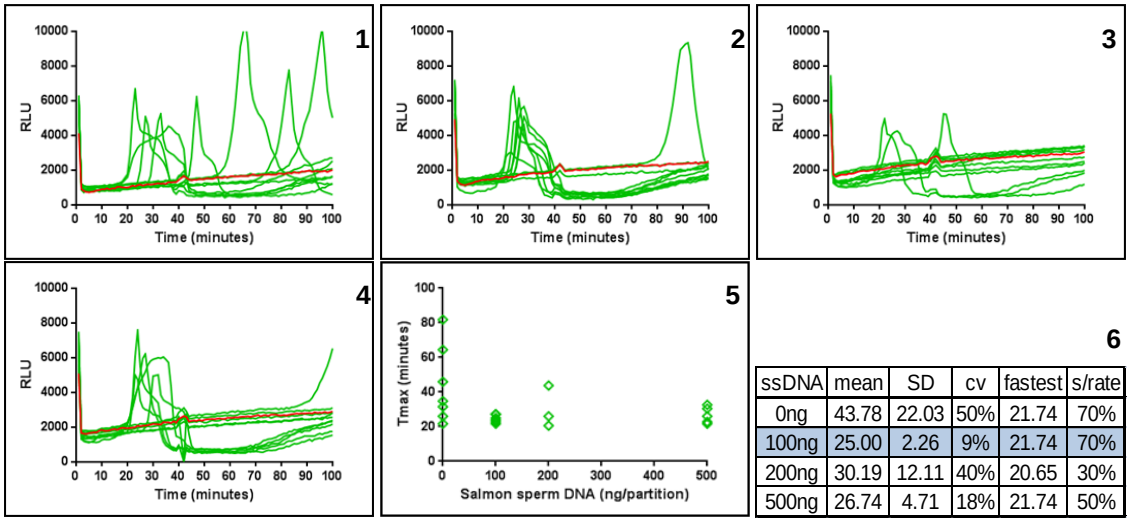
Figure 3.17: GM-LAMP-BART 35Sp assay with new primers of 10 copies of linearised plasmid with increasing concentration of salmon sperm DNA (1) without additional DNA (2) with 50ng salmon sperm DNA per partition (3) with 500ng salmon sperm DNA per partition (4) with 5µg salmon sperm DNA per partition (5) with 10µg salmon sperm DNA per partition (6) average T_{max} for each assay plotted against the carrier DNA (concentration of salmon sperm DNA in ng/partition)

The first two LAMP-BART assays for 10 copies of the linearised plasmid with 0ng and 50ng salmon sperm carrier DNA are of similar peak height, average T_{max} and variance. The variance and the average T_{max} increases for the samples with 500ng carrier DNA per partition and the peak heights appear reduced. At 5µg of carrier the background light is increased and the BART peaks are barely detectable above this level. The average T_{max} is approximately 30 minutes slower than the previous samples, but the variation between replicates is similar to the 500ng per partition samples and the

amplification frequency remains at 100%. At 10µg the LAMP-BART assay is unsuccessful. Although the high concentration slows the time-to-peak values, the variation between replicates increases only slightly. If the carrier acted to restrict the initiation of amplification an increased range of time-to-peak times would be likely. The low variation appears to be the result of reduced efficiencies of the enzymes in the LAMP-BART assay.

3.4.7.3 Effect of non-specific DNA on transgene detection of 500 copies per partition using LAMP-BART with NOSt primers

In this experiment, an alternative primer set was used to assay non-linear plasmid DNA with LAMP-BART with a range of salmon sperm DNA concentrations from 0ng to 500ng. The improved LAMP primer set for the NOS terminator sequence of the plasmid pUC35S ADH1 was used (Figure 3.18). The aim of this experiment was to use primers for a different target sequence and an alternative conformation of plasmid template DNA to assess the amplification frequency, variability between replicates and time-to-peak values for the increasing concentration of non-target DNA in the LAMP-BART assays.



ssDNA	mean	SD	cv	fastest	s/rate
0ng	43.78	22.03	50%	21.74	70%
100ng	25.00	2.26	9%	21.74	70%
200ng	30.19	12.11	40%	20.65	30%
500ng	26.74	4.71	18%	21.74	50%

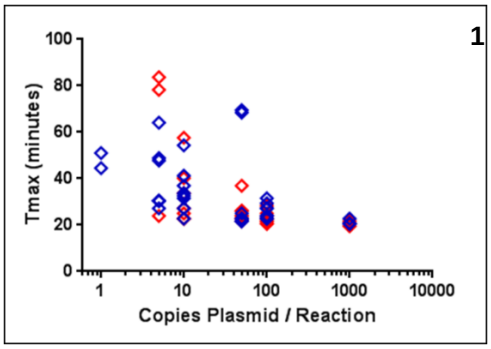
Figure 3.18: GM-LAMP-BART NOSt assay of 500 copies non-linear plasmid pUC35S ADH1 with increasing concentration of salmon sperm DNA (1) without additional DNA (2) with 100ng salmon sperm DNA per partition (3) with 200ng salmon sperm DNA per partition (4) with 500ng salmon sperm DNA per partition (5) time-to-peak values for each assay plotted against the carrier DNA (6) summary of results

The amplification frequency for the 0ng and 100ng per partition were both 70% for this assay using the NOSt primer set. The higher concentrations of carrier had lower amplification frequencies. At 100ng per partition of carrier DNA, the average T_{max} was

the fastest of the four assays with the lowest variation between replicates. All four assays had similar fastest T_{max} values which indicates that the presence of carrier DNA does not make the LAMP-BART assay faster, but does improve the reproducibility of the assay which as a consequence reduces average T_{max}. The optimal concentration of non-target DNA for this LAMP-BART assay was 100ng per partition, which is within the range of 30ng to 110ng per partition observed with previous results.

3.4.8 Effect of salmon sperm DNA on LAMP-BART sensitivity

The small size of the linearised plasmid pART7 makes it possible for initial quantitation and subsequent determination of copy number by Agilent Bioanalyzer microchip technology. The initial quantitation can be accurately calculated with this technology (and compared to quantification values from NanoDrop spectrophotometry) and the sample titrated to low copy numbers per partition. In this experiment the sensitivity of a LAMP-BART assay without carrier DNA was compared to an identical assay with 50ng per partition salmon sperm carrier DNA (Figure 3.19). The aim of this experiment was to investigate the sensitivity of the LAMP-BART assay with the addition of non-target DNA at a concentration within the range 30 to 110ng per partition.



2

no carrier DNA

copies	mean	SD	cv	fastest	s/rate
1					0%
5	61.95	33.05	53%	23.92	38%
10	32.77	12.66	39%	22.83	88%

50ng/partition carrier DNA

copies	mean	SD	cv	fastest	s/rate
1	47.83	4.60	10%	44.57	25%
5	39.91	13.91	35%	27.18	88%
10	35.06	9.61	27%	22.83	100%

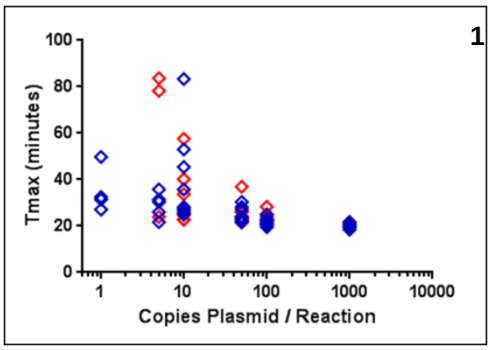
Figure 3.19: GM-LAMP-BART 35Sp assay of linearised plasmid at 1000, 100, 50, 10, 5 and 1 copy per partition (1) time-to-peak against template copy number for (red) without carrier DNA and (blue) with 50ng salmon sperm carrier DNA (blue) (2) summary tables for 10, 5 and 1 copy per partition without carrier DNA and with carrier DNA

The control LAMP-BART 35Sp assay of linearised plasmid template without additional non-template DNA, failed to amplify any of the replicates of 1 copy per partition. However the LAMP-BART assay with salmon sperm carrier DNA successfully amplified from two of the eight partitions. At 5 and 10 copies per partition the amplification frequency was higher for assays with 50ng per partition of non-template DNA.

In these LAMP-BART assays there was an increase in sensitivity resulting from the addition of carrier DNA to the template.

3.4.9 Effect of maize genomic DNA on LAMP-BART sensitivity

In a final experiment on non-target DNA in LAMP-BART assays, the previous assay was repeated using the linearised pART7 plasmid template and 35Sp primers but this time with maize genomic DNA added to the assay instead of salmon sperm carrier DNA. Maize genomic DNA from the 0% GM CRM Bt11 extract was used to increase the genome load of the test samples (Figure 3.20).



no carrier DNA

copies	mean	SD	cv	fastest	s/rate
1					0%
5	61.95	33.05	53%	23.92	38%
10	32.77	12.66	39%	22.83	88%

2

50ng/partition genomic carrier DNA

copies	mean	SD	cv	fastest	s/rate
1	35.21	10.02	28%	27.09	50%
5	29.04	5.38	19%	27.18	63%
10	40.49	20.10	50%	24.93	100%

Figure 3.20: GM-LAMP-BART 35Sp assay of linearised plasmid at 1000, 100, 50, 10, 5 and 1 copy per partition (1) time-to-peak against template copy number for (red) without carrier DNA and (blue) with 50ng maize genomic carrier DNA from 0% GM Bt-11 ERM (blue) (2) summary tables for 10, 5 and 1 copy per partition without carrier DNA and with genomic carrier DNA

The control LAMP-BART 35Sp assay of linearised plasmid template without additional non-template DNA, failed to amplify any of the replicates at 1 copy per partition. However the LAMP-BART assay with genomic DNA successfully amplified with a frequency of 50% of partitions. As was previously observed using salmon sperm carrier DNA, at 5 and 10 copies per partition the amplification frequency was higher for assays with 50ng per partition of non-template DNA.

The addition of non-target DNA to LAMP-BART assays within a range of approximately 30 to 110ng per partition improves amplification frequency, variation between replicates and time-to-peak values to a minimum fastest time. At 50ng per partition DNA from different sources and of different sizes improved the LAMP-BART results with different primer sets and different manifestations of DNA template. The effect of the non-target DNA on LAMP-BART assays appears to be more evident at template copy numbers near to the limit of detection. The concentration of carrier DNA for all subsequent LAMP-BART assays was set at 100ng per partition.

3.5 Effect of altering LAMP-BART components for quantitation by T_{max}

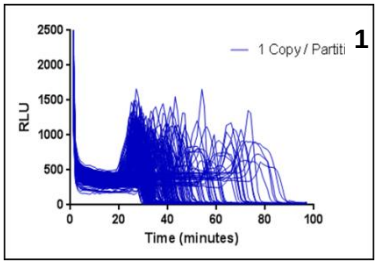
3.5.1 Introduction

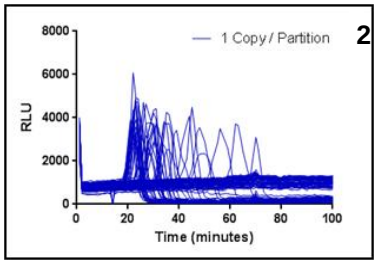
As a result of the previous section, one of the components of the LAMP-BART mix is salmon sperm carrier DNA at a concentration of 100ng per partition. This has been incorporated into both BARTmaster and non-BARTmaster assays. The other enzymes and chemicals that make up the mix are described by Gandelman (2010) and are detailed in Chapter 2.

BARTmaster is a freeze-dried preparation of all the components for a LAMP-BART assay, with the exception of an appropriate isothermal buffer, MGW, LAMP primers and DNA template. BARTmaster is ideal for assays in which all the components are standardised, but for assays in which the concentration of a component requires alteration, preparation of all the components in the BART mastermix is required.

3.5.2 BARTmaster and non-BARTmaster mastermixes

Comparisons of assays that are based on BARTmaster with those that are prepared from individual components have been favourable. In this example a BARTmaster LAMP-BART 35Sp assay of the linearised plasmid pART7 using robotic loading and 384 well plates was compared to an identical LAMP-BART assay prepared from individual components for loading to a 96 well plate (Figure 3.21).





3

BARTmaster

copies	mean	SD	cv	fastest	s/rate
1	35.22	12.01	34%	23.62	62%

Non-BARTmaster

copies	mean	SD	cv	fastest	s/rate
1	30.60	11.41	37%	20.78	48%

Figure 3.21: LAMP-BART 35Sp assays of single copy of linearised pART7 template per partition (1) BARTmaster assay with 384 partitions (negative results not shown) (2) non-BARTmaster assay with 96 partitions (3) summary table of results

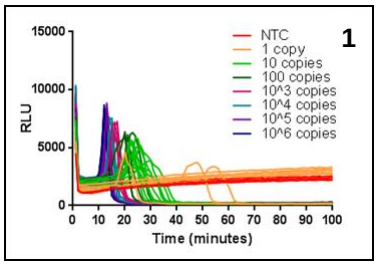
For single copy detection both of these assays achieve high values of amplification frequency and the small variations between results are likely to be stochastic and not due to problems with BARTmaster or non-BARTmaster LAMP-BART assays.

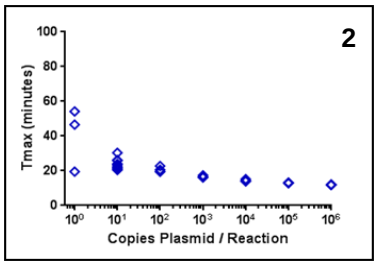
3.5.3 LAMP Primers

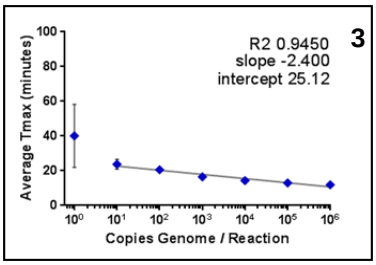
The LAMP primer set is composed of two displacement primers, two hairpin forming LAMP primers and two hairpin loop primers to accelerate the production of the inorganic pyrophosphate. The initiation of loop-mediated amplification requires the strand invasion of the double stranded DNA target by the LAMP primers, extension from these primers, the binding of displacement primers upstream of the LAMP primer binding and displacement by a displacing polymerase. The concentration of the LAMP primers is higher than the loop and displacement primers to ensure that the amplification initiation is likely to occur. This concentration of LAMP primers may be sufficient for these primers to act as displacers in the absence of displacement primers. By using deficient LAMP primer combinations and mismatched displacement primers the aim is to understand in more detail the role that these displacement and LAMP primers have in LAMP amplification.

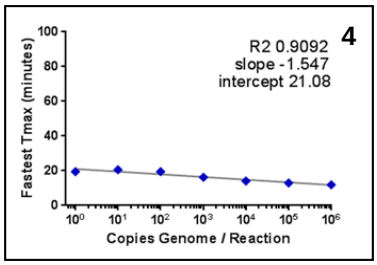
3.5.3.1 Standard 35Sp primer mix combination

In this first experiment the 35Sp LAMP-BART assay of linearised pART7 used the full set of HPLC 35Sp primers that were redesigned for the plasmid template (Figure 3.22). This assay forms the benchmark for comparison with sensitivity, amplification frequency, limit of quantitation, average T_{max} and variation at low copy number.









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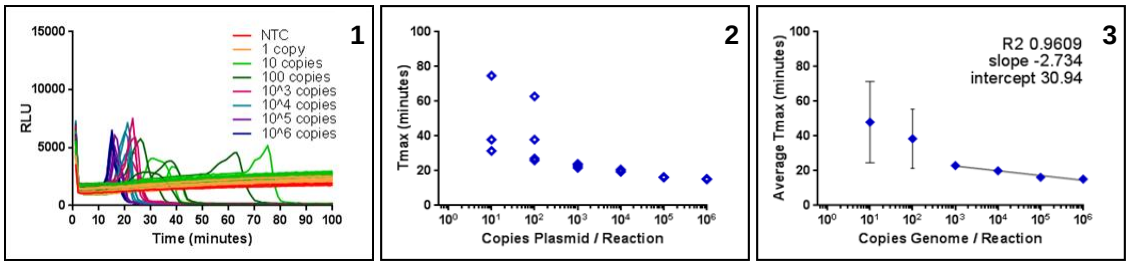
Copies	Mean	SD	cv	fastest	s/rate
1	40.11	18.22	45%	19.53	25%
10	23.77	2.90	12%	20.61	100%
100	20.61	1.53	7%	19.53	100%
1000	16.55	0.54	3%	16.28	100%
10000	14.39	0.54	4%	14.12	100%
100000	13.03	0.00	0%	13.03	100%
1000000	11.95	0.00	0%	11.95	100%

Figure 3.22: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with optimised primer set (1) light output against time for the serial dilution of 10^6 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

With this assay there is 25% amplification frequency for 1 copy per partition with high variation between the three positive replicates (from a total of twelve). The amplification frequency for 10^6 copies per partition to 10 copies per partition was 100% with increasing variation between replicates from zero to a standard deviation of 2.9 minutes at 10 copies per partition. The average T_{max} for 10^6 copies per partition was just under 12 minutes and for 10 copies approximately 24 minutes. The limit of quantitation appears to be between 10 and 100 copies where the variance increases.

3.5.3.2 Omission of both displacement primers

In the preparation of the primers for this assay both the displacement primers were replaced with MGW (Figure 3.23). The assay was otherwise identical to the one in section 3.4.3.1.



Copies	Mean	SD	cv	fastest	s/rate
1					0%
10	48.04	23.37	49%	31.43	25%
100	38.47	17.11	44%	26.01	100%
1000	23.04	1.04	5%	21.68	100%
10000	20.06	0.63	3%	19.52	100%
100000	16.27	0.00	0%	16.27	100%
1000000	15.19	0.00	0%	15.19	100%

5

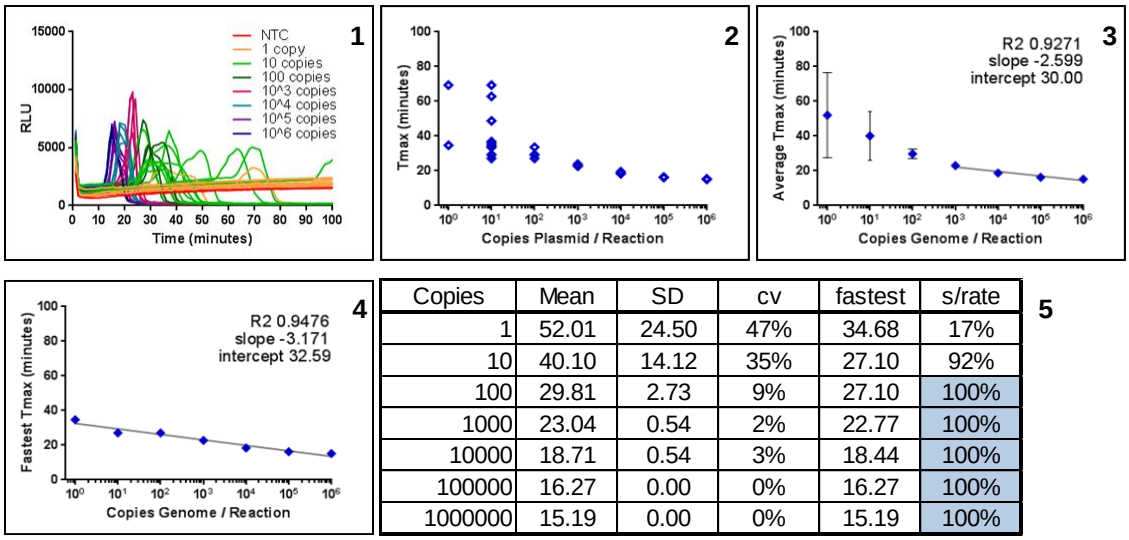
Figure 3.23: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with both displacement primers omitted (1) light output against time for the serial dilution of 10^6 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

The removal of the displacement primers is not catastrophic to the LAMP amplification. The amplification frequency for 1 and 10 copies is reduced to 0% and 25% respectively and the sensitivity is compromised by the lack of positive replicates at 1 copy per partition. For 100 copies per partition to 10^6 copies per partition the amplification frequency remains at 100% indicating that LAMP amplification can occur without displacement primers. The limit of quantification is reduced to 1000 copies per partition and the average T_{max} for copy numbers below this value increases rapidly. The average T_{max} for 10^6 copies per partition is just above 15 minutes which is three

minutes slower than the assay with displacement primers. The average T_{max} for 1000 copies per partition is about 23 minutes which is over six minutes slower. The results of this assay suggest that the initiation of LAMP amplification is less efficient without displacement primers and there is a delay to this initiation which is increasing apparent at lower copy numbers.

3.5.3.3 Omission of F3 version 3 displacement primer

The F3 displacement primer was omitted in this 35Sp LAMP-BART assay and replaced by molecular grade water (Figure 3.24). The assay therefore has the B3 displacement primer version 3 which was redesigned for the mismatch of the original primer with the sequence of the pART7 target.



Copies	Mean	SD	cv	fastest	s/rate
1	52.01	24.50	47%	34.68	17%
10	40.10	14.12	35%	27.10	92%
100	29.81	2.73	9%	27.10	100%
1000	23.04	0.54	2%	22.77	100%
10000	18.71	0.54	3%	18.44	100%
100000	16.27	0.00	0%	16.27	100%
1000000	15.19	0.00	0%	15.19	100%

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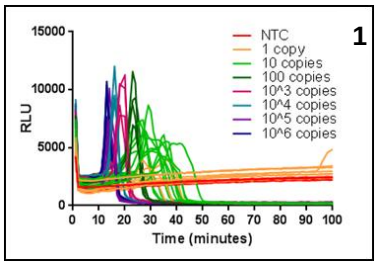
Figure 3.24: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with F3 version 3 displacement primer omitted (1) light output against time for the serial dilution of 10^6 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

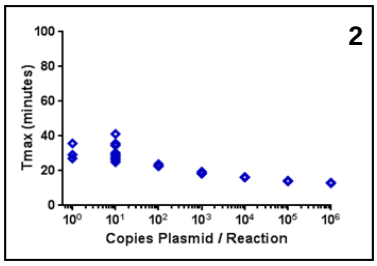
The amplification frequency of the 1 and 10 copies per partition samples is improved when compared to the assay without displacement primers but lower than the control assay. For 1 copy per partition there were two of the twelve replicates positive and this increased to 11 out of the 12 replicates for 10 copies per partition. The amplification frequency for the other samples remains at 100%. The sensitivity of the assay has improved with the addition of one of the displacement primers. Variability between replicates remains low from 10^6 to 1000 copies per partition and the limit of quantitation is approximately 1000 copies per partition. The average T_{max} for 10^6 copies per partition is approximately 15 minutes which is slower than the control and the same as the

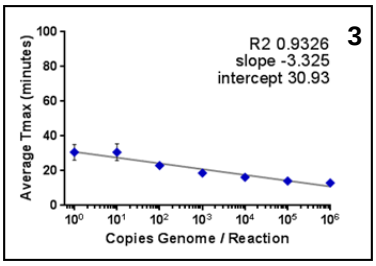
assay without displacement primers. The average T_{max} for 1000 copies per partition is about 23 minutes which is the same as the assay without displacement primers and over six minutes slower than the control. The initiation of LAMP amplification may be less efficient without a full complement of displacement primers causing a delay which is increasingly apparent at lower copy numbers. The inclusion of one of the displacement primers enhanced the LAMP-BART assay sensitivity.

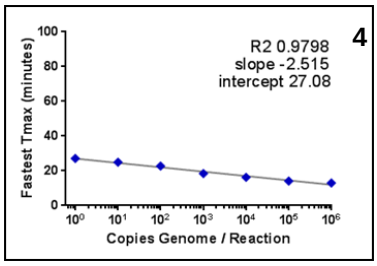
3.5.3.4 Omission of B3 version 3 displacement primer

The B3 version 3 displacement primer was omitted in this 35Sp LAMP-BART assay and replaced by molecular grade water (Figure 3.25). This primer was redesigned for the mismatch of the original primer with the sequence of the pART7 target. The assay therefore has one displacement primer; the F3 version 3 displacement primer.









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Copies	Mean	SD	cv	fastest	s/rate
1	30.73	4.51	15%	27.11	25%
10	30.73	4.85	16%	24.95	100%
100	23.05	0.54	2%	22.78	100%
1000	18.72	0.54	3%	18.45	100%
10000	16.28	0.00	0%	16.28	100%
100000	14.11	0.00	0%	14.11	100%
1000000	13.03	0.00	0%	13.03	100%

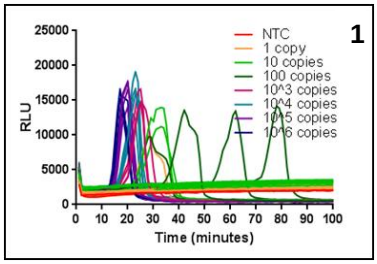
Figure 3.25: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with B3 version 3 displacement primer omitted (1) light output against time for the serial dilution of 10^6 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

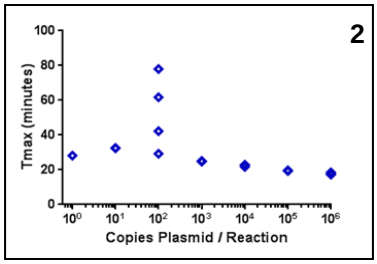
The omission of the B3 displacement primer to leave the F3 version 3 displacement primer in the assay has resulted in the same amplification frequencies for the samples as the control assay. The standard deviation for the three positive replicates at 1 copy per partition is lower than that seen with the control assay but this is probably fortuitous due to the low number of comparative replicates. At 10 copies per partition the standard deviation is higher than the comparable value from the control assay. The sensitivity of the assay has improved with the addition of one of the displacement primers. As with the control assay the limit of quantitation appears to be between 10

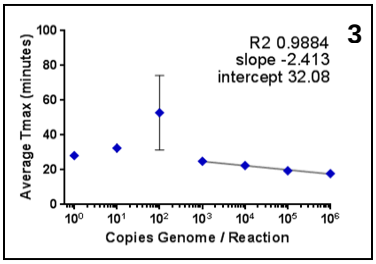
and 100 copies where the variance increases. The average T_{max} at 10^6 copies per partition is approximately 13 minutes which is only a minute slower than the control. At 100 copies per partition the average T_{max} is a couple of minutes slower than the control. These small differences could be due to inter-assay variation.
It appears that the F3 displacement primer is responsible for the improvement in sensitivity, average T_{max} times, limit of quantification, amplification frequency and variance between replicates that is observed from the assay without displacement primers to the control assay.

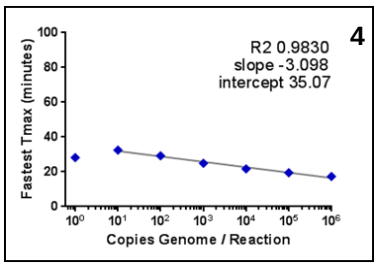
3.5.3.5 Omission of F3 version 3 inclusion of B3 version 1

The redesigned B3 displacement primer version 3 replaces version 1 which contains two mismatches within the last five nucleotides of the 3' end of the primer. In this assay the F3 displacement primer is omitted and the B3 displacement primer replaced with the mismatching primer to investigate primer mismatching events that may result from single nucleotide polymorphisms (Figure 3.26).









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Copies	Mean	SD	cv	fastest	s/rate
1	28.20	0.00	0%	28.20	8%
10	32.54	0.00	0%	32.54	17%
100	52.86	21.47	41%	29.29	100%
1000	24.95	0.00	0%	24.95	100%
10000	22.51	0.54	2%	21.70	100%
100000	19.53	0.00	0%	19.53	100%
1000000	17.90	0.63	3%	17.36	100%

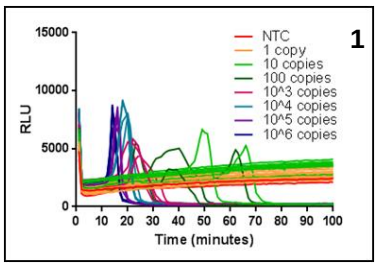
Figure 3.26: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with F3 version 3 displacement primer omitted and B3 version 3 replaced with B3 version 1 (1) light output against time for the serial dilution of 10^6 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

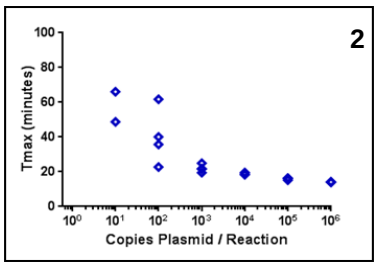
At 1 and 10 copies per partition the amplification frequency is reduced from 17% to 8% and from 92% to 17% respectively when compared to the assay with the matching B3 displacement primer and without the F3 displacement primer. The amplification frequency for the 100 copies to 10^6 copies per partition remains at 100%. The values for LOD and LOQ are the same as for the comparable matching B3 displacement

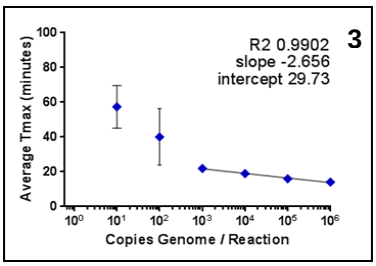
primer assay. The average T_{max} for this assay at 10^6 copies per partition is approximately 18 minutes which is almost 3 minutes slower than the matching assay and about 6 minutes slower than the control. At 1000 copies per partition the difference between this assay and the matching assay is about 2 minutes slower and approximately 8.5 minutes slower than the control. Mismatches in the displacement primer do decrease amplification frequency and increase time-to-peak times when compared to a matching displacement primer. The time-to-peak values were slower than those observed from the assay without displacement primers. This suggests that a mismatched displacement primer interferes with LAMP amplification more than not having a displacement primer at all.

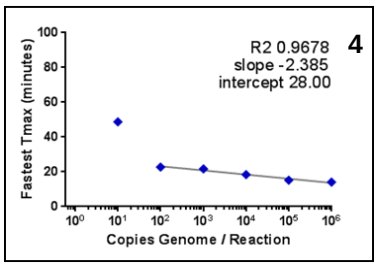
3.5.3.6 Omission of displacement primers and carrier DNA

All the 35Sp LAMP-BART assays in section 3.4.3 have had 100ng per partition salmon sperm carrier DNA as part of the LAMP-BART assay mastermix. In this assay the displacement primers are omitted and the carrier DNA replaced with MGW (Figure 3.27). In section 3.3 carrier DNA was shown to enhance amplification frequency, sensitivity and time-to-peak values.









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Copies	Mean	SD	cv	fastest	s/rate
1					0%
10	57.46	12.26	21%	48.79	17%
100	40.12	16.22	40%	22.79	100%
1000	21.98	2.23	10%	21.71	100%
10000	19.00	0.63	3%	18.46	100%
100000	16.02	0.54	3%	15.20	100%
1000000	14.12	0.00	0%	14.12	100%

Figure 3.27: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with both displacement primers omitted and no carrier DNA (1) light output against time for the serial dilution of 10^6 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

The amplification frequency at 10 copies per partition is slightly lower than with the comparable assay with salmon sperm carrier DNA. The sensitivity of the assay remains the same with limit of detection of 10 copies per partition. The variance and average

T_{max} values for the two assays have a similar profile as does the quantification. The addition of carrier DNA to the displacement primer deficient assays doesn't appear to enhance the assay results.

In summary, the absence of both displacement primers did not prevent LAMP amplification from initiating. Displacement primers are not required once the initial LAMP primers have been displaced and these results suggest that LAMP primer can act as displacement primers. However the absence of displacement primers reduced the amplification frequency and increased the variation between replicates at low copy number. In the optimised set of 35Sp primers, one of the displacement primers has more utility than the other and a mismatching displacement primer can interfere with LAMP amplification more than not having that displacement primer present in the primer set. The addition of carrier DNA to the assay without displacement primers did not improve the sensitivity, amplification frequency or variability between replicates of the deficient LAMP-BART assay.

3.5.4 Effect of APS concentration on LAMP-BART assays

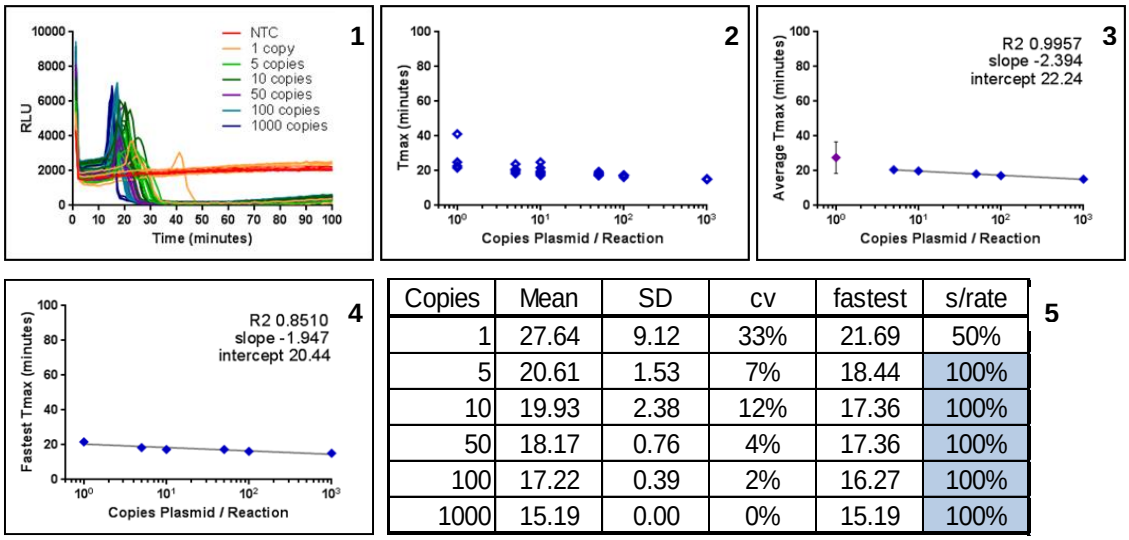
In a LAMP-BART assay the peak of light produced corresponds to the consumption of the substrate APS in combination with inorganic pyrophosphate by ATP sulphurylase which leads to the build up of pyrophosphate produced from the LAMP reaction. At this point no more ATP is produced for the luciferase/luciferin reaction and the excess of pyrophosphate is inhibitory to luciferase. The peak is therefore followed by a rapid decline in light output.

The concentration of APS in the reaction mix is therefore critical to the time the peak of light (T_{max}) is reached. Moreover, at low copy number the concentration of APS will be more important; a high concentration will take a long time to be consumed as the amount of pyrophosphate required will be high. The slowing of the T_{max} values for low copy number while maintaining high copy number T_{max} values, will hypothetically spread the range of times improving the differentiation between the low copy number samples tested.

A non-BARTmaster mastermix was set up for the 35Sp LAMP-BART assay of linearised pART7 so that the concentration of APS could be adjusted. The concentration of APS in the assays was calculated to be 125, 250, 500, 750 and 1000µM. The data from the 250, 500 and 750µM APS assays is in the Appendix (Figures: App3.1 to App3.3).

3.5.4.1 Effect of 125µM APS assay concentration

In the first experiment, the concentration of APS in the 35Sp LAMP-BART assay was reduced to 125µM (Figure 3.28). The aim was to observe the effect of reduced APS concentration on the differentiation between the copy numbers assayed (the standard concentration of APS is 250µM).



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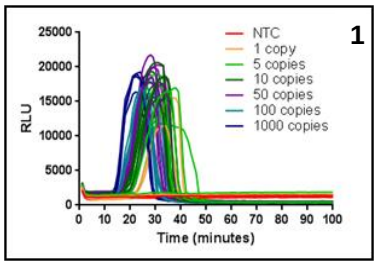
Copies	Mean	SD	cv	fastest	s/rate
1	27.64	9.12	33%	21.69	50%
5	20.61	1.53	7%	18.44	100%
10	19.93	2.38	12%	17.36	100%
50	18.17	0.76	4%	17.36	100%
100	17.22	0.39	2%	16.27	100%
1000	15.19	0.00	0%	15.19	100%

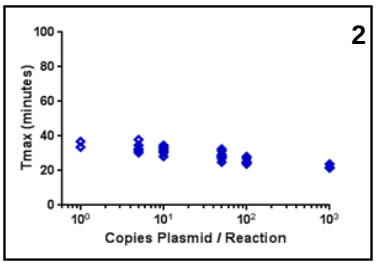
Figure 3.28: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with reduced concentration of APS (125µM) (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

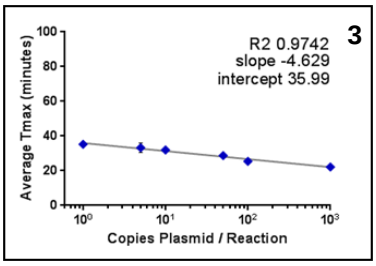
The amplification frequency for all copy numbers was high with the reduction in APS concentration. The amplification frequency for 1 copy per partition is within the typical range of values from 30 to 63% for this LAMP-BART assay. The gradient of the semi-logarithmic line correlating template copies to average T_{max} is shallow which impacts on the differentiation of copy numbers with this assay. The variation between replicates increases below 50 copies per partition, but there is still a strong correlation (R^2 of 0.996) between average T_{max} and copy number from 1000 to 5 copies per partition.

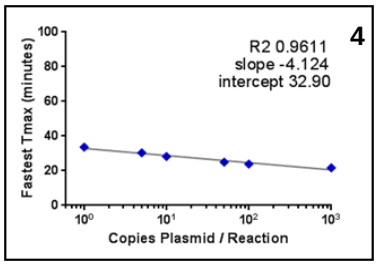
3.5.4.2 Effect of 1000µM APS assay concentration

In this experiment, the concentration of APS in the non-BARTmaster 35Sp LAMP-BART assay was increased to 1000µM (Figure 3.29). The aim of the experiment was to observe the effect of increased APS concentration on the differentiation between the copy numbers assayed without compromising sensitivity, variation between replicates and quantitation to very low copy number.









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Copies	Mean	SD	cv	fastest	s/rate
1	35.28	2.31	7%	33.65	25%
5	33.29	2.72	8%	30.39	75%
10	32.02	2.10	7%	28.21	100%
50	28.76	2.40	8%	24.95	100%
100	25.49	1.42	6%	23.86	100%
1000	22.23	1.09	5%	21.68	100%

Figure 3.29: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with four times the standard concentration of APS (1mM) (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

There is 100% amplification frequency from 1000 to 10 copies per partition, but the frequency is reduced for 5 and 1 copy per partition. The variance from all the positive replicates at each copy number level is low and the correlation to the semi-logarithmic lines of fastest and average T_{max} against copy numbers per partition are high (R^2 of 0.96 and 0.97). The gradient of the slopes is steeper than previously observed which allows for greater differentiation between copy numbers. The average T_{max} for 1000 copies per partition is approximately 7 minutes slower than the result from the 125µM APS assay and for 10 copies per partition this gap is approximately 13 minutes. This widening gap at lower copy number accounts for the increasingly steep slope. Visually the BART peaks are wider than previously observed and the average peak height has increased when compared to the 125µM APS assay.

3.5.4.3 Summary of the effect of APS assay concentration

The fastest T_{max} data from the five assays was plotted in a series of graphs for each copy number per partition against the concentration of APS in the LAMP-BART assay (Figure 3.30).

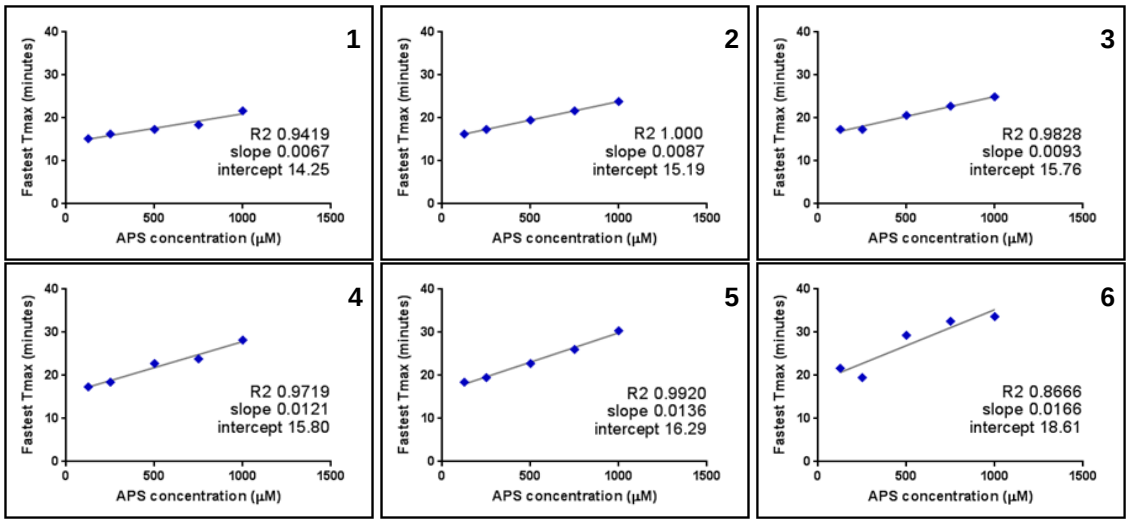


Figure 3.30: GM-LAMP-BART 35Sp assays of linearised plasmid pART7 with APS concentration of 125µM to 1mM, fastest T_{max} against APS concentration for (1) 1000 copies per partition (2) 100 copies per partition (3) 50 copies per partition (4) 10 copies per partition (5) 5 copies per partition (6) 1 copy per partition

The fastest T_{max} plots with increasing concentration of APS for each copy number per partition show that the gradient of the linear models becomes increasingly steep as the copy number reduces. At 1000 copies per partition the increase in fastest T_{max} with increasing APS concentration is shallow, but the increased concentration does clearly slow the LAMP-BART assay. This slowing of time to peak seems to be a result of the kinetics of the BART detection of the LAMP reaction and not the LAMP amplification. The point of inflection of a BART peak remains largely unaffected by the increasing APS concentration whereas the time-to-peak is affected by the additional peak height which is a result of the continued processing of inorganic pyrophosphate to ATP until APS is consumed.

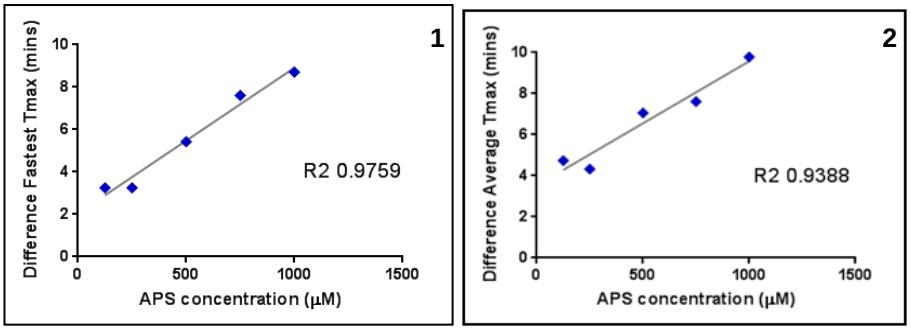


Figure 3.31: Improved differentiation between copy numbers in 35Sp LAMP-BART assay of pART7 template by increased APS concentration (1) difference between the fastest T_{max} values of 5 and 1000 copies per partition for each concentration of APS (2) difference between average T_{max} values between 10 and 100 copies per partition for each concentration of APS

There is a linear relationship between increasing APS concentration and the time difference between the fastest T_{max} values for 1000 and 5 copies per partition (Figure

3.31). This is also observed with the time difference between the average T_{max} values for 1000 and 10 copies per partition. The difference increases from approximately four minutes for the standard assay APS concentration of 250µM to approximately nine minutes at 1mM. Increasing the APS concentration can therefore improve the differentiation between copy numbers and improve the 35Sp LAMP-BART quantitation of the pART7 linearised plasmid. However, amplification frequency may be compromised.

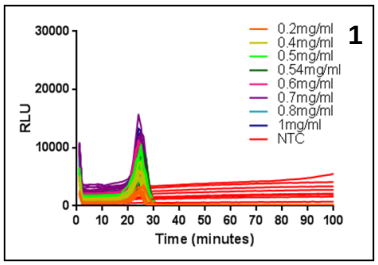
3.5.5 Luciferase

In these experiments two batches of Promega's Ultra -Glo® luciferase (**OLD** batch: E140X 25724903 and **NEW** batch: E140X 29457706) were assessed for robustness for LAMP-BART assays over a range of concentrations and different preparation conditions. The thermostability of the new luciferase batch in LAMP-BART assays is investigated in section 3.6.2.

3.5.5.1 Effect of luciferase concentration on LAMP-BART time-to-peak

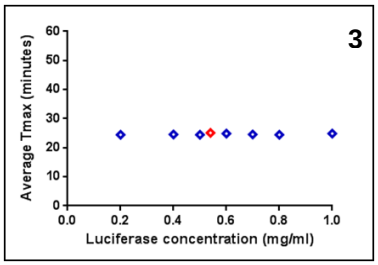
In this first experiment, the robustness of the old batch of Promega Ultra-Glo® luciferase at 5.4mg/ml batch E140X 25724903 was investigated over a range of luciferase concentrations. The aim of the experiment was to observe the effect of altered luciferase concentration on average T_{max}, variability between replicates and average peak height. Higher concentrations of luciferase may be required when BART peaks are close to the baseline light output, for example in the reduction of total assay volume. Higher concentrations of luciferase should theoretically increase the peak height of BART curves.

Thawed stock of luciferase was diluted to aliquots with concentrations of 0.2 to 1.0mg/ml using MGW, DTT and ThermoPol buffer as detailed in Chapter 2. Eight individual multimixes were prepared containing separately the eight luciferase concentrations. The 35Sp LAMP-BART assay was prepared to detect 100 copies per partition of the linearised plasmid template pART7 (Figure 3.32).



Copies	Luciferase	Mean	SD	cv	fastest	s/rate
100	0.2mg/ml	24.46	0.83	3%	23.91	100%
100	0.4mg/ml	24.59	0.57	2%	23.91	100%
100	0.5mg/ml	24.46	0.83	3%	22.82	100%
100	0.54mg/ml	25.14	0.91	4%	23.91	100%
100	0.6mg/ml	24.87	0.91	4%	23.91	100%
100	0.7mg/ml	24.59	0.57	2%	23.91	100%
100	0.8mg/ml	24.46	0.58	2%	23.91	100%
100	1.0mg/ml	24.87	0.70	3%	23.91	100%

2



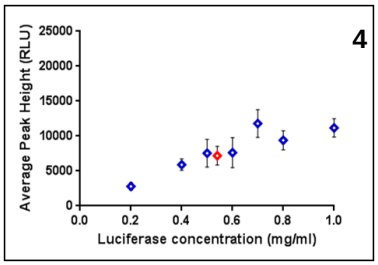
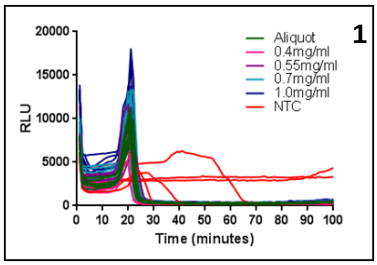


Figure 3.32: (1) light output against time for the concentrations of Ultra-Glo® luciferase (5.4mg/ml batch E140X 25724903) in the LAMP-BART 35Sp assay of 100 copies pART7 per partition (2) summary table of results (3) average T_{max} against concentrations of Ultra-Glo® luciferase at 100 copies pART7 per partition (4) average peak height against luciferase concentration

The first graph (panel 1) shows average T_{max} values of 24 to 25 minutes for all of the assays with concentrations of luciferase from 0.2mg/ml to 1.0mg/ml. Also, the variation between replicates for all of the assays is low. The average peak height for the BART curves shows a general increase with luciferase concentration, although the value for 0.7mg/ml luciferase in the assay appears to be higher than the value from 1.0mg/ml. The data suggests that for this batch of luciferase, the concentration of luciferase in this range does not affect LAMP-BART average T_{max} or variance. The maximum light output of the BART peaks increases with increasing luciferase concentration.

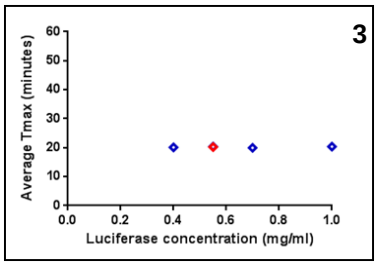
3.5.5.2 Effect of luciferase concentration on LAMP-BART time-to-peak using a new luciferase batch

In a repeat of the previous experiments, a range of luciferase dilutions were prepared between 0.4 and 1.0 mg/ml. The aim of this experiment was to investigate the effect of altered luciferase concentration on average T_{max}, variability between replicates and average peak height using a new batch of Ultra-Glo® luciferase. Five individual multimixes were prepared containing separately the four luciferase dilutions of 0.4, 0.55, 0.7 and 1.0mg/ml and one of the pre-prepared frozen luciferase aliquots, also at 0.55mg/ml. The LAMP-BART assay was run to detect 100 copies per partition (Figure 3.33).



Copies	Luciferase	Mean	SD	cv	fastest	s/rate
100	0.4mg/ml	20.11	0.82	4%	18.47	100%
100	Aliquot	20.38	0.50	2%	19.56	100%
100	0.55mg/ml	20.24	0.56	3%	19.56	100%
100	0.7mg/ml	19.97	1.00	5%	18.47	100%
100	1.0mg/ml	20.38	0.50	2%	19.56	100%

2



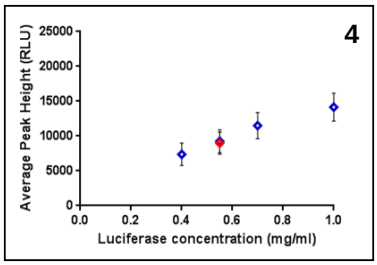


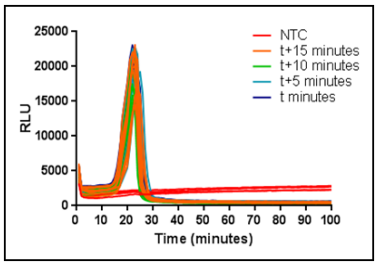
Figure 3.33: (1) light output against time for the concentrations of Ultra-Glo® luciferase (5.5mg/ml batch E140X 29457706) in the LAMP-BART 35Sp assay of 100 copies pART7 per partition (2) summary table of results (3) average T_{max} against concentrations of Ultra-Glo® luciferase at 100 copies pART7 per partition (4) average peak height against luciferase concentration

The average T_{max} values between luciferase dilutions were approximately 20 minutes for all the assays with low variation. The average peak heights at T_{max} increased with increasing luciferase concentration.

Both these batches of luciferase have consistent average time-to-peak values with the alteration of luciferase concentration. The new batch of luciferase has faster average T_{max} times and was selected for further testing. The inter-assay variation between the average T_{max} values from these assays could be related to the batch of luciferase used, but may also be associated with variation between aliquots of the same batch of Bst polymerase or other factors.

3.5.5.3 Effect of luciferase dilution preparation on LAMP-BART time-to-peak

To investigate the impact of thawing time on the stock luciferase before dilution, a number of dilutions were prepared at time intervals after the stock luciferase had fully defrosted. The first dilution was prepared directly after defrosting of the luciferase, followed by a further one at 5 minutes, 10 minutes and finally 15 minutes. Four individual multimixes were prepared containing separately the four luciferase aliquots. The LAMP-BART assay was set up to detect 100 copies per partition of the linearised plasmid template pART7 (Figure 3.34).



1

Copies	Luciferase	Mean	SD	cv	fastest	s/rate
100	t+15 minutes	22.78	0.82	4%	21.69	100%
100	t+10 minutes	22.24	0.58	3%	21.69	100%
100	t+5 minutes	22.78	1.16	5%	21.69	100%
100	t minutes	22.51	0.50	2%	21.69	100%

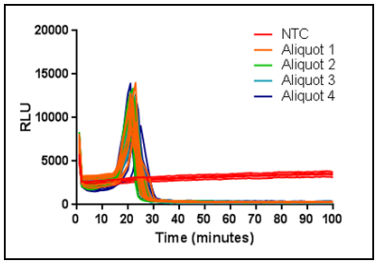
2

Figure 3.34: (1) light output against time for the preparations of Ultra-Glo® luciferase in the LAMP-BART 35Sp assay of 100 copies pART7 per partition (2) summary table of results

All the assays of 100 copies per partition pART7 with these luciferase preparations resulted in consistent average T_{max} between 22 and 23 minutes. This assay suggests that the handling of the luciferase does not affect the LAMP-BART assay

3.5.5.4 Effect of luciferase single-use aliquots on LAMP-BART time-to-peak

In a final experiment, a number of diluted aliquots of 0.55mg/ml were prepared and kept frozen. Four of these aliquots were selected at random and used in this experiment to assess the average T_{max} and variability between aliquots. Four individual multimixes were prepared containing separately the four luciferase aliquots. Once again the LAMP-BART assay was set up to detect 100 copies per partition of the linearised plasmid template pART7 (Figure 3.35).



1

Copies	Luciferase	Mean	SD	cv	fastest	s/rate
100	Aliquot 1	21.70	1.30	6%	19.52	100%
100	Aliquot 2	21.42	0.50	2%	20.61	100%
100	Aliquot 3	21.70	0.82	4%	20.61	100%
100	Aliquot 4	22.10	1.42	6%	20.61	100%

2

Figure 3.35: (1) light output against time for the aliquots of Ultra-Glo® luciferase in the LAMP-BART 35Sp assay of 100 copies pART7 per partition (2) summary table of results

The single-use aliquots of diluted luciferase from the new batch resulted in consistent time-to-peak results at 100 copies of the linearised plasmid template per partition. Single-use luciferase aliquots were therefore used for all LAMP-BART assays subsequently to contribute towards reducing inter-assay variation.

In summary, the concentrations of luciferase did not affect the time-to-peak results from the LAMP-BART assays of 100 copies per partition. The average peak height of the BART curves did increase with the increasing concentrations of luciferase which could

be beneficial to LAMP-BART assays with reduced total assay volume. The length of time that the luciferase stock was defrosted during preparation of dilutions did not affect time-to-peak values. Also the preparation of multiple aliquots of diluted luciferase for single use showed consistent results.

3.6 Effect of altering LAMP-BART assay parameters for quantitation by time-to-peak

3.6.1 Negative controls (anti-contamination)

Fundamental to the development of quantitation at low copy number and for accuracy in quantification, is the control of contamination. To set up LAMP-BART and PCR assays a clean area was defined and dedicated equipment used. Other laboratory areas were assumed to be contaminated and precautions taken to separate these areas. Areas with amplified product such as associated with the BART hardware and gel electrophoresis were assumed to be highly contaminating. The transfer of an unamplified assay in a 96 well plate necessitated a clear seal to cover the wells. After amplification this seal was only removed if non template samples were tested to identify contamination from non-specific primer interaction by visualising any LAMP ladder pattern, otherwise the plates were placed in grip seal bags and disposed by autoclaving (it should be noted that autoclaving does not destroy DNA and incineration of bleach would have been more effective). A benefit to using individual traceable aliquots in non-BARTmaster assays was to aid troubleshooting should contamination occur. Each LAMP-BART assay contained a number of non-template controls to identify contamination. Periodically background checks were made with the 35Sp primer set especially before a series of digital BART reactions. In the unlikely event that contamination should occur, a deep clean was instigated followed by a background check LAMP-BART assay. In this way contamination was controlled and baseline results were observed for NTCs.

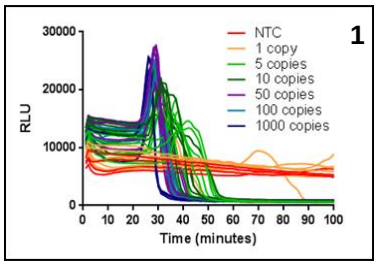
3.6.2 Assay temperature

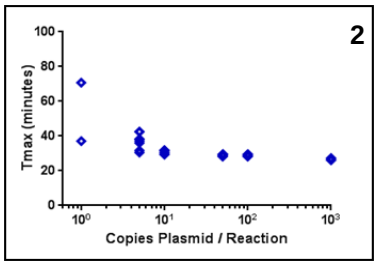
As the temperature of the assay increases, so does the ‘breathing’ of the target DNA sequence, increasing the likelihood of LAMP amplification initiation. The activities for the Bst polymerase, ATP sulphurylase and Ultra-Glo® luciferase in the reaction mix will be affected by the change in temperature as will the binding of the primers to their target sequences. With the aim of quantification at low copy number the optimum

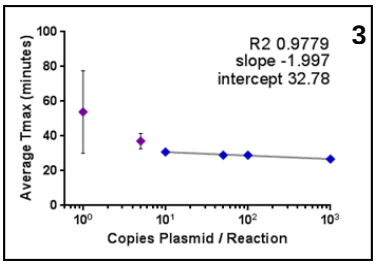
assay temperature will be a balance of a number of factors. The luciferase will undoubtedly become less efficient with increasing temperature, whereas higher temperatures will favour amplification initiation which may be crucial to low copy number detection with high amplification efficiency.

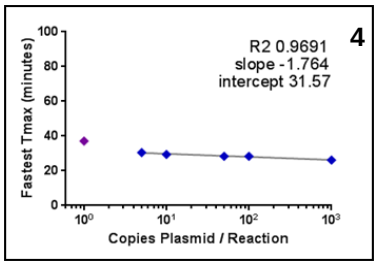
3.6.2.1 52°C Assay Temperature

In this first experiment the 35Sp LAMP-BART assay of linearised pART7 was set up as a standard non-BARTmaster assay with 100ng/partition salmon sperm carrier DNA. The programme of the LUCY hardware was adjusted to perform the assay at 52°C (Figure 3.36). This assay forms a starting point for comparison with sensitivity, amplification frequency, limit of quantitation, average T_{max} and variation at low copy number to optimise the assay for low copy number quantification.









5

Copies	Mean	SD	cv	fastest	s/rate
1	53.94	23.78	44%	37.12	25%
5	37.11	4.39	12%	30.58	100%
10	30.85	0.97	3%	29.48	100%
50	29.07	0.56	2%	28.39	100%
100	29.07	0.56	2%	28.39	100%
1000	26.76	0.63	2%	26.21	100%

Figure 3.36: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 at 52°C assay temperature (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average T_{max} against template copies per partition (4) fastest T_{max} against template copies per partition (5) summary table of results

Variation between replicates is low (2% to 3%) for 1000, 100, 50 and 10 copies per partition with and associated 100% success rate for each of these. The limit of quantitation using average T_{max} is 10 copies per partition. Lower copy numbers per partition have increasing variation between the replicates. However the differentiation between the individual copy numbers from 1000 to 10 copies per partition could be poor due to the shallow slope representing the correlation between copy number and average T_{max}. Peak heights for the 52°C assay for 1000, 100 and 50 copies per partition were in the region of 24500 RLU. In contrast, peak heights for the standard assay temperature of 60°C are typically in the region of 7000 RLU. The NTCs

remained clear throughout the assay, however a slight increase was observed with one of them suggesting that non-specific primer interactions could be an issue at low assay temperature.

The results from the repeat LAMP-BART assays at 55, 57, 60, 63 and 65°C are in the Appendix (Figures: App3.8 to App3.12).

3.6.2.2 Summary of Temperature Range 52°C to 65°C

Light Output (RLU) with Increasing Temperature

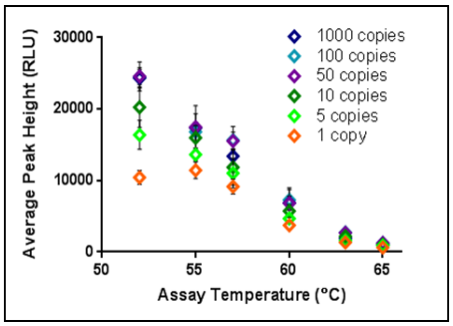


Figure 3.37: Average peak height against assay temperature

The assay results suggest that the optimal assay temperature for Ultra-Glo™ luciferase is 52°C or possibly below. The light output from the average T_{max} peaks for 1000 copies per partition shows a steep decline from 52°C to 65°C (Figure 3.37). The average peak height values for 1000 copies per partition were higher than the 1 copy per partition values suggesting that average peak height may be useful for quantitation. This is more relevant at the lowest assay temperature of 52°C where the range of average peak heights was greatest. At 65°C the light output was very low, but time-to-peak values could still be determined from the baseline of luminescence. Any further increase in assay temperature could make time-to-peak determination difficult to ascertain and less certain.

Amplification Frequency with Increasing Temperature

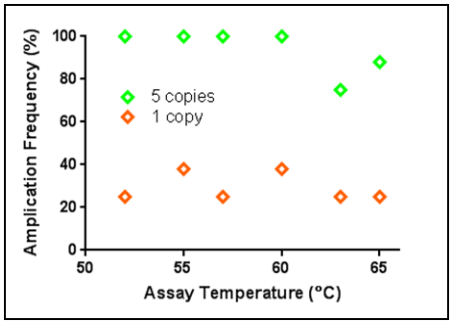


Figure 3.38: Amplification frequency against assay temperature

Between 1000 copies and 10 copies per partition the amplification frequency is 100% for all assay temperatures. There is very little change in the amplification frequency for single copy detection which fluctuates in the range 20 to 40%, but at 5 copies per partition the success rate drops below 100% with temperatures of 63°C and above (Figure 3.38). At the higher assay temperatures conditions should be favourable for the Bst polymerase, ATP sulphurylase and for the 'breathing' of the double stranded DNA template. It is possible that the reduction in amplification frequency at the lowest copy numbers at these high temperatures is related to the optimal conditions for the LAMP primers.

Time to Peak (T_{max}) with Increasing Temperature

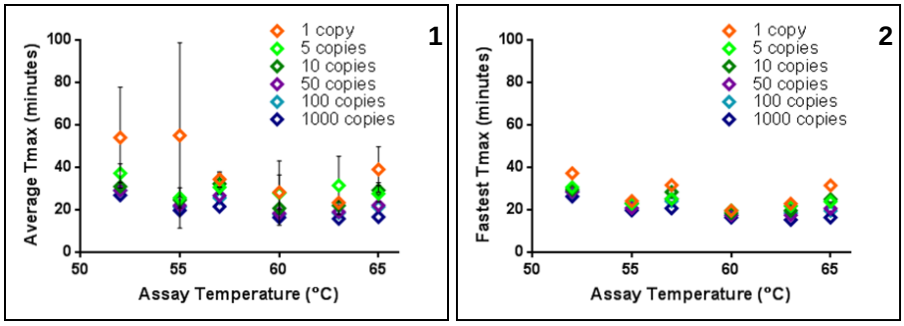


Figure 3.39: (1) average T_{max} against assay temperature (2) fastest T_{max} against assay temperature

For 1000 copies per partition the average and fastest T_{max} values are approximately 25 minutes at 52°C, 20 minutes for 55°C and 57°C and reaching a minimum time of about 15 minutes at 60°C and above. It appears that the optimal conditions for the initiation of LAMP amplification increase with temperature reaching a maximum level in the region of 60°C (Figure 3.39). The increasing spread of values above 60°C may be the result of suboptimal conditions for the various enzymes in the assay at high temperature.

Time to Peak v Copy Number

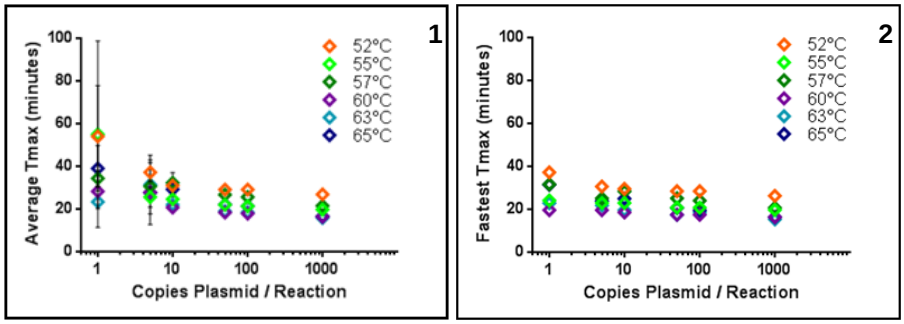


Figure 3.40: (1) average T_{max} against copy number per partition (2) fastest T_{max} against copy number per partition for each assay temperature

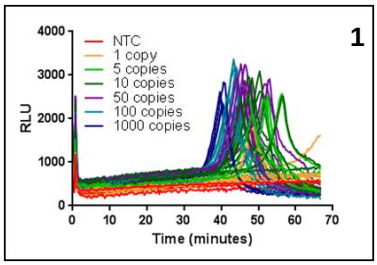
The fastest time to peak times were observed with the assays at 60°C and above (Figure 3.40). The slowest assay times were observed at 52°C.
The light output, amplification frequency and fast reaction times are favourable for the assay temperature for the 35S LAMP-BART assay of linearised pART7 template to remain at 60°C.

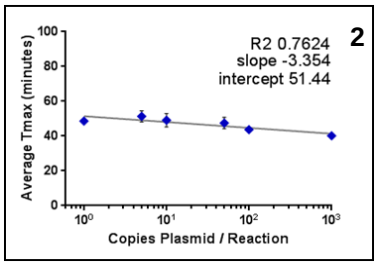
3.6.3 Hardware settings

The BART platform used in the Cardiff University laboratory is of a type called LUCY. Assay parameters can be programmed using the software associated with the T-Robot thermal cycler. Assay temperature, total length of assay and the time integrals in which light output is accumulated can be set. Routinely the time integrals are set to one minute at which time point a value for the accumulated light will be recorded. As a result time-to-peak values will be at defined time points which may be the closest time point to the actual peak. To investigate possible inaccuracies in time-to-peak values from the hardware settings the LUCY was set to 15 second time integrals.

3.6.3.1 Assay set for 15 second time integrals

A 35Sp LAMP-BART assay of the linearised pART7 template was used to provide data for 1000 copies to 1 copy per partition (Figure 3.41). The assay was slower than normal due to the issues with a batch of Ultra-Glo® luciferase that was affecting assays at the time.





3

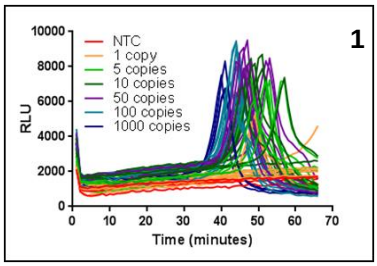
copies	mean	SD	cv	fastest	s/rate
1	48.62	0.00	0%	48.62	25%
5	51.34	3.23	6%	47.29	75%
10	49.10	3.87	8%	45.29	88%
50	47.54	3.25	7%	43.95	100%
100	43.70	1.09	2%	41.62	100%
1000	40.20	0.42	1%	39.62	100%

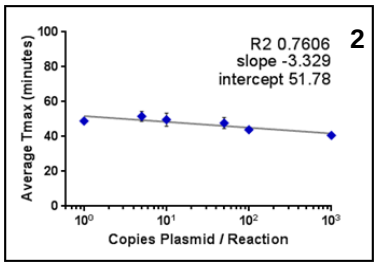
Figure 3.41: LAMP-BART 35Sp assay of linearised pART7 with LUCY time integral parameter set to 15 seconds (1) light output against assay time (2) average T_{max} against copy numbers per partition of the pART7 template (3) summary table of results

The morphology of the majority of BART peaks for the 15 second setting is a sharp peak. Those peaks that have a flattened top are not wide at the top and are usually sloped in favour of one of the two time points. The flat top of a peak implies that the actual peak time was between the two time points. The standard deviation between the replicates at 1000 copies is very low at 0.42 minutes with the average T_{max} of 40.20 minutes.

3.6.3.2 Data extrapolated to 1 minute time integrals

The data from the LAMP-BART assay was used to create time integrals of 1 minute (Figure 3.42).





3

copies	mean	SD	cv	fastest	s/rate
1	48.96	0.00	0%	48.96	25%
5	51.62	2.87	6%	47.96	75%
10	49.67	3.82	8%	45.95	88%
50	47.83	3.09	6%	43.95	100%
100	43.95	1.07	2%	41.95	100%
1000	40.70	0.50	1%	39.95	100%

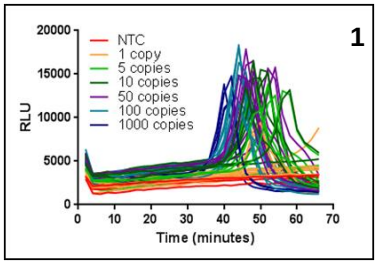
Figure 3.42: LAMP-BART 35Sp assay of linearised pART7 with LUCY time integral parameter set to 15 seconds, data accumulated to 1 minute integrals (1) light output against assay time (2) average T_{max} against copy numbers per partition of the pART7 template (3) summary table of results

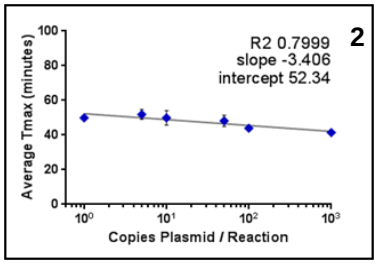
A number of the peaks which were previously shown to have flattened tops are now wider at the apex favouring one side or the other. The increasing width should impact on the accuracy of the time-to-peak value assuming that the 'true' peak is between those two values. Indeed the standard deviation of the replicates at 1000 copies per partition has increased but this is only from 0.42 minutes to 0.50 minutes. At 100 copies per partition the standard deviation decreased from 1.09 to 1.07 minutes. Using average T_{max} as against individual time-to-peak values appears to even out any inaccuracies originating from integral time settings. The use of individual peak times in

the case of the fastest T_{max} may lose accuracy but then this method of quantification is prone to inaccuracy as discussed in section 4.5.1 of Chapter 4.

3.6.3.3 Data extrapolated to 2 minute time integrals

In a final review of the results from the LAMP-BART assay the data was accumulated to create integrals of 2 minutes (Figure 3.43).





3

copies	mean	SD	cv	fastest	s/rate
1	49.96	0.00	0%	49.96	25%
5	51.95	2.83	5%	47.96	75%
10	49.95	4.16	8%	45.95	88%
50	48.2	3.28	7%	43.95	100%
100	43.95	1.07	2%	41.95	100%
1000	41.45	1.00	2%	39.95	100%

Figure 3.43: LAMP-BART 35Sp assay of linearised pART7 with LUCY time integral parameter set to 15 seconds, data accumulated to 2 minute integrals (1) light output against assay time (2) average T_{max} against copy numbers per partition of the pART7 template (3) summary table of results

The flattening of many of the peaks by the width of the time points has resulted in a further increase in the standard deviation for 1000 copies per partition from 0.50 to 1.00 minutes. The standard deviation for 100 copies per partition remains the same as the previous interpretation of the results. The time-to-peak values are affected by the increasing size of the time integrals, but the average T_{max} continues to be a good quantitation measurement.

3.7 Discussion

The aim of this chapter was to improve the sensitivity and variation between replicates in LAMP-BART assays of various templates and to be able to quantify over a wide range of template concentrations using average T_{max}. A LAMP-BART assay using 35Sp primers successfully detected 50 copies of the genomic DNA target within a background of non-target genomic DNA. At this copy number the amplification frequency was less than 100% and quantification using average T_{max} was not possible. To understand the factors that influence the quantification of genomic DNA, an artificial template of linearised plasmid DNA of small size was selected based on LOD before optimisation of <10 copies per partition and LOQ of approximately 100 copies per partition. The benefit of using the pART7 linearised DNA as a template is the accuracy of initial quantification by Agilent Bioanalyzer and NanoDrop spectrophotometer which

are either unsuitable or inaccurate for genomic DNA. Also the NanoDrop appears to indicate that the purity of the plasmid DNA is higher than the genomic template and could therefore be less affected by inhibitory impurities.

The concentration of non-target DNA has important implications to molecular diagnostics whether PCR or isothermal amplification based. The ERM maize GM experiments showed that the sensitivity of the LAMP-BART assays could be improved by increasing total genome load to between 30 and 110ng DNA. Furthermore the reproducibility and success rate were improved by the presence of genome carrier to within this range. However, large amounts of non-target DNA had a negative impact on LAMP-BART kinetics with increased time to peak values. One possible explanation for the beneficial concentration of non-target DNA could be from providing a large amount of alternative DNA for deoxyribonucleases that may be present in the sample which would afford some protection to the target DNA. Alternatively the non-target DNA could restrict the loss of target copy number to the plasticware or possibly act to bring reactants together for amplification. The high concentrations of carrier DNA increased the time-to-peak values with an increase in variation between replicates. This suggests that the availability of the template to LAMP primers is hampered physically by the excess DNA resulting in time delays in the initiation of amplification.

Although salmon sperm carrier DNA was shown to improve sensitivity in LAMP-BART assays, it was unsuitable for use with qPCR due to amplification of the carrier. Non-template genomic DNA above a low level (approximately 4ng per partition) reduced the amplification efficiency of the qPCR reaction. Above approximately 110ng per partition, the SYBR green-based qPCR assay failed to differentiate target amplicon from the background fluorescence generated by the excess of non-template DNA. The lack of enhancement to qPCR suggest that the assay is either already optimised or that the improvement to LAMP-BART assays is related to the LAMP amplification mechanism. To investigate the LAMP mechanism, displacement primers were removed from the LAMP-BART assay of linearised plasmid template. The assay showed that LAMP amplification can still continue without displacement primers albeit with reduced amplification frequency and increased variation between replicates at low copy number. From the published details of the LAMP mechanism (Notomi 2000), displacement primers are required for the initial step of displacing the LAMP primer from the template. After the formation of the ‘dumbbell’ structure, displacement primers are no longer required (except for the displacement of further LAMP primers from the template in an assay). The data suggests that the loop-forming LAMP primers not only

initiate amplification but can also act as displacing primers. However, for an optimised LAMP assay, displacement primers appear to be required to achieve high sensitivity with low variation between replicates. In the 35Sp primer set only one of the displacement primers was required to achieve this and the presence/absence of the other displacement primer was ineffectual. A mismatching displacement primer interfered with LAMP amplification possibly by restricting the displacement activity of the LAMP primers that appeared to occur in the absence of displacement primers. The optimisation of a LAMP primer set could be achieved by selecting the loop-forming LAMP primers first in the absence of displacement primers, before increasing assay sensitivity and the utility of using average T_{max} for quantification by decreasing variation between replicates, by adding individually the displacement primers. These experiments also highlight the importance of primer design for the sensitivity and reproducibility of LAMP-BART assays.

One of the components of BART is adenosine phosphosulphate (APS) which is a substrate with inorganic pyrophosphate for the production of adenosine triphosphate (ATP) catalysed by ATP sulphurylase. Increasing APS concentration has been shown (Gandelman 2010) to increase light intensity over a range of tested concentrations and to have a small effect on peak time. The semi-logarithmic correlation between average T_{max} and template copy number is typically shallow, which is a disadvantage to quantification using this method. If the correlation could be steeper (creating a wider time integral between for example 1000 and 1 copy per partition) then there could potentially be increased discrimination between copy numbers in the LAMP-BART assay. The experiments with increasing APS concentration in this chapter showed that at 1 copy per partition the time-to-peak could be slowed to a greater extent than higher copy numbers at 1000µM APS assay concentration, producing a wide time integral and steeper correlation between average T_{max} and copy number. However there was the potential for the amplification frequency to be compromised at low copy number. A further benefit to a higher concentration of APS is the increased light output above the baseline luminescence, which could be useful to low total volume assays.

Another component of BART, critical to light output is the thermostable Ultra-Glo® luciferase. Troubleshooting inter-assay variation between comparable LAMP-BART assays had suggested that luciferase could be a source of this. The robustness of a new batch of luciferase was assessed and multiple aliquots of pre-diluted and frozen luciferase had consistent average time-to-peak results. The possibility of inter-assay variation from BART components is only relevant to non-BARTmaster assays. One

feature of the increased concentration of luciferase was the increase in average peak height of the BART curves, which could be beneficial to LAMP-BART assays with reduced total assay volume.

The LAMP-BART assay temperature for the 35Sp LAMP primer amplification of linearised pART7 was investigated to find the optimal temperature for the various components. The isothermal assay temperature ranged from 52°C to 65°C. The light intensity of BART peaks was highest at 52°C at which temperature the range of average peak height values was highest between the replicates at 1 copy per partition and 1000 copies per partition. There was a clear separation of average peak height for 1 copy and 5 copies per partition indicating that average peak height could be used for quantification at very low copy number. However at the low temperature assays of 52, 55 and 57°C there was some evidence from the NTCs that non-specific primer interactions could occur, which were not observed at the higher temperatures. The 35Sp primers would require stringent redesigning for low temperature assays. The slowest average T_{max} times were observed at 52°C which suggests that at low temperatures initiation of amplification is delayed possibly by a reduction in the 'breathing' of the double stranded target DNA preventing LAMP primer invasion. Slow assay times would not be a desirable characteristic in molecular diagnostics but this could be ameliorated by the potential of the 52°C assay to differentiate individual copy numbers at very low copy number.

The lowest temperature assay could be sub-optimal for the Bst polymerase and ATP-sulphurylase but the steepness in the BART curve from the inflection time to the peak suggests that the production of inorganic pyrophosphate and subsequently ATP remains rapid. Of the six assay temperature experiments, catalysis of luciferin by luciferase was highest at 52°C. The stability and activity of the enzymes at lower temperatures raises questions about the interactions between the components in the LAMP-BART assay before the start of the experiment. In the field conditions, such as in Africa and Australia, the temperature could approach 50°C and the assay could potentially start before it is transferred to the BART hardware. This could cause inaccurate quantification results using time-to-peak unless appropriate calibrating template was exposed to the same conditions.

The sensitivity of the assay is unaffected over the range of temperatures; single copy detection ranges in amplification frequency from 25% to 38%, the variation of which may be stochastic. The assay temperature results suggest that quantification at low copy number using average T_{max} down to 10 copies per partition was marginally better for the assays at 60°C and below. However, the likelihood of non-specific primer

interactions at the lower temperatures suggests that 60°C is the optimal temperature for this LAMP-BART assay.

The BART hardware settings can be adjusted to determine the length of the assay, temperature of the block and the time integrals for gathering light intensity from each partition. The light measurements are usually recorded every minute (Gandelman et al. 2010) by the camera to give sufficient light intensity for analysis. Reducing the time integral reduced the peak heights but the BART curves remained interpretable from the baseline luminescence. Time-to-peak values were affected by the decreased time integral defined by the LUCY settings. Visually there were fewer peaks with flattened tops, caused by two adjacent time points having similar light intensities, and this implies that the peak time was more accurate. However, when multiple replicates are used this slight time difference does not result in an alteration to the average time-to-peak measurement. One minute time integrals do provide greater light intensity and this could be important should smaller assay volumes be required for example with the development of microfluidic partitions and digital BART.

In conclusion quantification of DNA template by LAMP-BART using average time-to-peak is affected by LAMP and BART components, template and non-template and the settings of the hardware, but when optimised can achieve single copy sensitivity (LOD <10 copies per partition). The linear dynamic range for the LAMP amplicon assay was greater than 10 orders of magnitude and suggests that a wide range of template concentrations can be assayed using LAMP-BART; wider than the normal linear dynamic range achieved with qPCR (Bustin et al. 2009). The limit of quantification using average time-to-peak was in the range of 10 to 100 copies per partition. The increasing variation at low copy number prevented lower LOQ values. Alternative quantitation strategies to average time-to-peak could reduce these values further.

Chapter 4
Ultra-quantification

4.1 Introduction

The aim of this chapter was to use a simple target to obtain low copy number quantification. The work in chapter 3 showed that this remained elusive with genomic templates. The choice of template was based on the observation that linearised plasmid DNA from earlier experiments with the pUC35S GUS plasmid (Figure 4.1) showed an improvement in sensitivity in a 35Sp LAMP-BART assay when compared to the circular plasmid. This has also been observed with PCR amplification whereby circular DNA is less efficiently amplified (Chen et al. 2007). Poor results due to the supercoiled form of plasmid DNA has led to the recommendation to use the linear form for qPCR standards (Hou et al. 2010). The choice of a linearised plasmid template is also supported by its stability when stored at 4°C (Aguilera et al. 2008) after rehydration with molecular grade water. Storing the rehydrated plasmid at -20°C would inevitably increase the incidence of mechanical shearing by ice crystals of the DNA and subsequent disruption of complete target sequences.

The term ultra-quantification is used here to describe the quantification of a low copy number target typically in the range 1 copy per partition to 20 copies per partition. For an optimised assay, the variability of the time to peak (T_{max}) is low above 20 copies of linearised plasmid template as is shown in this chapter, so samples above this level are appropriate for quantification using average T_{max}. For copy numbers below 20 copies per partition alternative strategies are shown to be required. The aim of this chapter was to explore the limit of quantitation (LOQ) using the standard T_{max} approach, and to explore the possibility of using other parameters for quantification. Also to define the limit of detection (LOD) by the 95% positive replicates approach with LAMP-BART assays of 1, 2, 3, 4 and 5 copies per partition for comparison with the qPCR theoretical LOD (Bustin et al. 2009).

4.2 Linearised plasmid template

The template chosen for the following experiments was the linearised form of the cloning vector pART7 (Gleave 1992) which contains the cauliflower mosaic virus 35S

promoter, a multiple cloning site and the transcriptional termination region of the octopine synthase gene (Figure 4.1). For calculations of copy number the size of the pART7 plasmid was assumed to be 4.9kb, which is the value from Gleave (1992).

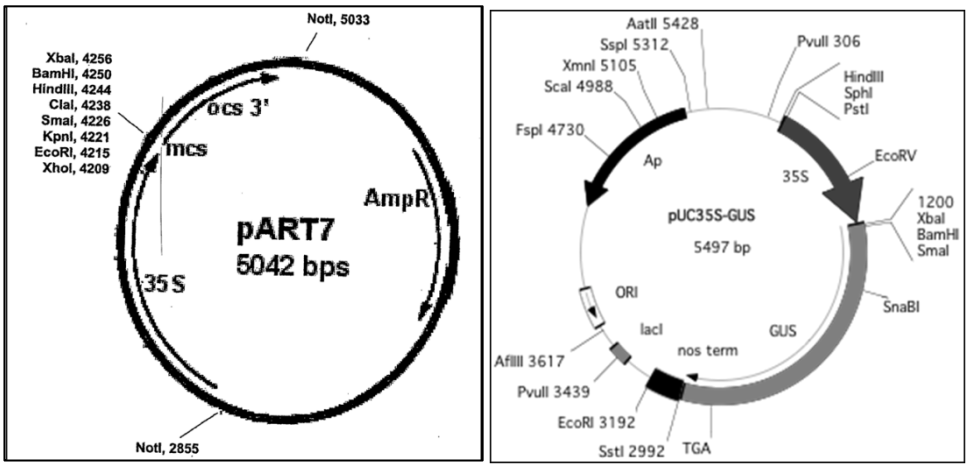
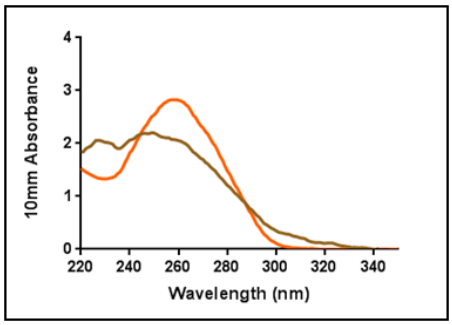


Figure 4.1: (1) circular map of the plasmid pART7 from patent EP1723244 B1 (2) circular map of the pUC35S-GUS plasmid

4.2.1 Initial quantification of the linearised pART7 plasmid template

Before the preparation of dehydrated aliquots of the pART7 target the original linearised sample was assayed at Lumora in Ely, Cambridgeshire by Dr Guy Kiddle and by me at the Murray Lab in Cardiff University for an accurate starting concentration.

Firstly Thermo Fisher's NanoDrop™ spectrophotometer was used to test a microlitre of the homologous sample (Figure 4.2).



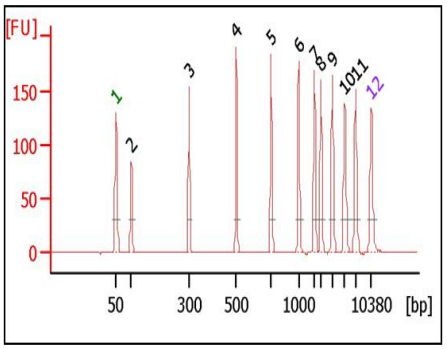
Peak table for pART7

Sample ID	Conc. [ng/µl]	260/280	260/230	Observations
pART7	102.77	1.70	1.02	1µl undiluted sample
pART7	140.91	1.90	2.12	Repeat

Figure 4.2: (L to R) Thermo Fisher NanoDrop™ results for undiluted pART7 sample (sample1, sample 2) (1) graphical representation of the 10mm absorbance with increasing wavelength (2) calculated concentration of pART7 sample

The average NanoDrop™ result from the sample and device in Cardiff of 122ng/µl followed a result of 118ng/µl from the NanoDrop™ at Lumora in Ely.

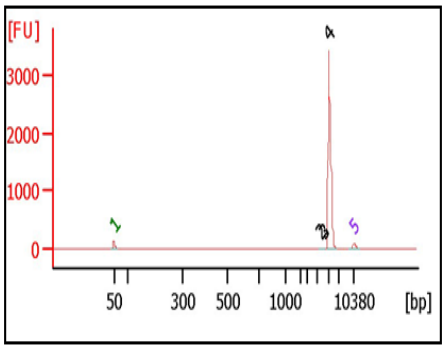
Following this, Agilent's Bioanalyzer in Cardiff University was used on the undiluted linearised pART7 sample and also a 10^{-1} diluted sample (Figures 4.3 to 4.5).



Peak table for Ladder

Peak	Size [bp]	Conc.ng/µl]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
2	100	4.00	60.6	Ladder Peak
3	300	4.00	20.2	Ladder Peak
4	500	4.00	12.1	Ladder Peak
5	700	4.00	8.7	Ladder Peak
6	1,000	4.00	6.1	Ladder Peak
7	1,500	4.00	4.0	Ladder Peak
8	2,000	4.00	3.0	Ladder Peak
9	3,000	4.00	2.0	Ladder Peak
10	5,000	4.00	1.2	Ladder Peak
11	7,000	4.00	0.9	Ladder Peak
12	10,380	4.20	0.6	Upper Marker

Figure 4.3: (L to R) Agilent Bioanalyzer results for (1) electropherogram of the internal ladder (2) size and concentration of the internal ladder

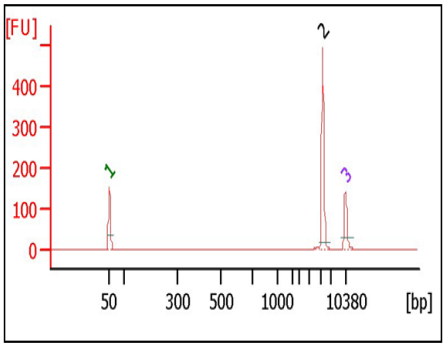


Peak table for pART7

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
4	4,977	93.77	28.5	pART7
5	10,380	4.20	0.6	Upper Marker

Figure 4.4: (L to R) Agilent Bioanalyzer results for (1) electropherogram of the undiluted linearised pART7 plasmid, peaks 1 and 5 represent the lower and upper ladder markers (2) size and concentration of the undiluted linearised pART7 plasmid (peak 4)

The Agilent Bioanalyzer assay of the undiluted sample resulted in a concentration of 93.77ng/µl which, from the instrument maker's guidelines, falls outside the optimum concentrations of 0.5 to 50ng/µl for accurate measurement although the concentration 93.77ng/µl was very close to the value from Lumora of 92.29ng/µl from the undiluted sample on their Agilent Bioanalyzer.



Peak table for pART7

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	50	8.30	251.5	Lower Marker
2	5,387	11.09	3.1	pART7 1/10
3	10,380	4.20	0.6	Upper Marker

Figure 4.5: (L to R) Agilent Bioanalyzer results for (1) electropherogram of the 10^{-1} dilution of linearised pART7 plasmid, peaks 1 and 3 represent the lower and upper ladder markers (2) size and concentration of the 10^{-1} dilution of the linearised pART7 plasmid (peak 2).

The concentration from the diluted sample was shown to be higher than those derived from the undiluted sample with a value of 110.9ng/µl when the dilution factor is taken into consideration. This value falls in the middle of the two NanoDrop concentrations from Cardiff and Ely.

These values were converted into copy numbers/µl using an on-line calculator (http://cels.uri.edu/gsc/cndna.html) giving $1.8x10^{10}$ copies/µl for the undiluted pART7 samples from the Agilent Bioanalyzers in Cardiff and Lumora. This rises to $2.1x10^{10}$copies/µl when the sample was diluted before analysis. The NanoDrop result in Cardiff corresponds to $2.3x10^{10}$copies/µl whereas the Lumora NanoDrop is lower at $2.2x10^{10}$copies/µl. Disregarding the measurement of the undiluted pART7 Agilent Bioanalyzer result, the other results give a mean value of $2.2x10^{10}$ copies/µl with a standard deviation of $0.1x10^{10}$ copies/µl which is a 5% coefficient of variation.
The low variation between these two different quantification techniques for the linearised pART7 plasmid permit confidence in the low copy number assays that follow. The linearised pART7 sample was subsequently diluted to $4x10^{8}$ copies, lyophilised and stored at -20°C by Dr Guy Kiddle in Lumora.

4.3 Primers

The primers used for the analysis of the 35S promoter sequence using genomic template in chapter 3 were those detailed in Kiddle (2012) and originally designed by Lee (2009), and shown in chapter 2. These LAMP, displacement and loop primers were designed to the CaMV 35S promoter reference sequence stored on the National Center Biotechnology Information website (http://www.ncbi.nlm.nih.gov/genbank/) database GenBank (Benson et al. 2014) accession numbers V00141 and X79465. The 35Sp primers used in chapter 3 were the ‘pure and simple’ primers supplied by Sigma-Aldrich® with reverse-phase cartridge purification.

4.3.1 Sequencing Results of the 35S promoter in pART7

The sequence of the 35S promoter in the pART7 plasmid was first checked to confirm a match to the original 35Sp LAMP primers (Figure 4.6).

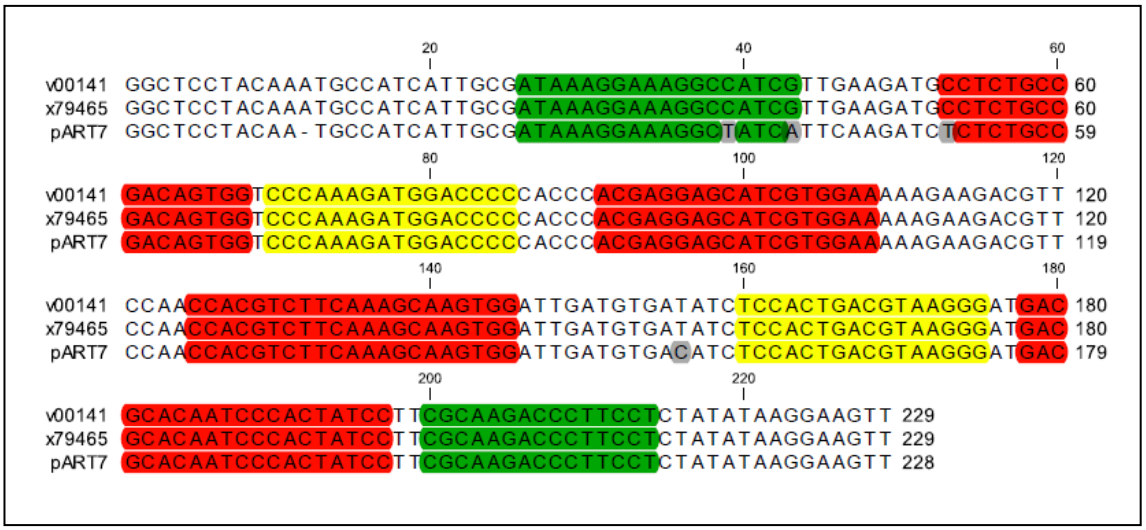


Figure 4.6: Sequencing result for the plasmid pART7 compared to database 35Sp reference sequences (sequencing data courtesy of Guy Kiddle). The sequences in green denote the displacement primers, red represents the lamp primers and the yellow sequences are loop primers.

The sequencing of pART7 highlighted a number of mismatches to the CaMV 35S promoter reference sequence. Firstly the displacement primer B3 has two mismatches to the pART7 complementary sequence within the last 5 nucleotides at the 3' end of the primer. Secondly the BIP LAMP primer has a mismatch at the 5' end of the primer. Mismatches at the 3' end of a primer has been shown to have a far greater deleterious effect on amplification than at the 5' end (Stadhouders et al. 2010) as such mismatches can interfere with the nearby polymerase active site. It was therefore important for the displacement primer B3 in particular to be redesigned for the pART7 template.

4.3.2 Redesigned primers

The LAMP displacement primer B3 for the CaMV 35S promoter sequence was modified to complement the pART7 sequence. Both displacement primers were also modified to balance length, melt temperature and GC content with each other. The melt temperature for the displacement primers was increased to 56°C due to the greater stability of the primer-template DNA duplex between the melting temperature range of 52°C to 58°C. The LAMP primer FIP was also redesigned to balance the melt temperatures of the F1 and F2 sequences separated by a linker consisting of four thymidine nucleosides. The BIP mismatch at the 5' end of the B2 sequence was not deemed important due to the proximity of the thymidine linker which is in any case non-complementary. Therefore the function of the BIP LAMP primer should be unaffected by this mismatch.

The details of the original and redesigned primers are shown in chapter 2. The redesigned primers were compared to the original versions using linearised pART7 as the template (Figure 4.7).

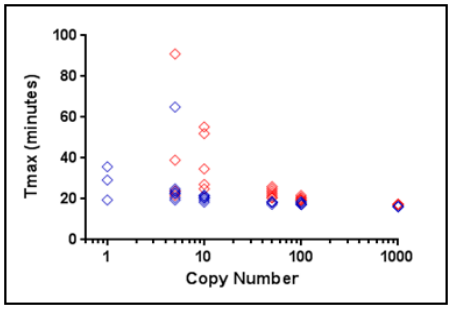


Figure 4.7: Comparison between the original 35Sp LAMP primer set (in red) and the new primer set (in blue) containing redesigned displacement primers and the FIP LAMP primer

At 1000 copies per partition of the pART7 target the reproducibility of the T_{max} values for both primer sets is excellent, but the original primers produce a slower T_{max} value. As the copy number reduces to 100 and 50 copies per partition, the variance in T_{max} for the new primer set assay remains low, but the standard deviation for the 50 copies with the original set has increased to 2.5 minutes. At 10 copies per partition the standard deviation has climbed to 14 minutes with only 5 of the 8 partitions positive for the original primer set, this variance rises to 29.5 minutes at 5 copies per partition with the same number of positive results. At 1 copy per partition there are no positive results for the original primer set. At 10 copies per partition for the new primer set the standard deviation is 1 minute for the 8 positive repeats; this increases to 15 minutes from the 8 positive repeats at 5 copies per partition due mainly to one outlying T_{max} value of 65 minutes. There are 3 of the 8 repeats positive at 1 copy per partition which equates to an amplification frequency of 38%.
The mismatches in the B3 displacement primer and the melt temperatures of the original 35Sp primer set therefore impact on sensitivity, reproducibility and the success rate of the assays at low copy number. This demonstrates the importance of primer match for the reproducibility of T_{max} values.

4.3.3 Primer quality

From the previous section the impact of the primers on assay reproducibility, sensitivity and amplification frequency is evident. This section aims to investigate the importance of primer quality in optimising the 35Sp LAMP-BART assay of pART7 template (Figure 4.8). The first of these experiments compares the levels of purification applied to newly

synthesised oligonucleotides by the manufacturer. All primers manufactured by Sigma-Aldrich are first de-salted to remove residual by-products that form in the manufacturing process. Reverse-phase cartridge purification separates truncated sequences from complete primers due to the hydrophobicity of full length products which contain a dimethoxytitryl group (5'-DMT). The suitability of this purification approach decreases with larger primers such as the LAMP primers FIP and BIP. For these primers high-performance liquid chromatography (HPLC) purification is recommended. Therefore in a LAMP-BART assay improved results should be seen with HPLC grade primers due to the reduced purity of LAMP primers with the reverse-phase cartridge purification. The increased concentration of truncated oligonucleotides may increase non-specific interactions reducing amplification efficiency or inhibiting amplification. The concentration of full length primers will be reduced due to the reduced purity with a possible impact on the critical initiation of LAMP amplification by LAMP and displacement primers.

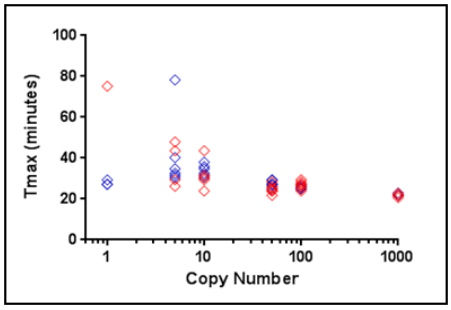


Figure 4.8: Comparison between reverse-phase cartridge purified 35Sp LAMP primers in red and HPLC purified primers in blue

At 1000 copies per partition of the pART7 template both primer purities produced similar T_{max} results with low variation between the repeats. The variation for the reverse-phase cartridge purified increase from 100 copies to 10 copies to a greater extent than the HPLC primers and the amplification frequency is reduced. Therefore HPLC grade primers provide more reliable amplification and are more appropriate for low copy number quantification of the linearised pART7 plasmid.

To establish whether significant differences were found between the primer suppliers the HPLC purified primers of the original documented version (therefore with mismatches to the pART7 template) from Sigma-Aldrich and Eurofins MWG Operon were compared (Figure 4.9).

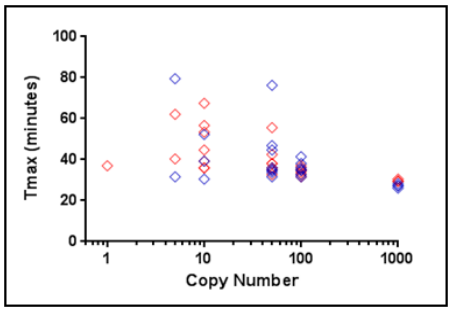


Figure 4.9: HPLC purified primers from Sigma-Aldrich in blue and Eurofins MWG Operon in red. Assay of copy number per partition concentrations of pART7 template between 1000 and 1 copy

The HPLC primers from Sigma and Eurofins both provided low variance for the 1000 and 100 copies per partition assays but although both were highly variable with reduced success rates for 10 copies per partition and below, no consistent difference was seen between these primer sets from these two suppliers.

The improvements made in the previous chapter to the reagent preparations and the storage and optimisation of carrier DNA concentration were then combined with the redesigned primers of higher quality to provide an optimised LAMP-BART 35Sp assay for the linearised plasmid pART7 (Figure 4.10).

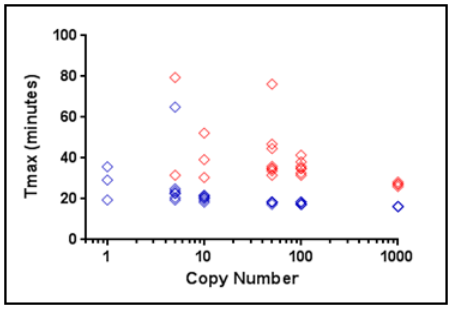


Figure 4.10: HPLC purified Sigma-Aldrich 35Sp primer pART7 LAMP-BART assays. Suboptimal conditions in red and optimised assay with salmon sperm DNA carrier in blue

When this optimised assay results are plotted against the sub-optimal assay with Sigma HPLC primers from the previous experiment, it is apparent how much improved are the low variation and high success rates seen down to low copy number.

4.4 Detection of single copies of target DNA

In the experiments described so far with linearised pART7 plasmid and modified 35Sp primers, only eight replicates of 1 copy per partition were assayed, resulting in two or three positive results observed. Therefore the number of wells for 1 copy per partition

was increased to 76 (a convenient number on a microtitre plate with controls) to assess the success rate and distribution of T_{max} values at this level and to investigate the suitability of average T_{max} as a quantitation method for assays below 100 copies per partition (Figure 4.11).

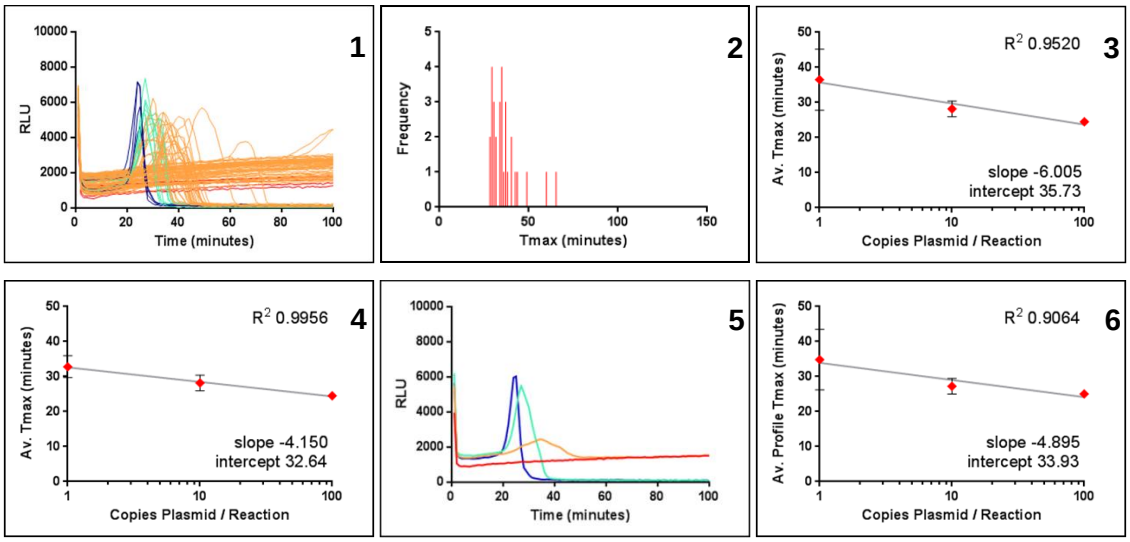


Figure 4.11: (L to R) Optimised 35Sp LAMP-BART assay of linearised pART7 (1) BART curves for 100 copies in blue, 10 copies in green and 1 copy per partition in orange (2) frequency distribution of T_{max} results for 1 copy per partition (3) average T_{max} for the 100, 10 and 1 copy per partition (4) average T_{max} for the 100, 10 and 1 copy per partition with the fastest 75% of 1 copy per partition T_{max} values only (5) average BART curve profile for the three assays (6) T_{max} values of the average BART curve profiles

All four of the wells containing 100 copies and the eight wells containing 10 copies were positive with low standard deviations of 0.63 and 2.21 minutes respectively. From the 76 partitions for single copy detection 30 were positive giving an amplification frequency of 39%. The standard deviation for these assays rose to 8.66 minutes.

The frequency distribution of T_{max} values for the 1 copy per partition showed a cluster of values around the average T_{max} of 36.5 minutes. Over 75% of the positive results were in a ten minute window between the fastest T_{max} of 28.3 minutes and the single result at 38.1 minutes. Although the majority of T_{max} results are in this window, there is a spread of values up to 65 minutes. These values increase the time for average T_{max} and contribute to the high standard deviation. The usefulness of the variance at low copy number will be investigated later in this chapter.

The plot of the average T_{max} and standard deviation for the three assays fits a semi-log linear model with an R^2 of 0.95. However it appears that the line would fit better to this model if the average T_{max} for 1 copy per partition was lower by a couple of minutes. Indeed considering the average T_{max} derived from the fastest 75% of T_{max} results the time would reduce from 36.5 minutes to 32.8 minutes with a standard deviation of 3.1 minutes. The fit to the semi-log linear model is improved (R^2 = 0.9956). From these

observations of quantification down to single copy number it appears that using only the majority of T_{max} results give the best average T_{max} measurement and therefore the variation at low copy number is an undesirable feature of LAMP-BART.
By taking the average of each time integral for the three assay sets to get an average profile for each copy number, we get an average BART curve for each. The time to peak for these curves is therefore associated with the time at which the total light output from all the partitions is at its highest for each copy number and this approach should therefore favour the majority of results with reduced effect from outlying values to the overall T_{max}. The shape of the BART curve for this average profile reflects the increasing variance observed with the three copy number assays; the 100 copies per partition profile is steep sided with a narrow base and high peak height, the 1 copy per partition profile is flatter with a wide base and lower peak height.
The amplification frequency of 39% for the 1 copy per partition derived from 76 partitions is similar to the 37.5% amplification frequency of 3 positive results from 8 partitions which were seen in previous assays. The variability of T_{max} results at single copy number indicates a requirement for a large number of repeats for quantification. The number of repeats required will depend on the approach to quantification and this will be investigated later on in this chapter.

4.4.1 Assessment of template variation

So that a large number of repeats of the same copy number per partition can be compared it is important to assess the variation that may be associated with the individual lyophilised aliquots of linearised pART7 and their handling.
The first experiment investigated the differences in T_{max} and variation of assays of eight hydrated aliquots ranging from freshly hydrated and homogenised to an aliquot that had been stored at 4°C for almost 4 months (116 days). The aliquots were assayed undiluted therefore at a concentration of $5x10^6$ copies per partition (Figure 4.12). The aim was to ensure low variation between aliquots at high copy number before addressing the potentially more variable results at low copy number.

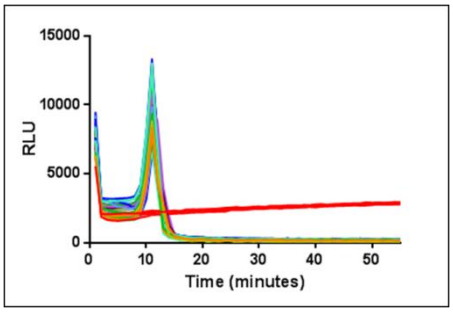


Figure 4.12: 35Sp LAMP-BART assay of eight aliquots of linearised pART7 at $5x10^6$ copies per partition

All eight aliquots had average T_{max} values of 10.86 minutes with no variation between the four repeats from each. Therefore at high copy number there is no difference in the LAMP-BART assay of a freshly prepared pART7 aliquot to an aliquot that has been stored at 4°C for almost 4 months.

Three recently hydrated pART7 aliquots stored at 4°C were assayed at low copy number to investigate the consistency between aliquots at 100, 10 and 1 copy per partition (Figure 4.13).

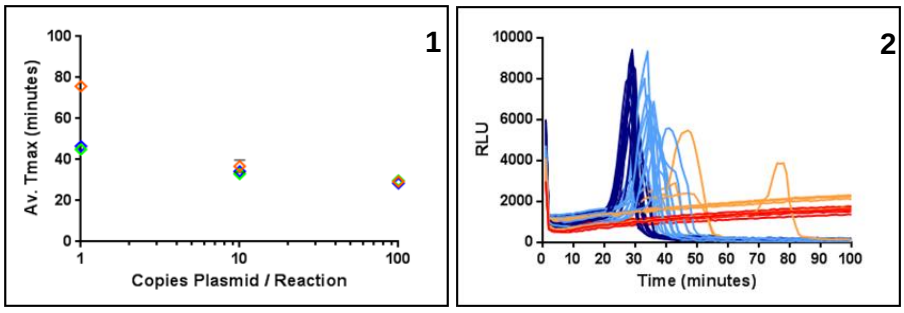


Figure 4.13: (L to R) Average T_{max} for 100, 10 and 1 copy per partition of pART7 (1) the three template aliquots A, B and C marked in orange, green and blue (2) assay results combined to show all results from the three templates.

Each of the three templates had coefficients of variation below 10% for both the 100 and 10 copies per partition and for both of these copy numbers and for all three templates there was a 100% success rate. All three templates successfully had at least one positive result at 1 copy per partition. At 1 copy per partition, assuming a 40% amplification frequency, either 1 or 2 positive partitions would be expected from the 4 repeats, and this was observed for each template.

The impressive sensitivity and reproducibility at 10 and 100 copies are indicative of a low variation between these pART7 aliquots, and demonstrates that storage for extended periods did not affect DNA quality in amplification.

4.4.2 Inter-assay variation

Although the time to peak is proportional to the concentration of template, there are a number of variables that can influence the T_{max} which are discussed in chapter 3. Control of these variables is essential to limit inter-assay variation and the aim of this experiment is to assess the variation in T_{max} between two replicated 35Sp LAMP-BART assays of linearised pART7 (Figure 4.14).

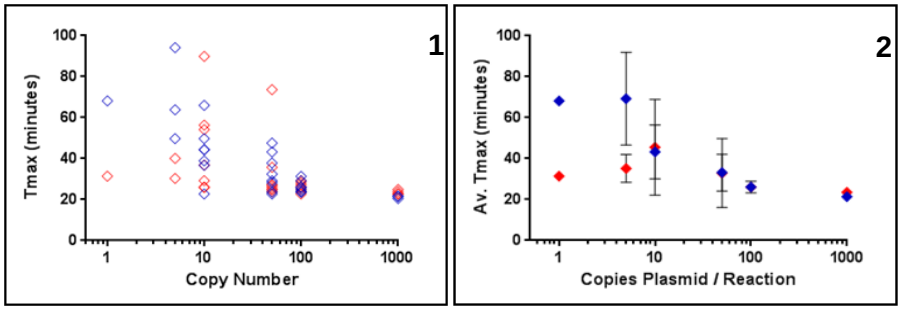


Table 4.14: (1) T_{max} results from two identical assays without carrier DNA of the linearised pART7 template at 1000, 100, 50, 10, 5 and 1 copy per partition, each assay had eight repeats of 1, 5, 10, 50 and 100 copies per partition and four repeats of 1000 copies per partition (2) average T_{max} of the data

No consistent difference was observed between the two repeat assays and the results for average T_{max} from 1000 copies to 10 copies were closely matched. Increasing variability in T_{max} results is evident for decreasing copy number until the success rate starts to decline at less than 10 copies per partition whereupon there are insufficient positive results for calculations of average T_{max} and standard deviation. The results demonstrate the consistency between assays in the range 10 to greater than 1000 copies per partition.

4.4.3 Detailed analysis of amplification at 60, 30, 6 and 3 copies

A more detailed analysis at different copy levels was carried out using four sets of 96 well 35Sp LAMP-BART assays of the linearised plasmid pART7. The aim was to investigate the reproducibility of T_{max} results with decreasing copy number and to generate data for other potential analysis methods that could be explored. It has been noted in the earlier experiments that at very low copy numbers, slower peaks can be observed. Due to the complexity of the LAMP reaction it may be possible that amplification may take alternative pathways from the formation of the dumbbell structure with the possibility that one pathway will be less efficient. An outcome of a less efficient pathway could be the clustering of slow T_{max} results away from the more

favourable amplification route. Therefore the frequency distribution data from these template dilutions were also scrutinised for evidence of this. These assays were set up in a highly defined manner to ensure reproducibility.

A fresh aliquot of the plasmid was prepared each time from frozen stock and diluted to the appropriate copy number concentration for the assay. Carrier DNA at a concentration of 100ng/µl was incorporated into the LAMP-BART mix before the addition of template. The modified 35Sp primer set was used at the HPLC level of purity for all assays and the reagent batches were kept constant. The LUCY was set to assay the samples at 60°C for a total of 92 time integrals which equates to a 100 minute assay time.

4.4.3.1 60 copies per partition

In the first of these experiments 60 copies per partition in a total of 88 partitions was assayed with four NTCs and four positive controls (Figure 4.15).

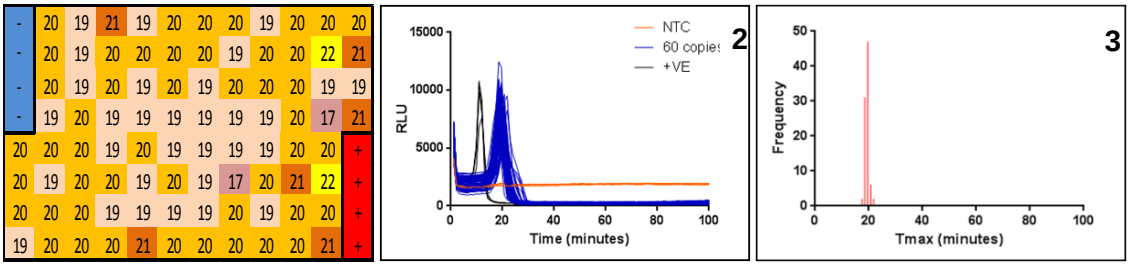


Figure 4.15: (L to R) 60 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of $5x10^6$ copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 17.44 minutes and a highest peak of 47 positive results at 19.63 minutes.

It is remarkable how closely grouped the T_{max} values are at this copy number with no outlying values beyond that initial group in a 100 minute assay showing a range of 17.44 to 19.63 minutes. 47 of the positive results have the same T_{max} value of 19.63 minutes and another 31 of the results at 18.53 minutes. As a consequence the standard deviation is only 0.8 minutes and the average T_{max} is 19.32 minutes. Such low variation allows the average T_{max} method of quantitation to be applied that has been successfully applied to higher copy number samples. No evidence of slower T_{max} peaks is seen at this copy number level.

4.4.3.2 30 copies per partition

A fresh pART7 aliquot was diluted to give 30 copies per partition with sufficient volume to partition over 88 wells (Figure 4.16).

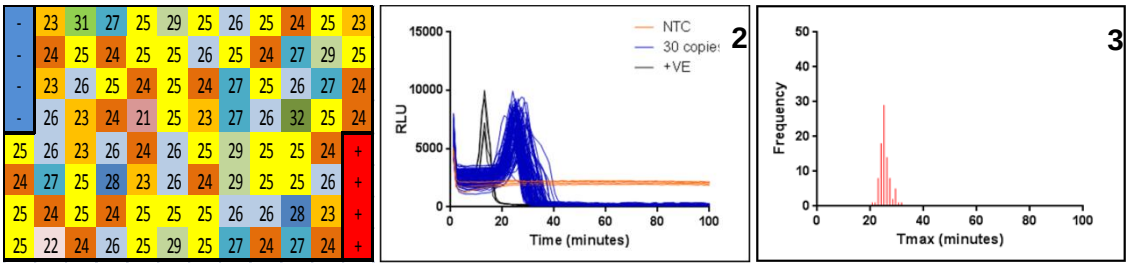


Figure 4.16: (L to R) 30 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of $5x10^6$ copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 20.73 minutes and a highest peak of 29 positive results at 25.11 minutes.

All the partitions were positive and no template controls (NTCs) remained free from contamination. As with the 60 copy per partition assay there were no outlying values after the initial group which is centred on 29 results at 25.11 minutes. The average T_{max} is 25.44 minutes with a standard deviation of 1.9 minutes. The increased variance is apparent from the morphology of the frequency distribution data both in terms of the decreased peak height and the increased range of T_{max} results to approximately 11 minutes. The previous 60 copies per partition assay had a spread of T_{max} results of just over 4 minutes. Again there is no evidence of a cluster of slower T_{max} results.

4.4.3.3 6 copies per partition

At 6 copies per partition (Figure 4.17) the importance of accurate dilution and thorough mixing are increased. To combat these issues, maximum recovery pipette tips and non-stick tubes were used to prepare the dilution. Inevitably some partitions will contain more or less copies than 6 copies per partition, and this will therefore be a factor in the variance.

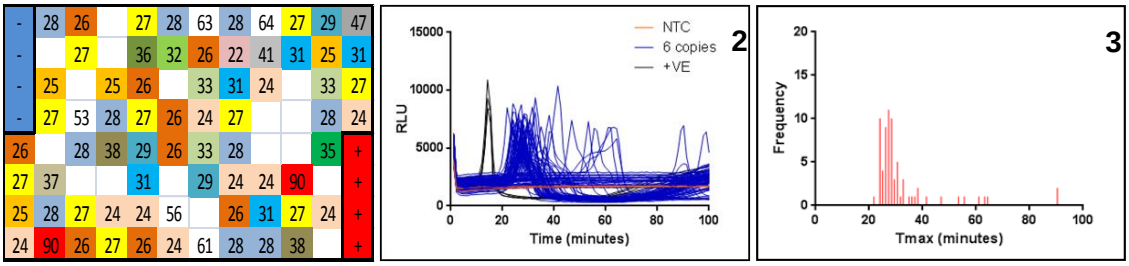


Figure 4.17: (L to R) 6 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of 5x10^6 copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 21.78 minutes and a highest peak of 11 positive results at 27.23 minutes.

At 6 copies per partition there is a reduction in the success rate to 81% with negative results from 17 of the partitions. There were two positive results very late in the assay at a T_{max} of 90.37 minutes; therefore it is possible that some of the negative partitions might have been positive if the total assay time were greater than 100 minutes. 80% of the positive results are in an 11 minute range from the fastest T_{max} of 21.78 minutes. The average T_{max} is 28 minutes with a standard deviation of 13.5 minutes due to the two positive results at 90.37 minutes. Although there are many T_{max} results away from the main cluster they do not themselves appear to be clustered, but rather the frequencies appear to represent a Poisson distribution.

4.4.3.4 3 copies per partition

A fresh aliquot was diluted to 3 copies per partition (Figure 4.18). From the previous experiment it was expected that the success rate will drop further and therefore the number of positive results to analyse would be reduced.

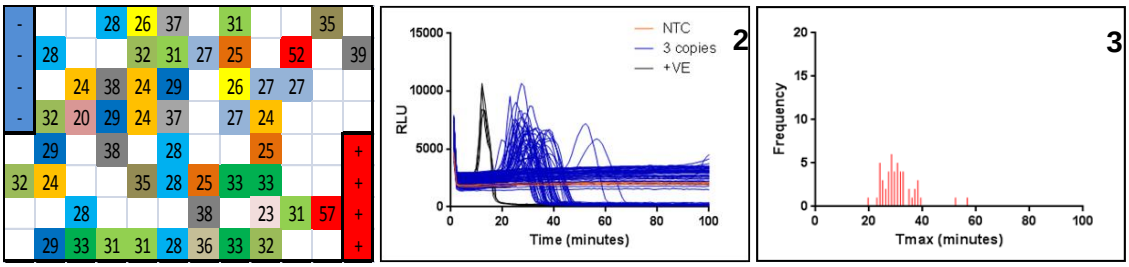


Figure 4.18: (L to R) 3 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of 5x10^6 copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 19.63 minutes and a highest peak of 6 positive results at 28.35 minutes.

Indeed there were thirty eight negative partitions giving only 57% amplification frequency. Possibly due to this lower number of positive partitions there are only two outlying values between forty minutes and the assay cut-off at a hundred minutes. In comparison to the previous assay more variation might have been anticipated, but forty-eight of the fifty positive results are in a twenty minute range from the fastest T_{max} time of 19.63 minutes. The average T_{max} for this assay is 30.80 minutes with a standard deviation of 6.7 minutes.

The data from the sixty, thirty, six and three copies per partition showed a reduction in success rate and an increase in average T_{max} together with increased variance for the main cluster of T_{max} results. At sixty and thirty copies per partition every test partition was positive and all the T_{max} results were closely grouped, but at low copy number the assays became more variable and are therefore investigated further by analysing six assay sets at ten, five, four, three, two and one copy per partition.

4.4.4 Detailed analysis of amplification at 10, 5, 4, 3, 2 and 1 copies

A more detailed analysis at different copy levels below 10 copies per partition was carried out using six sets of 96 well 35Sp LAMP-BART assays of the linearised plasmid pART7. The aim of this set of assays was to provide quality data at low copy number to investigate possible ultra-quantification strategies that can discriminate between individual copy numbers. Secondary to this was to observe the T_{max} frequency distribution patterns for the change from a normal distribution to a Poisson distribution – the calculation of standard deviation for a Poisson distribution is the square root of the mean whereas the calculation of standard deviation for a normal distribution is the square root of the average of the squared differences of the values from the mean. Therefore it is important to know at what copy number this occurs to make that switch or alternatively assume for standard deviation calculations that all the T_{max} frequency distribution data is normally distributed. Thirdly was the potential clustering of frequencies as evidence for alternative LAMP amplification pathways.
As with the assays in section 4.4.5 carrier DNA at a concentration of 100ng/µl was incorporated into the LAMP-BART mix before the addition of template, the modified 35Sp primer set was used at the HPLC level of purity for all assays and the reagent batches were kept constant. The samples were assayed at 60°C for a total of 92 time integrals which equates to 100 minute assay time.

4.4.4.1 10 copies per partition

The first experiment was with ten copies per partition of pART7. A fresh aliquot of the plasmid was diluted for this assay (Figure 4.19).

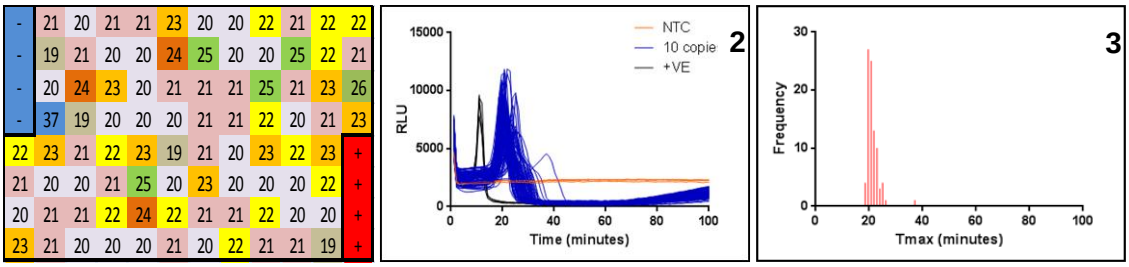


Figure 4.19: (L to R) 10 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of $5x10^6$ copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 18.54 minutes and a highest peak of 27 positive results at 19.63 minutes.

The frequency distribution at ten copies per partition has four positive results at the fastest T_{max} of 18.54 minutes immediately followed by twenty six positives at the next time point of 19.63 minutes and by twenty five positive results at 20.73 minutes. The majority of positive results are in a small time range of approximately 8 minutes. There is one isolated T_{max} value at 37.07 which increases the average T_{max} from 21.08 minutes to 21.26 and the standard deviation from 1.7 minutes to 2.4 minutes. All eighty eight test partitions were positive at this level and the NTCs were negative.

4.4.4.2 5 copies per partition

The next experiment was with five copies per partition of pART7 (Figure 4.20). A fresh aliquot of the plasmid was used for this assay.

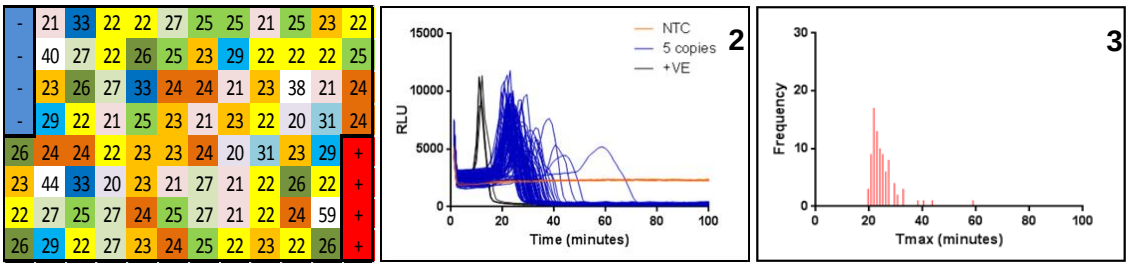


Figure 4.20: (L to R) 5 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of $5x10^6$ copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 19.63 minutes and a highest peak of 17 positive results at 21.82 minutes.

As with the ten copies per partition all the 88 test partitions were positive and the NTCs were uncontaminated. At five copies per partition the fastest T_{max} was a minute slower than the ten copies per partition at 19.63 minutes. The highest frequency of seventeen positive results refers to the T_{max} value of 21.82 minutes. The average T_{max} is 25.16 with a standard deviation of 5.63 minutes if a normal distribution is assumed. There are three T_{max} values above 40 minutes and the shape of the distribution appears flatter and more spread out than the previous assay.

4.4.4.3 4 copies per partition

The following assay uses the same aliquot of linearised pART7 as the five copies per partition assay and has one copy fewer at four copies per partition (Figure 4.21).

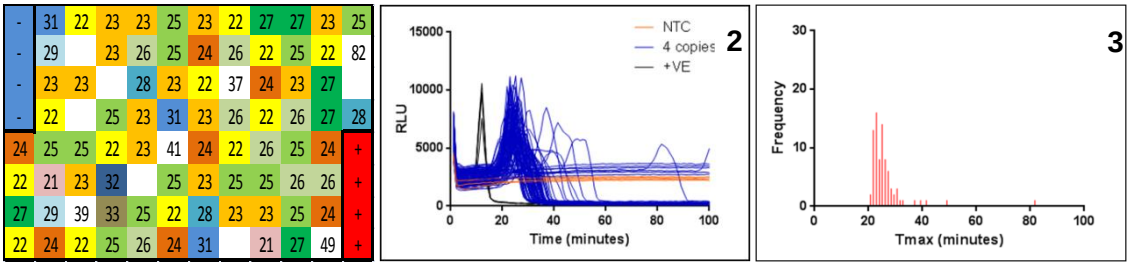


Figure 4.21: (L to R) 4 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of $5x10^6$ copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 20.72 minutes and a highest peak of 16 positive results at 22.91 minutes.

The fastest T_{max} time is at 20.72 minutes which is approximately a minute slower than the five copies per partition assay. The number of positive test partitions has now dropped to 82 from the 88 which is a percentage of 93%. The highest frequency for this assay is lower than the previous one at sixteen positive results at 22.91 minutes and the main cluster of positive results covers an interval of approximately 12 minutes. There are three values greater than 40 minutes and one of these is at 81.65 minutes. As a consequence the standard deviation is higher still at 7.72 minutes and the average T_{max} is 26.31 minutes. The NTCs continue to be contamination free.

4.4.4.4 3 copies per partition

The following assay (Figure 4.22) is a repeat of the three copies per partition assay and uses the same aliquot of linearised pART7 as the previous two assays (stored at 4°C between uses).

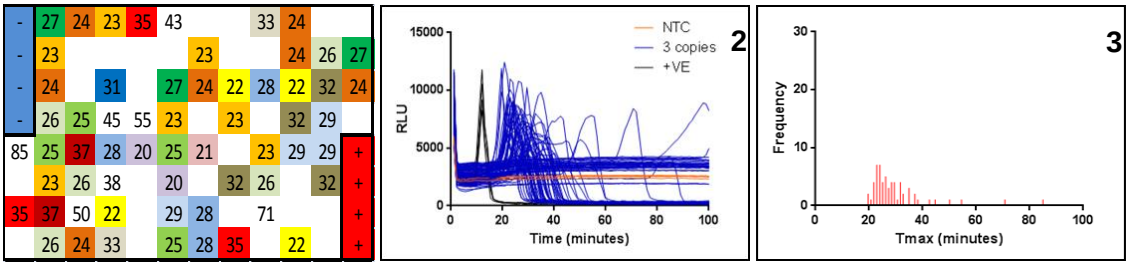


Figure 4.22: (L to R) 3 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of $5x10^6$ copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 19.63 minutes and two adjacent highest peaks of 7 positive results at 22.91 and 24.00 minutes.

The fastest T_{max} is faster than the previous assay and the same as the five copies per partition assay. At these low copy numbers it may be that those particular partitions contained five copies and not three copies just through stochastic variation. The highest frequency of 7 refers to two T_{max} values of 22.91 and 24.00 minutes, the highest frequency for the previous three copies per partition assay was 6 for a higher T_{max} value. There are six positive results above 40 minutes one of which is at 85 minutes and as result the standard deviation is the highest of the assays so far at 11.43 minutes with an average T_{max} of 30.13 minutes. The number of positive partitions has reduced to a success rate of 68% which is higher than the 57% for previous three copies per partition assay.

4.4.4.5 2 copies per partition

The next assay is of two copies per partition (Figure 4.23). The same aliquot of pART7 is used as the previous three assays.

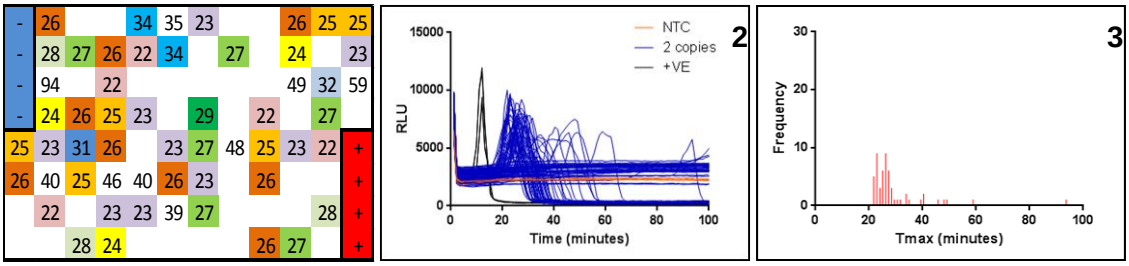


Figure 4.23: (L to R) 2 copies of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of 5x10^6 copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 21.82 minutes and two highest peaks of 9 positive results at 22.91 and 26.19 minutes.

The number of positive results has dropped further to fifty five of the eighty eight total partitions and a success rate of 63%. There are seven positive results above 40 minutes one of which is at 93.69 minutes; the standard deviation is slightly higher than the previous assay at 11.71 minutes. The average T_{max} is 29.67. The highest frequency is for two peaks at 22.91 and 26.19 minutes with a frequency of 9.

4.4.4.6 1 copy per partition

The last in this group of assays is one copy per partition (Figure 4.24). Again the template was the same pART7 aliquot used for the previous four assays. This assay required a new batch of Bst polymerase (NEB).

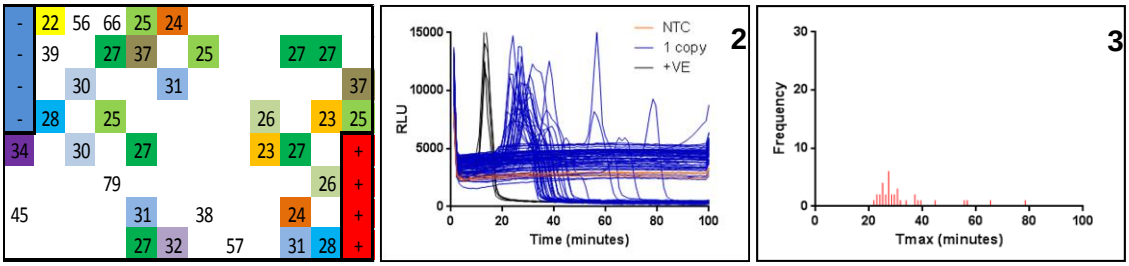


Figure 4.24: (L to R) 1 copy of linearised plasmid pART7 per partition with NTCs (light blue) and positive control of 5x10^6 copies per partition (red), times for T_{max} results at each partition (arbitrary colours used to show groups of T_{max} values) (1) positions of positive results and associated T_{max} values (2) the spread of T_{max} results and the clear NTCs (3) the frequency distribution of T_{max} values indicating a fastest T_{max} value of 21.86 minutes and a highest peak of 6 positive results at 27.33 minutes.

The first notable observation with these assays is the assay peak heights, which are generally higher than the previous assays and is presumably the result of the new batch of Bst polymerase. The success rate has continued to drop with only thirty five positive results which equates to a success rate of 40% which is higher than that previously calculated from seventy six partitions. The highest frequency of coinciding

peaks is six for the T_{max} value of 27.33 minutes and there are five positive results above 40 minutes with the highest at 78.65 minutes. The standard deviation is the highest of all the assays at 12.8 minutes. The average T_{max} is 33.14 minutes.

From the frequency data it appears that the distribution resembles increasingly a Poisson distribution and is not normally distributed between ten and six copies per partition as the variance increases.
As with the previous group of assays there was no evidence of secondary clusters of peaks that might suggest that LAMP amplification can follow distinct less efficient alternative pathways. The spread of results at low copy number is therefore more probably linked to the stochastic initiation of the LAMP amplification by strand invasion by the LAMP primers as a limiting step. Slow initiation of a number of templates in a partition would be expected to become more of an issue as the copy number of the template reduces. An outcome of slow initiation at low copy number will be higher T_{max} values. A few slow initiations at high copy number will have minimal impact on the T_{max} value. It is therefore likely that the increasing variance as the number of template copies reduces to one is due to this amplification initiation event. Notable from these experiments is that different and distinct results were obtained as copy numbers reduced from 5 copies to 1 copy by single steps.

4.5 Data analysis of LAMP-BART time to peak (T_{max})

In this section, different approaches to data analysis are explored for quantitative determination of DNA copy number. Quantification using BART has focused on time-to-peak (Gandelman et al. 2010). This is because, as discussed before, the production of inorganic pyrophosphate, a by-product of DNA synthesis, is proportional to the concentration of the template and therefore to the time to peak observed in a BART reaction. The measurement of time to peak (T_{max}) is therefore the point at which the concentration of inorganic pyrophosphate inhibits the enzyme luciferase resulting in a rapid decline in bioluminescence (Figure 4.25).

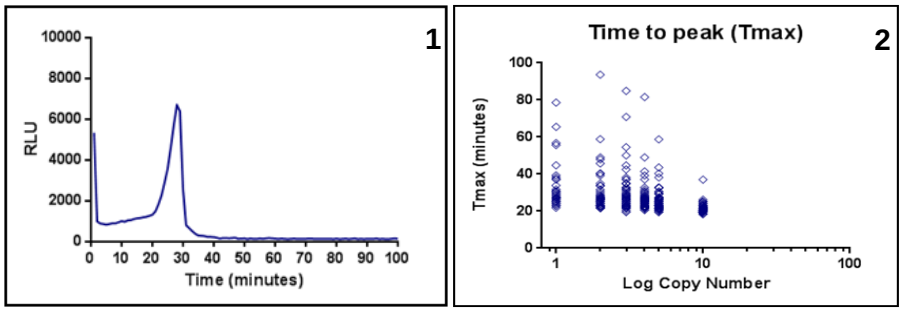


Figure 4.25: (1) BART peak (2) T_{max} values for 10, 5, 4, 3, 2 and 1 copy per partition of the linearised plasmid pART7

Plotting the data generated in section 4.4.4 as individual T_{max} results shows the variation in values at these copy numbers. Also the fastest T_{max} for each assay gradually increases with reducing copy number. For this reason, further approaches to determining copy number were considered using the data of the previous section (4.4).

4.5.1 Data Analysis - Fastest T_{max}

From the data for the fastest T_{max} from the six assays ranging from 10 to 1 copy per partition there is a relationship between the fastest T_{max} values and copy number (Figure 4.26). However the inherent problem with this measurement is the probability that the fastest T_{max} value will be present in a dataset. If the definition of fastest T_{max} is the optimum amplification of each and every template in a partition to give the fastest time to peak, then this may not occur from the replicates. Stochastic variations in copy number to a higher number of target copies present in a partition may give a faster fastest T_{max} value. This will be less significant at higher copy numbers but is a problem with this method for ultra-quantification.

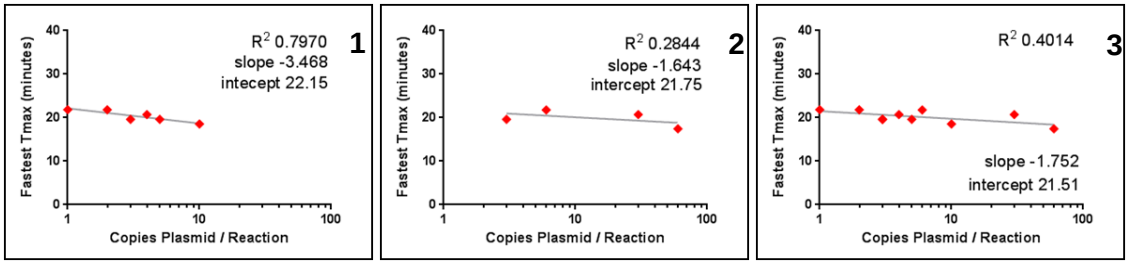


Figure 4.26: (L to R) Fastest T_{max} results for (1) 10, 5, 4, 3, 2 and 1 copy per partition (2) 60, 30, 6 and 3 copies per partition linearised plasmid pART7 (3) combined results from 60 copies to 1 copy per partition

The downward trend of the fastest T_{max} with increasing copy number for the ten to one copies per partition showed that the gradient is gradual giving at best discrimination between orders of magnitude and not between single copies. For the sixty to three

copies per partition the values are more erratic due to single isolated fastest T_{max} values observed for both the six and the three copies per partition data.

One way to disregard isolated fastest T_{max} values is to redefine the fastest T_{max} as the fastest value from a proportion of the results clustering around the mean. Therefore by assuming normal distribution of the data around the mean, the fastest T_{max} can be calculated to exclude the first 5% of positive results (Figure 4.27).

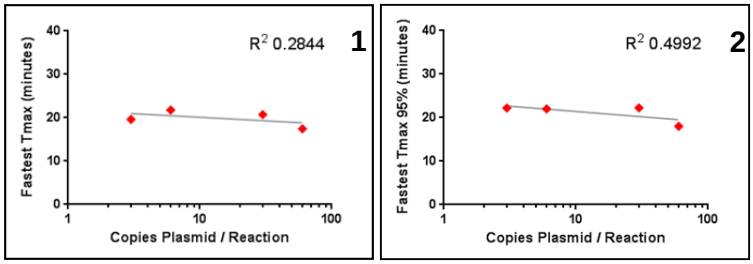


Figure 4.27: (L to R) Fastest T_{max} from (1) all of the data from 60, 30, 6 and 3 copies per partition (2) the data with the first 5% of positive results removed

The fastest T_{max} with the first 5% of positive results removed has an improved R^2 value of 0.4992, but this best fit to the semi-log model highlights the slow value from the 30 copies per partition data. This could be due to the whole assay being slower due to an uncontrolled variable or to the possibility that the faster T_{max} values weren't in that dataset.

Overall this approach to low copy number quantification showed that there is a correlation between fastest T_{max} and reducing copy number but the gradient is too shallow to discriminate between individual copy numbers and the correlation to a sensitivity model is relatively imprecise.

4.5.2 Data Analysis - Average T_{max}

Quantification using the average of the T_{max} values at different copy numbers is described in Gandelman (2010) for the quantification of *Chlamydia* and for GM targets in maize in Kiddle (2012).

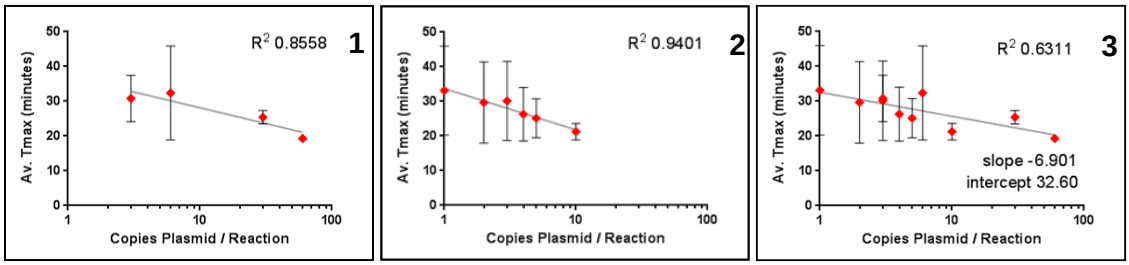


Figure 4.28: (L to R) Average T_{max} results for (1) 60, 30, 6 and 3 copies per partition and (2) 10, 5, 4, 3, 2 and 1 copy per partition linearised plasmid pART7 (3) combined results

Average T_{max} for the sixty to three copies per partition data displays a general increase with decreasing copy number (Figure 4.28). However the variability for the six and three copies per partition assays is high. The average T_{max} for three copies per partition is lower than six copies per partition and the variation is lower. As the increased variance is from T_{max} values towards the end of the assay time the average T_{max} is higher as a result.

Average T_{max} for the ten to one copy per partition data again shows a general increase in average T_{max} with decreasing copy number. The variance also increases with decreasing copy number and with it the confidence in the average T_{max} result. The points fit the semi-log line with an R^2 of 0.9401, but the 3 copies per partition result is higher than the two copies per partition. Although average T_{max} is a good quantification method for copy numbers where the variation is low it appears to breakdown at low copy number where there is also poor discrimination between copy numbers.

The two datasets combined indicate a certain amount of inter-assay variation accounting for the reduced R^2 value and this is most evident with the six, ten and thirty copies per partition average T_{max}. However, the gradient does indicate an improved discrimination between copies when compared to the fastest T_{max} data.

4.5.3 Data Analysis - Average T_{max} <40 minutes

Shortening the total assay time to 40 minutes removes all T_{max} values between 40 and 100 minutes. This will focus the average T_{max} and variance on the majority of T_{max} values (Figure 4.29). As with the 50 minute cut-off, this will not change the average T_{max} for sixty, thirty and ten copies per partition where the variance is low and there are no T_{max} values above 40 minutes. The aim is therefore to reduce the variance for those assays below ten copies per partition for a more accurate average T_{max}. The thirty copies per partition assay continues to appear isolated from the other assays in terms of average T_{max} as the measurement is slower than that from ten copies per partition. This may be due to inter-assay variation, although tightly controlled, and shows a

problem with using time to peak. The average T_{max} value for the positive control for the thirty copies per partition was over two minutes slower than the corresponding positive control used for the ten copies per partition. The majority of positive controls had an average T_{max} of eleven to twelve minutes. Those that were slower were the six copies at 13.5 minutes, thirty copies at 13.1 minutes and one copy per partition at 13.1 minutes. The thirty and six copies per partition average T_{max} values are too high. Calibrating samples are therefore required to compare average T_{max} results between assays.

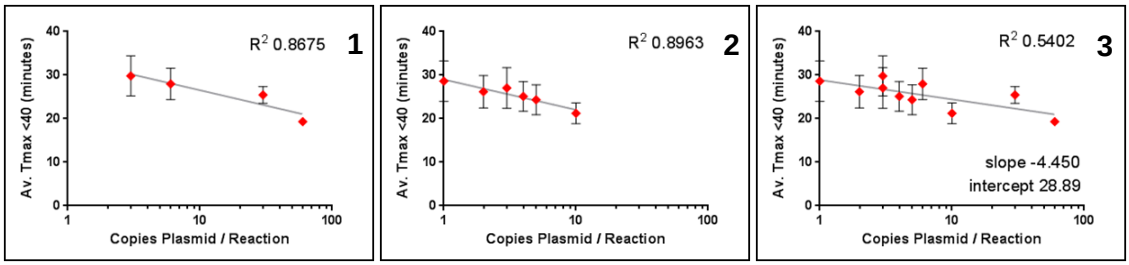


Figure 4.29: (L to R) Average T_{max} results for T_{max} results less than 40 minutes for (1) 60, 30, 6 and 3 copies per partition and (2) 10, 5, 4, 3, 2 and 1 copy per partition linearised plasmid pART7 (3) combined results

For those assays below ten copies per partition the reduction in assay time has reduced the variance, but standard deviation for all of these assays remains over three minutes. The R^2 values for all the graphs from the 40 minutes assay time cut-off is reduced still further and the values for 3 copies per partition are slower than the two copies per partition. To reduce the variance further would be to investigate the mode or the median of the data rather than the mean.

4.6 Analysis of LAMP-BART peak morphology

As previously discussed, the shape of the BART peak is formed by the exponential increase in inorganic pyrophosphate production and light output followed by the inhibition of luciferase by either an excess of inorganic pyrophosphate or by the consumption of the limiting substrate APS. For a high concentration of DNA target this exponential increase in light output may be steep giving rise to a tall peak with narrow base, conversely a low concentration of target will have less simultaneous amplification and a less steep rise in light output. If this is indeed the case then the morphology of the peaks could indicate the concentration of template.

To some extent the shape of the BART peak is governed by the settings on the light detection equipment. The LUCY is routinely set to accumulate a light output value in one minute integrals. This can give the appearance of flat top peaks when one integral

has the same total light output value as the next integral – in this instance the peak height may be compromised.
In the previous chapter, it was observed that the concentration of APS could alter the shape of the BART curve from a sharp peak to a bell curve. Also with the ten to one copies per partition dataset the one copy assay required a new batch of Bst polymerase which resulted in a higher than expected peak height. Therefore the LAMP-BART reagents need to be controlled if peak morphology is to be a measurement of target copy number concentration.

4.6.1 Data analysis - Peak height

The average peak height result for 1 copy per partition has been removed due to the anomalous result from the new batch of Bst polymerase.

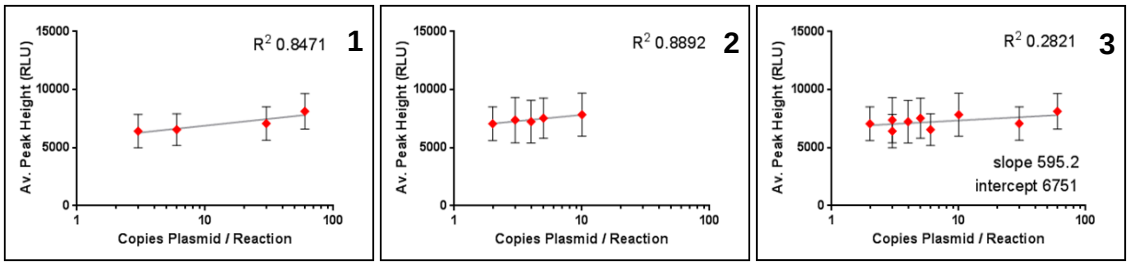


Figure 4.30: (L to R) Average peak height results for (1) 60, 30, 6 and 3 copies per partition and (2) 10, 5, 4, 3 and 2 copies per partition linearised plasmid pART7 (3) combined results

The average peak height (Figure 4.30) from both datasets decreases with decreasing copy number. This decrease is gradual and is associated with high variance even at 60 copies per partition. The combined datasets indicate the inter-assay variation and poor discrimination between orders of magnitude. The high variation in peak height, poor discrimination between copies and the difficulty in controlling inter-assay variation are factors against the use of average peak height for low copy number quantitation.
Peak height may also reduce as the T_{max} times increase due to the effect of the assay temperature on the thermolabile luciferase and not in relation to the target copy numbers.

4.6.2 Data analysis - Full width half maximum of BART peaks

Over the range of copy numbers assayed, the peak height reduces with decreasing copy number. This reduction may be associated with a broadening of the BART peak due to decreasing amplification with increasing assay time and/or reducing copy number. One way to measure the breadth of the peak is to calculate the full width half maximum (FWHM). The measurement could also be referred to as full duration half maximum (FDHM) when the interval is a period of time (Figure 4.31).

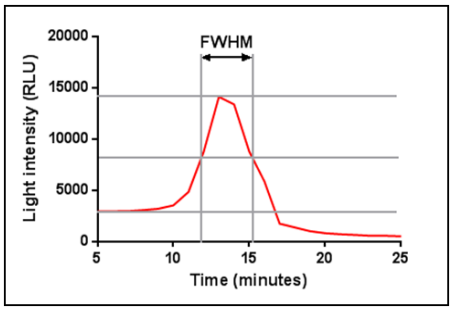
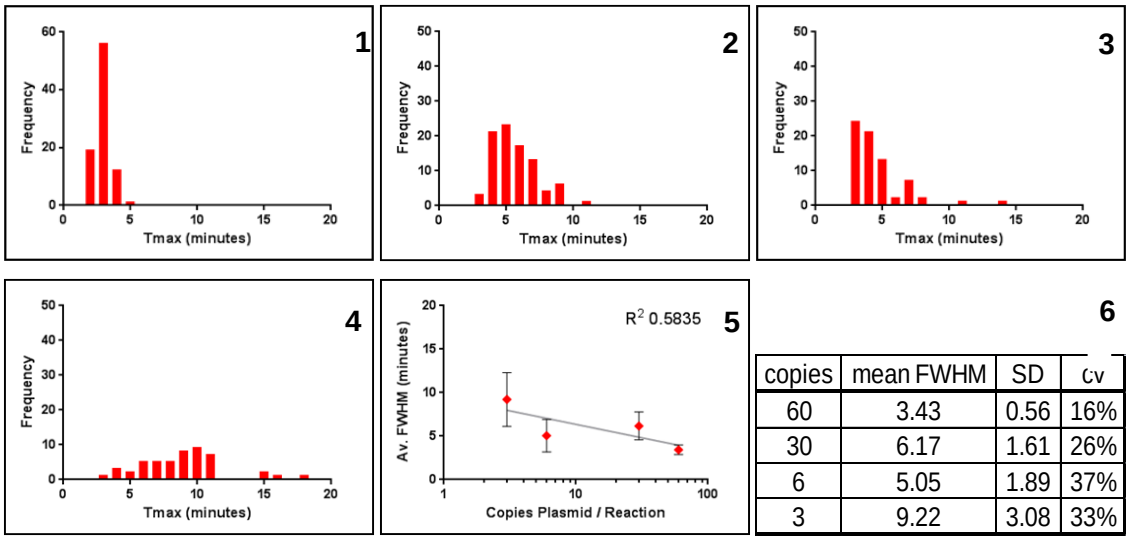


Figure 4.31: (1) Full width half maximum as applied to a BART curve

FWHM calculates the time between the two points on the peak where the light intensity is half of the maximum. For the BART curve the minimum light output in this investigation has been taken from the lowest RLU value between 1 and 9 minutes and not for the minimum after the peak. The half maximum light output was calculated by dividing the sum of the maximum and minimum values by two. The two times required were calculated by taking the light output: time coordinates for the light intensity values on either side of the half maximum light output values and defining the gradients and intercepts. The half maximum light output values, put into these equations give the two times from which the FWHM is calculated.

4.6.2.1 BART peak FWHM of 60, 30, 6 and 3 copies

Firstly the data generated from the T_{max} distribution experiments in section 4.4.3 were used to investigate FWHM for quantification (Figure 4.32).



6

copies	mean FWHM	SD	cv
60	3.43	0.56	16%
30	6.17	1.61	26%
6	5.05	1.89	37%
3	9.22	3.08	33%

Figure 4.32: Full width half maximum assigned to ranges i.e. 2.00 to 2.99, 3.00 to 3.99 etc. shown as frequency distributions (1) FWHM of 60 copies per partition (2) FWHM of 30 copies per partition (3) FWHM of 6 copies per partition (4) FWHM of 3 copies per partition (5) average FWHM (6) values of average FWHM and standard deviation

At sixty copies per partition the frequency distribution is centred on a peak between 3.00 to 3.99 FWHM. This peak shifts to 5.00 to 5.99 FWHM for thirty but then returns to 3.00 to 3.99 FWHM for six and rises to 10.00 to 10.99 for three copies per partition. Average FWHM has a poor correlation with copy number across the four individual assays for 60, 30, 6 and 3 copies per partition, but there is a big difference between 6 copies and 3 copies which is worth investigating with the data from section 4.4.4. The variation in FWHM values increases as the copy number decreases. From the distribution data the assay at 30 copies per partition appears to have larger than expected FWHM values when compared to the assays at 60 and 6 copies/rep and could be indicative of other factors that can influence peak morphology, such as APS concentration, creating inter-assay variation.

4.6.2.2 BART peak FWHM of 10, 5, 4, 3, 2 and 1 copy

Full width half maximum was calculated for all the data from the ten to one copy per partition data from section 4.4.4 (Figure 4.33).

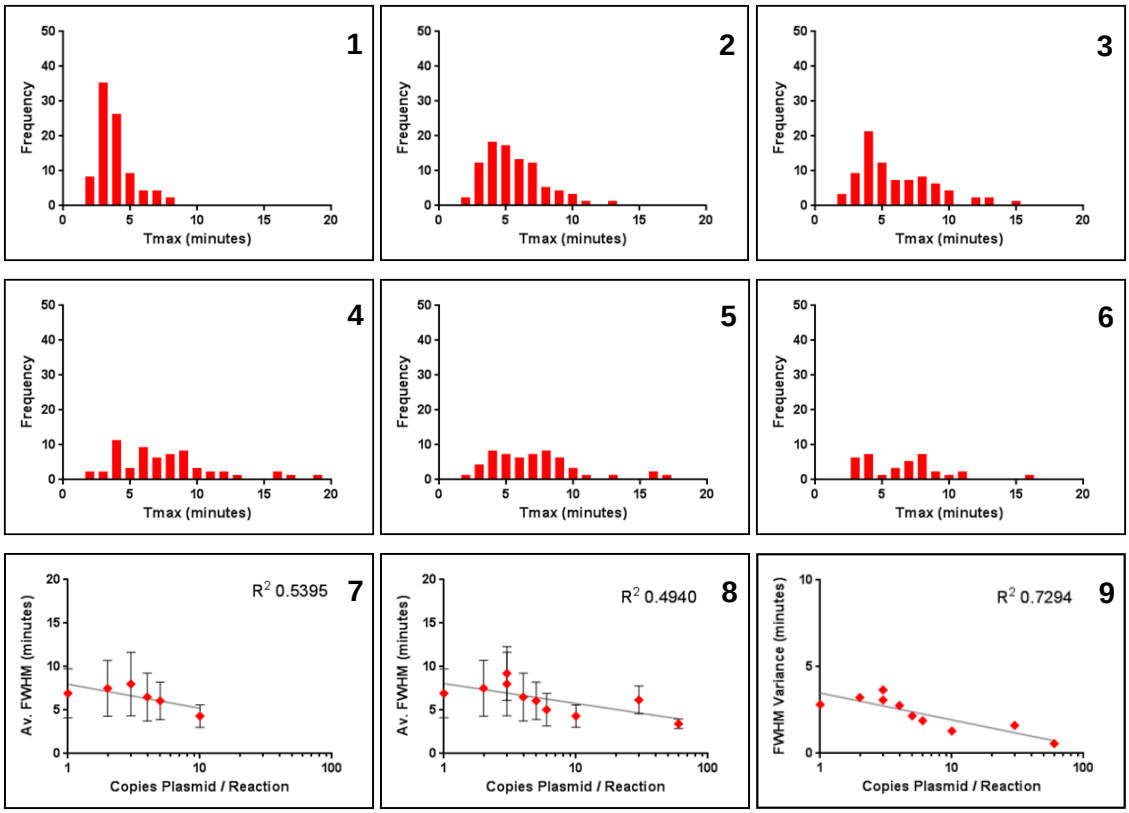


Figure 4.33: Full width half maximum assigned to ranges i.e. 2.00 to 2.99, 3.00 to 3.99 etc. shown as frequency distributions (1) FWHM of 10 copies per partition (2) FWHM of 5 copies per partition (3) FWHM of 4 copies per partition (4) FWHM of 3 copies per partition (5) FWHM of 2 copies per partition (6) FWHM of 1 copy per partition (7) average FWHM (8) average FWHM combined results (9) variance combined results

At ten copies per partition the frequency distribution is centred on a peak between 3.00 to 3.99 FWHM. This peak shifts to 4.00 to 4.99 FWHM for five, four and three copies and to 4.00 to 4.99 and 8.00 to 8.99 for two and one copies per partition. There is a gradual increase with average FWHM and the standard deviation for ten copies to 3 copies per partition but two copies has lower values and at one copy per partition the average FWHM and standard deviation are lower still. The range of FWHM values for 10 copies per partition is 2.00 to 8.99 this increases to 2.00 to 13.99 for five copies, 2.00 to 15.99 for four copies and 2.00 to 19.99 for three copies per partition, but then falls back with the two and one copy FWHM ranges.

The average FWHM results for both datasets shows some indication that average FWHM increases with decreasing copy number but the results from both the thirty and the three copy per partition assays from that first dataset are higher than predicted from the trend line. The low FWHM values for one and two copies per partition may have resulted from the low number of positive results for these assays. The variance observed for these two assays is also lower than for the three copies per partition assay.

Average FWHM is unsuitable for low copy number quantification due to lack of discrimination between copy numbers, the high variability and the susceptibility to anomalous results from inter-assay variation.

4.7 Data analysis of T_{max} Distribution

4.7.1 Frequency distribution of T_{max}

For copy numbers above 100, for the pART7 template with an optimised 35Sp assay protocol, the variation between T_{max} values of replicates partitioned is very low.

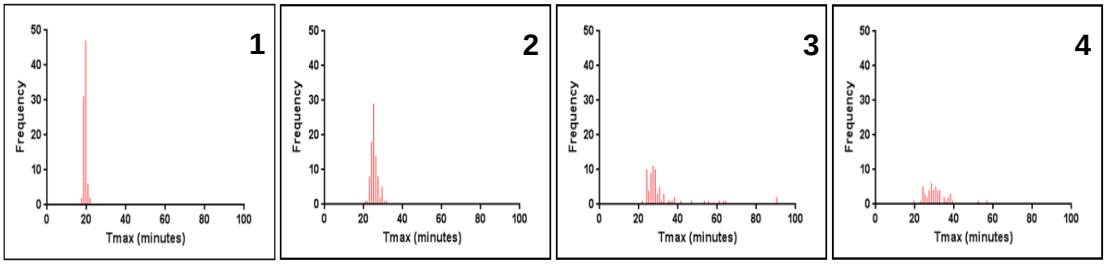


Figure 4.34: (L to R) Frequency distribution of T_{max} results from (1) 60 copies per partition (2) 30 copies per partition (3) 6 copies per partition (4) 3 copies per partition linearised pART7 template

For normal distribution the mode, mean and median should be equivalent and there should be symmetry on either side of the mean. At 60 copies per partition (Figure 4.34) the mode and median are 19.63 minutes and the mean is 19.32. The symmetry is skewed to the right with a value of 0.49. Comparisons of expected and observed frequencies for this data analysed with the chi-square test produces a p-value of 0.06 which is a low probability against the assumption of normal distribution.
At 30 copies per partition the mode and median are 25.11 minutes and the mean is 25.44 minutes and the skewness is 0.78. Again the probability against the assumption of normal distribution has a p-value of 0.06. Therefore the data for these two assays approximates to normal distribution.

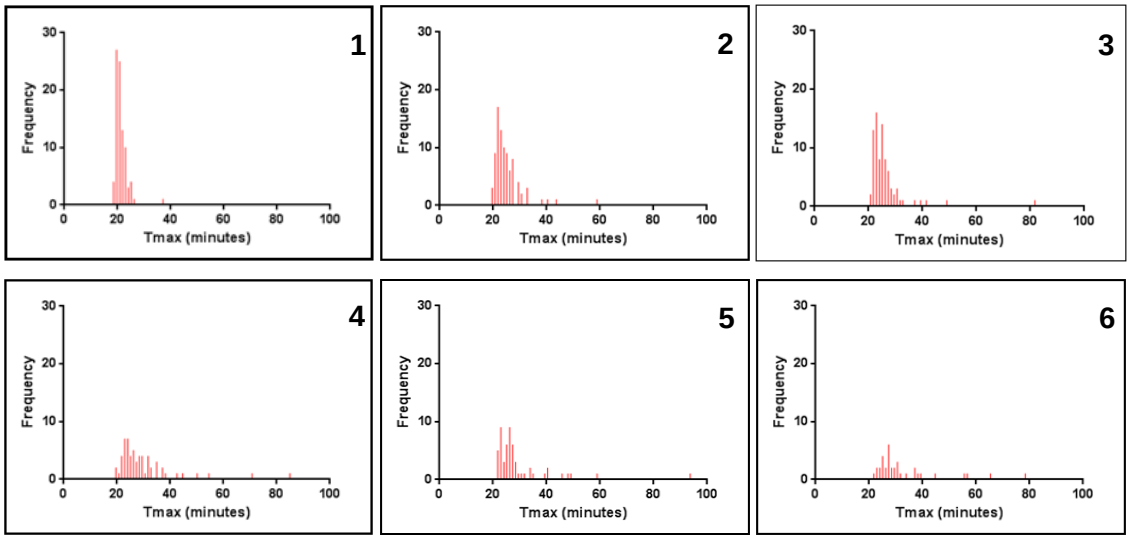


Figure 4.35: (L to R) Frequency distribution of T_{max} results from (1) 10 copies per partition (2) 5 copies per partition (3) 4 copies per partition (4) 3 copies per partition (5) 2 copies per partition (6) 1 copy per partition linearised pART7 template

At ten copies per partition (Figure 4.35) the mode is 19.63, median 20.73 and the mean is 21.26 minutes and the skewness is further away from symmetry at 3.56. Using chi-squared the p-value is calculated to be 0.00015 and therefore the dataset is different from normality. This is holds for all the data below ten copies per partition. Therefore the shift from normal distribution to Poisson distribution appears to be between 30 and 10 copies per partition for this assay.

The standard deviation for the data from ≤ 10 copies per partition should be recalculated as the square root of the mean for a more accurate value, but this value increases with the increasing mean and does not increase with the increasing variability in T_{max} values in the way that the standard deviation assuming normal distribution does. At 5 copies per partition the standard deviation is 5.63 and 5.01 assuming Poisson distribution, but at 1 copy the difference is larger with 12.80 and 5.76 for Poisson SD. The standard deviation assuming normal distribution is not adding bias to further statistical analysis and is therefore used in this form.

4.7.2 Standard deviation assuming normal distribution

The T_{max} frequency distribution data showed that the variance from the mean increases with decreasing copy number. At the highest copy number concentration of 60 copies per partition this variance is very low at 0.8 minutes and it follows that higher concentrations will have standard deviation values of between 0.0 and 0.5 as the range of T_{max} values for a higher copy number will be either one or two. For example 1000 copies per partition may have half the positive results at one T_{max} value and the other

half at the next T_{max} value. Therefore at a copy number concentration above 60 copies per partition the variance will reach a minimum level and therefore variance can only be used for low copy number quantification (Figure 4.36).

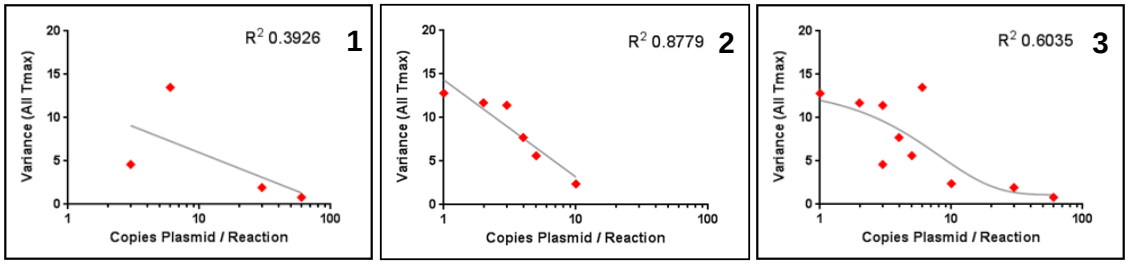


Figure 4.36: (L to R) Standard deviation from (1) 60, 30, 6 and 3 copies per partition pART7 T_{max} data and (2) 10, 5, 4, 3, 2 and 1 copy per partition pART7 T_{max} data (3) combined results to show inter-assay variation for this measurement and poor fit to semi-linear model when datasets are combined – likely to fit a sigmoidal model due to the maximum variance limiting at lower copy number and the low variance at copy numbers above 10.

Both datasets, with the exclusion of the 6 copies per partition, show a good fit to the semi-log linear model but the gradients of the lines are quite different. As already stated the variance will reach a minimum level at higher copy number. The variance increases exponentially with reducing copy number but will tend towards a maximum level; the combined data therefore fits a sigmoidal line with a logarithmic scale for the template copies. Two points that don't fit the model are for the two assay repeats for 3 copies per partition, one of which is too high and the other lower than the trend line. Another point that doesn't fit is the 6 copies per partition which has two outlying T_{max} values greater than 90 minutes. The exponential increase in the low copy number range benefits the quantification of individual copy numbers in the low copy number range. Therefore standard deviation has great potential for ultra-quantification.
A low number of positive results may not result in sufficient numbers of outlying values to increase the standard deviation for low copy numbers and therefore larger datasets may be required. This would be less desirable. The total assay time of 100 minutes to get the necessary high values of standard deviation for low copy numbers is also not ideal. Therefore the theoretical length of the assay was reduced from 100 to 50 minutes by disregarding peaks after this time to utilise the variance seen close to the mean for the datasets (Figure 4.37). This may reduce the discrimination at low copy number between single copy numbers.

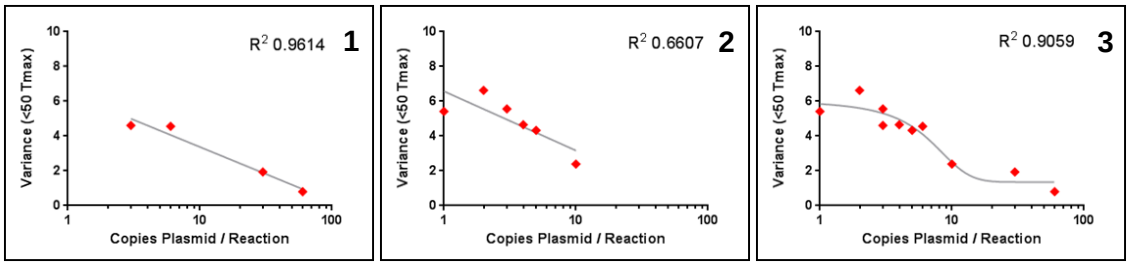


Figure 4.37: (L to R) Standard deviation after removal of partitions with T_{max} values >50 minutes (1) 60, 30, 6 and 3 copies per partition pART7 T_{max} data and (2) 10, 5, 4, 3, 2 and 1 copy per partition pART7 T_{max} data (3) combined results

The two assays at 3 copies per partition are brought closer together by reducing the assay length, but 1 copy per partition has a greatly reduced variance due to a high number of outliers above 50 minutes.

Reducing the assay time still further to 40 minutes will still further reduce the variance to that adjacent to the mean (Figure 4.38).

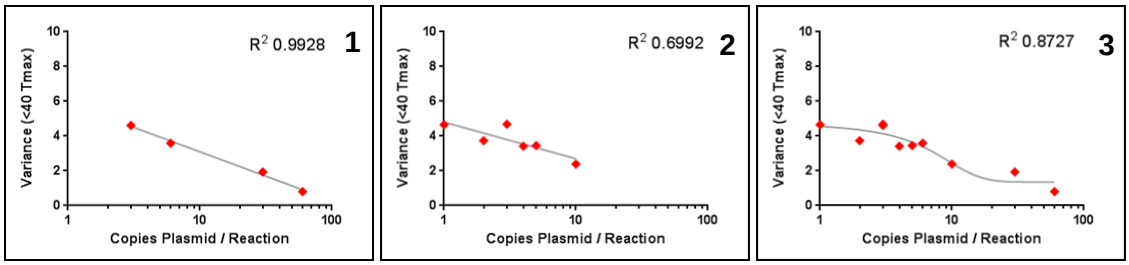


Figure 4.38: (L to R) Standard deviation after removal of partitions with T_{max} values >40 minutes (1) 60, 30, 6 and 3 copies per partition pART7 T_{max} data and (2) 10, 5, 4, 3, 2 and 1 copy per partition pART7 T_{max} data (3) combined results

As with the reduction in total assay time to 50 minutes the discrimination between copy numbers at low copy number is lost by capping the variance.

Therefore to determine copy number using variance requires the assay to run for at least 100 minutes to ensure that the variance for low copy number assays are not limited by the length of the assay. The amplification frequency at low copy number will also have an influence on the number of repeats that are required to ensure an appropriate value.

4.7.3 Modal T_{max}

From the T_{max} frequency distribution data the position of the highest frequency or modal T_{max} increased with decreasing copy number (Figure 4.39).

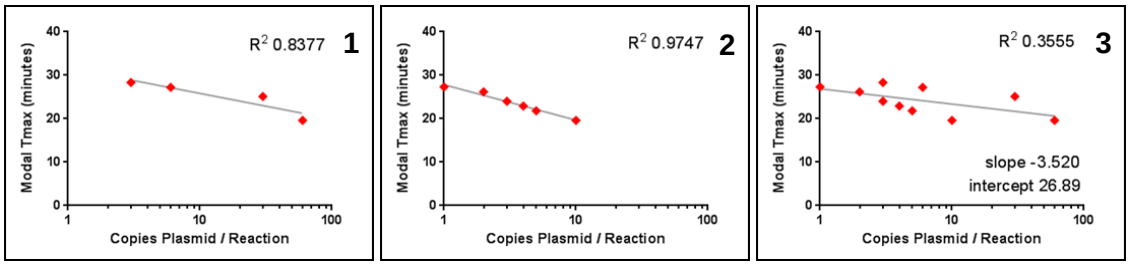


Figure 4.39: (L to R) Modal T_{max} values from (1) 60, 30, 6 and 3 copies per partitions (2) 10, 5, 4, 3, 2 and 1 copy per partition linearised pART7 template (3) combined results

The modal T_{max} for these two datasets both showed an excellent fit to the semi-log linear model and the gradient of the slopes indicate determination of single copies at low copy number. The benefit of modal T_{max} over other measurements is the short assay time – the assay in these examples only needs to run for 30 minutes for all the required data.

Another interesting phenomenon of the modal T_{max} and the frequency distribution data is the number of measurements contributing to the modal value (Figure 4.40).

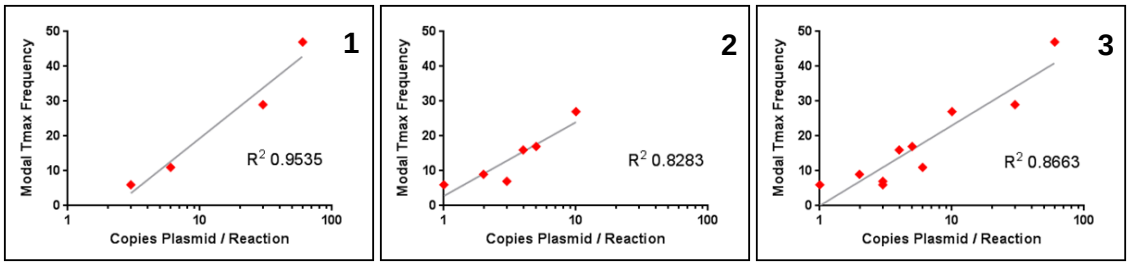


Figure 4.40: (L to R) Modal T_{max} frequencies from (1) 60, 30, 6 and 3 copies per partition linearised pART7 and (2) 10, 5, 4, 3, 2 and 1 copy per partition linearised pART7 (3) combined results

The frequency for the modal T_{max} for each of the assays from three to sixty copies shows an increase in frequency from 6 to 47. For the one to ten copies per partition the increase in frequency for the modal T_{max} goes from 6 to 27 but with an anomalous low frequency for three copies per partition due to two adjacent modal T_{max} times with the same frequency.

At low copy number the number of positive results drops and also the variance increases and both these factors operate to reduce modal T_{max} frequency. This should lead to discrimination between copy numbers but the problem with this approach is the dilution of the frequency by the presence of more than one modal T_{max}.

4.7.4 Median T_{max}

The T_{max} frequency distribution data showed an increase in variance with reducing copy number. So far two approaches to quantification have used values from within the main cluster of results; the average T_{max} and the modal T_{max}. Another method is the median T_{max} which finds the central T_{max} value between the highest and lowest halves of the T_{max} results (Figure 4.41).

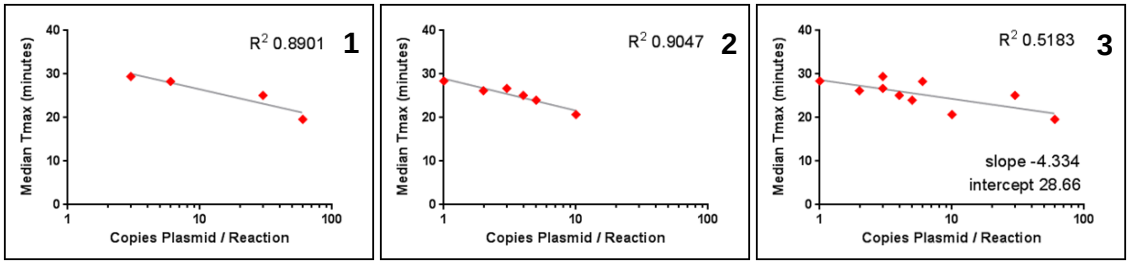
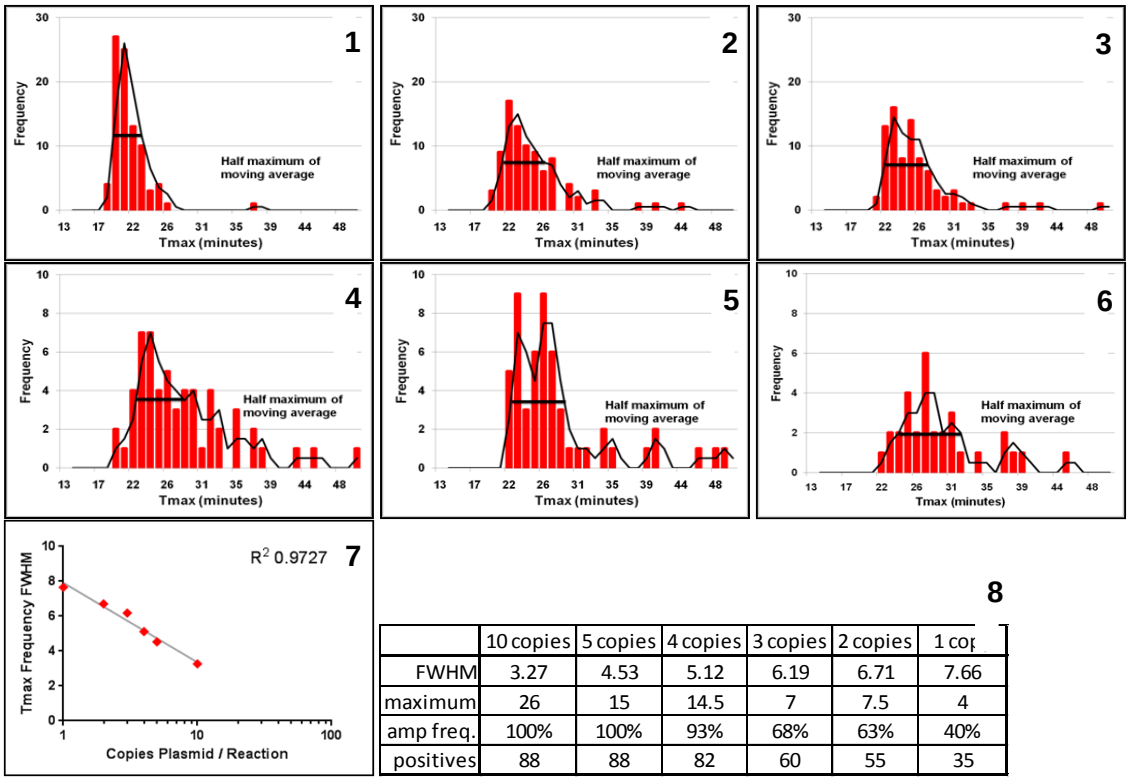


Figure 4.41: (L to R) Median T_{max} from (1) 60, 30, 6 and 3 copies per partition linearised pART7 and (2) 10, 5, 4, 3, 2 and 1 copy per partition linearised pART7 (3) combined results

The median T_{max} for both datasets showed an increase with reducing copy number. The method would not require a long assay time because the majority of positive results required would be achieved with a 40 minutes assay time. The number of assay repeats required may be reducible from the current 88 because the most frequent T_{max} values are below 40 minutes. The problem with median T_{max} and other measurements of T_{max} is the inter-assay variation as seen with the combined datasets. Comparisons between assays using T_{max} measurements, requires normalisation of results with suitable calibrators.

4.7.5 FWHM of the moving average of T_{max} frequency distribution

An approach to quantification at low copy number that uses the morphology of the frequency distribution data is the FWHM of the moving average of the T_{max} frequency distribution (Figure 4.42). The method calculates the two point moving average of the frequencies and then determines the full width half maximum of this calculation. This method only uses the main cluster of positive T_{max} results but is not reliant on the T_{max} timings for quantification and is therefore not as affected by inter-assay variation.



8

	10 copies	5 copies	4 copies	3 copies	2 copies	1 cop
FWHM	3.27	4.53	5.12	6.19	6.71	7.66
maximum	26	15	14.5	7	7.5	4
amp freq.	100%	100%	93%	68%	63%	40%
positives	88	88	82	60	55	35

Figure 4.42: (L to R) (1) Frequency distribution with two point moving average indicated by a black line and the FWHM by a black horizontal bar from (1) 10 copies per partition (2) 5 copies per partition (3) 4 copies per partition (4) 3 copies per partition (5) 2 copies per partition (6) 1 copy per partition linearised pART7 template (7) FWHM of the moving average of T_{max} frequency distribution against copy number (8) table of results with frequency of amplification

There is an excellent R^2 value of 0.9727 for the line connecting the copy number on a logarithmic scale with the frequency FWHM values. The steepness of the gradient discriminates between individual copy numbers. Frequency FWHM only requires the data from the first 40 minutes of the assay therefore the total assay time can be cut. At low copy number more repeats may be required to ensure a suitable number of positive results for analysis.

4.8 Data analysis of assay replicates

The volume of a sample may be limited and therefore a quantitation approach must show accuracy with a low number of repeats. This section therefore investigates the datasets from the 10, 5, 4, 3, 2 and 1 copy per partition and the 60, 30 6 and 3 copies per partition to assess the repeat requirements for the different measurement types. The aim is to find a method which can discriminate between copy numbers with the lowest number of repeats possible. Firstly the data was analysed by taking the T_{max} value for each repeat (88 repeats at each copy number) in the order in which they were loaded (Figure 4.43). For example, the first result plus the second result was used to

calculate the average T_{max} and other measurements, and these values therefore were associated with two assay repeats.

4.8.1 Average T_{max}

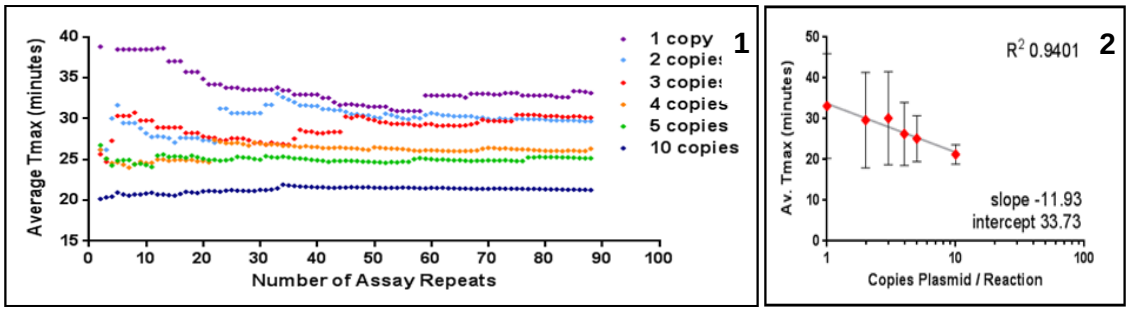


Figure 4.43: Average T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

The ultra-quantification using average T_{max} was in the correct order of copy numbers from 35 repeats until 75 repeats at which point the three copies and the two copies per partition switched. The average T_{max} range between ten copies and one copy per partition was maintained at over 10 minutes from just a few repeats and therefore there should be plenty of range for the other average T_{max} values to fit into.
The average T_{max} for the 4, 3, 2 and 1 copy per partition data are affected by outlying T_{max} values and removing these should give more consistent results from less replicates (Figures 4.44 and 4.45).

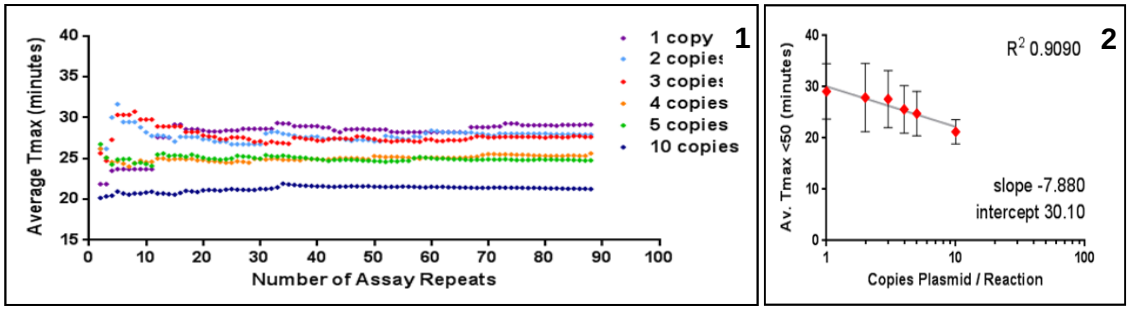


Figure 4.44: Average T_{max} for values less than 50 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

Reduction in the total assay time to 50 minutes results in lines that are straighter and more horizontal. The range of times between ten and one copy per partition average T_{max} is reduced which in turn reduces the discrimination of single copy numbers. From seventy repeats onwards there is discrimination between the copy numbers.

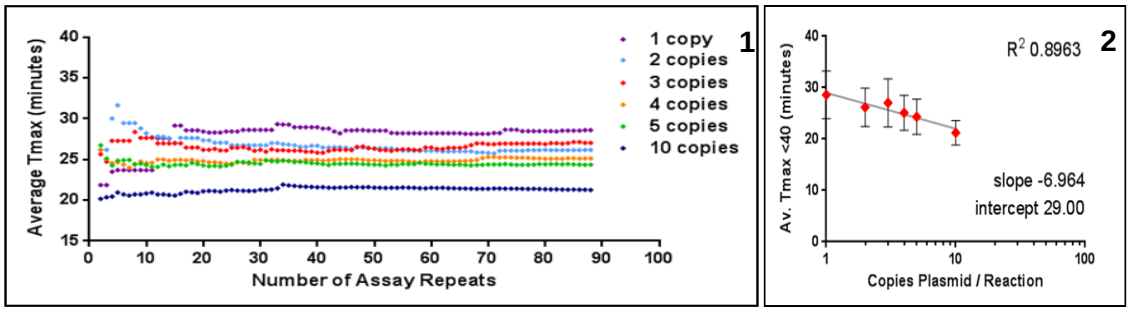


Figure 4.45: Average T_{max} for values less than 40 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

The removal of partitions with T_{max} values above 40 minutes gives straight horizontal lines but the separation between three and two copies and between four and five copies is not achieved correctly within the eighty-eight repeats.

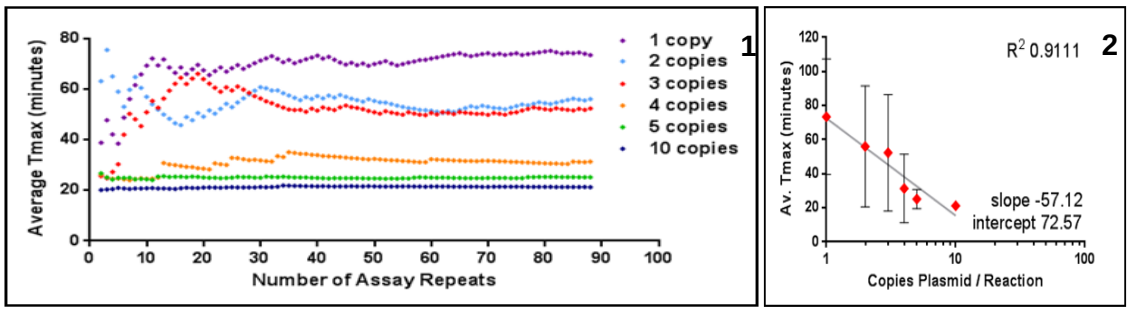


Figure 4.46: Average T_{max} for all data with negative results given a T_{max} value of 100 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

The combination of decreasing amplification frequency and increasing average T_{max} increases the range of times between ten and one copy per partition (Figure 4.46). There is still however poor separation between two and three copies per partition. But with this method the average T_{max} results are in the correct order from only 30 repeats.

4.8.2 Fastest T_{max}

The fastest T_{max} value for a copy number assay is the first T_{max} value recorded (Figure 4.47). Such a method is beneficial because the total assay time is reduced, however there is poor discrimination between copy numbers and an excess of copies in one partition will lead to a spurious result.

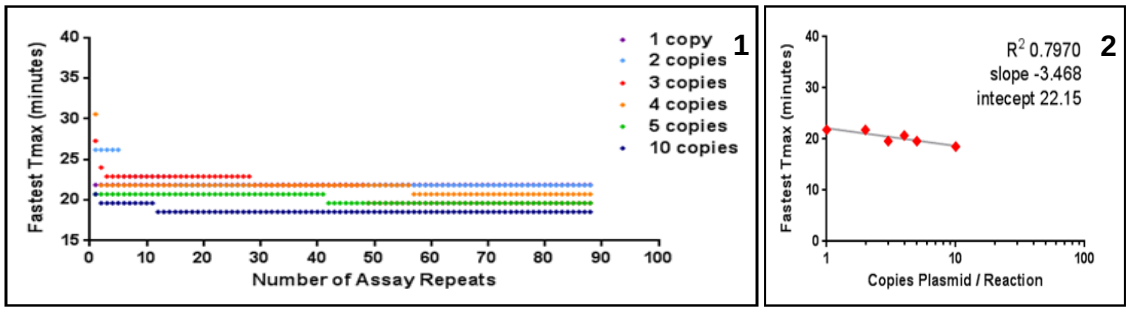


Figure 4.47: Fastest T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

For low copy number quantification the fastest T_{max} method shows insufficient separation between copy numbers. There is no separation between one and two or between three and four copies per partition regardless of the number of assay repeats.

4.8.3 Modal T_{max}

One of the benefits of using the modal T_{max} for quantification is the reduced total assay time (Figure 4.48). For the 35Sp LAMP-BART assaying of the linearised plasmid pART7 the total assay time can be reduced to 30 minutes and achieve low copy number detection.

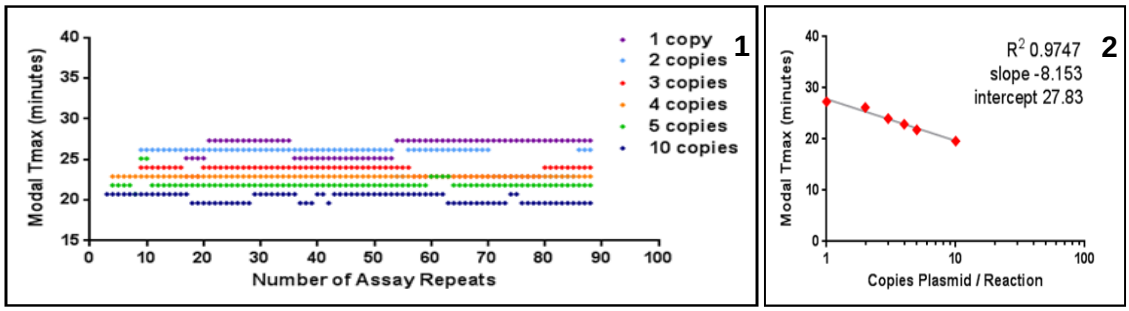


Figure 4.48: Modal T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

At ten copies per partition the value for modal T_{max} switches between two neighbouring T_{max} times (approximately one minute integrals defined by the BART software). The range of T_{max} times between the ten and one copy per partition modal T_{max} is at best 7 minutes and that means that there are only six T_{max} times in that range to separate eight copy numbers between one and ten copies per partition. Therefore some of the copy number assays will have the same modal T_{max} value. The range from five to one copy per partition is sufficient to allow separate modal T_{max} values for four, three and two copies per partition. This method is therefore only suitable for the discrimination of copy numbers between one and five copies.

From 86 to 88 repeats all the copy numbers are in the correct order and separated from each other. The linearity of this end-point can be seen on the graph for all replicates. As with all T_{max} quantification methods the inter-assay variation requires tight control.

4.8.4 Median T_{max}

The median T_{max} is derived from the main cluster of T_{max} results as described in section 4.7.4. Unlike the modal T_{max}, the median T_{max} will be affected by the variance and this will be increased by reducing copy number (Figure 4.49).

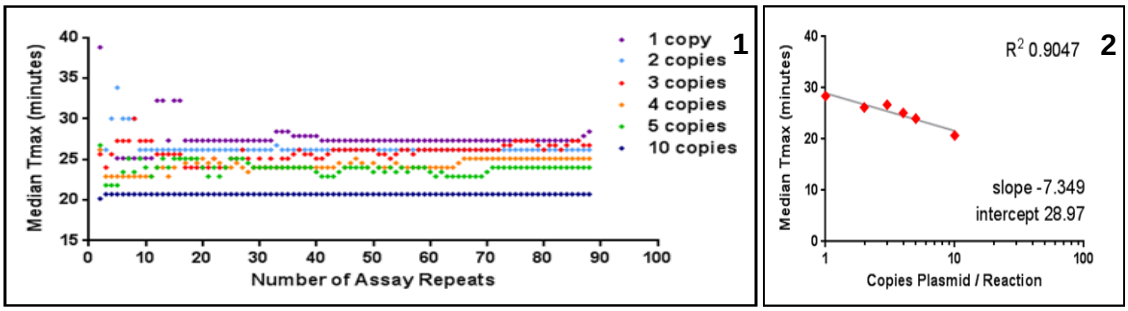


Figure 4.49: Median T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

Calculating the median T_{max} from all T_{max} values up to 100 minutes (total assay time) for the ten copies per partition gives the consistent value of 20.73 minutes from less than 5 replicates onwards. Comparing modal T_{max} and average T_{max} with this value shows the influence the variance has on the measurements on the assay time line. The lowest value is from the modal T_{max} at 19.63 minutes, next is the median at 20.73 minutes followed by the average T_{max} at 21.26 minutes. This order is repeated at one copy per partition with a modal T_{max} at 27.33 minutes, median T_{max} at 28.43 minutes and finally the average T_{max} at 33.14 minutes. The range of median T_{max} values between ten and one copy per partition is the same as the modal range of 7.7 minutes. However the position of the modal T_{max} for five copies is mid range reducing the time range for the four, three and two copies per partition but increasing the time range for nine, eight, seven and six copies per partition. Therefore the median T_{max} approach to determine individual copy numbers will have a larger range than the modal T_{max} but may be less suitable for very low copy numbers.

From all 88 replicates the two and three copies per partition are poorly separated and may be a result of the high variance at this level. The variance is reduced by discounting T_{max} values above 50 minutes (Figure 4.50).

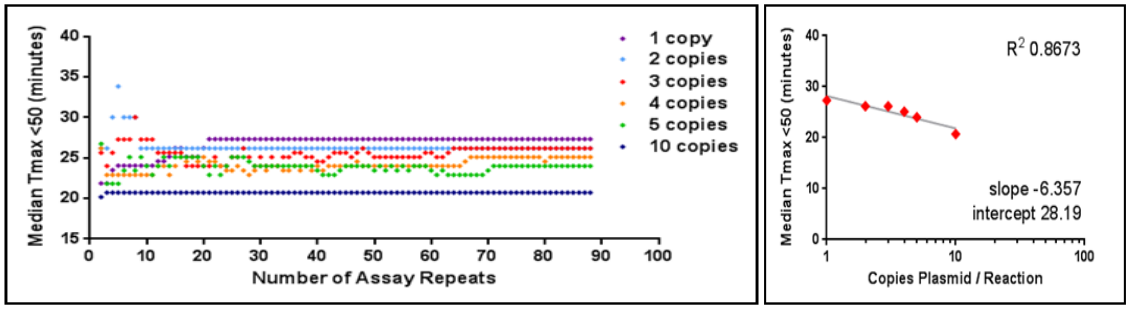


Figure 4.50: Median T_{max} for T_{max} values less than 50 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

Straighter horizontal lines are observed for the lower copy number assays but the reduction in variance brings a reduction in the range of median T_{max} values and the two and three copies per partition cannot be separated. The variance observed at very low copy number should therefore be utilised to give separation between individual copy numbers.

4.8.5 Standard deviation of T_{max} data

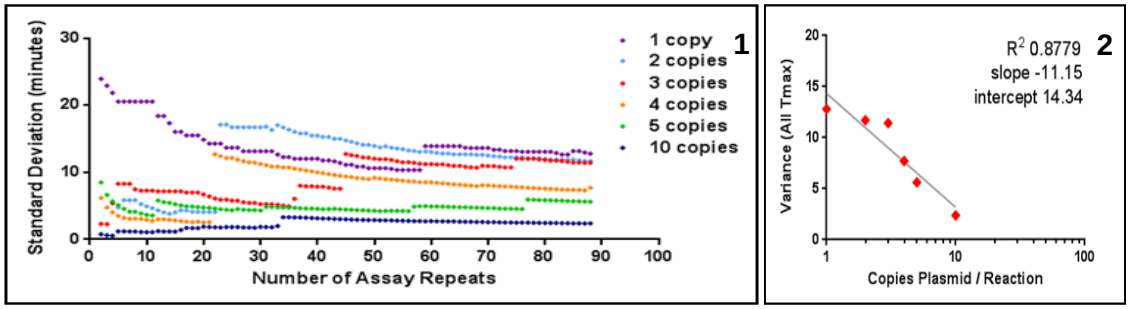


Figure 4.51: Standard deviation of average T_{max} times (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

Using the full assay time of 100 minutes the standard deviation separates the copy number levels in the correct order from approximately 60 partitions to the maximum of 88 partitions (Figure 4.51). There is a good range between five and one copy and clear separation between five and four copies but the three and two copies are almost superimposed.

Reducing the assay time to 50 minutes will remove the high T_{max} values and stabilise the variance (Figure 4.52).

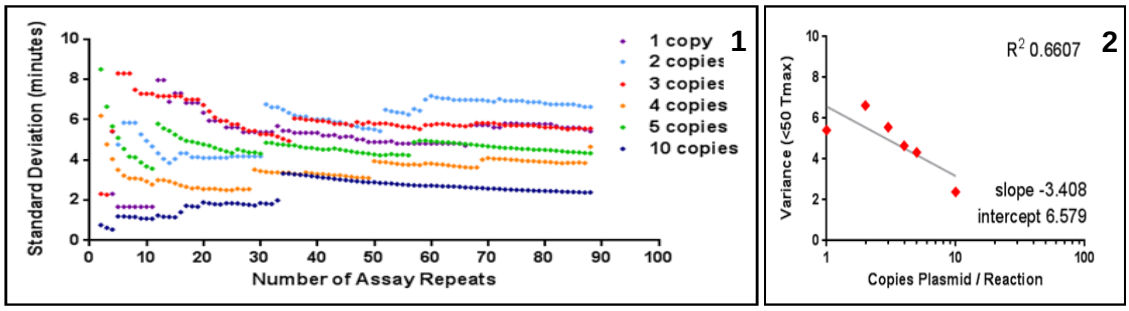


Figure 4.52: Standard deviation of average T_{max} times less than 50 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

The range between ten and one copy per partition in terms of standard deviation values is reduced by the total assay time to 50 minutes. The one copy number result with the lowest amplification frequency and therefore the least positive results is the dataset that drops off here. A greater number of positive results from increased amplification efficiency or number of replicates would be beneficial here. The discrimination between four and five copies is poor and only achieves the correct copy number order after 88 repeats.

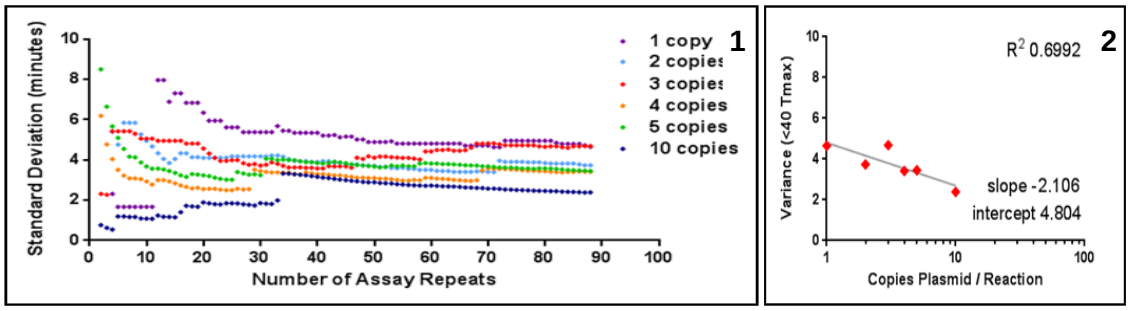


Figure 4.53: Standard deviation of average T_{max} times less than 40 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

Further reducing the total assay time to 40 minutes narrows the range of the standard deviation further by decreasing the variance of the one and two copies per partition (Figure 4.53). There is poor discrimination between copy numbers.

Using standard deviation for quantification at low copy number requires the longer assay time of 100 minutes to ensure high variance for the lowest copy numbers. For these copy numbers there is also an associated reduction in amplification frequency and the number of positive results needs to be increased to overcome this.

4.8.6 Average Peak Height

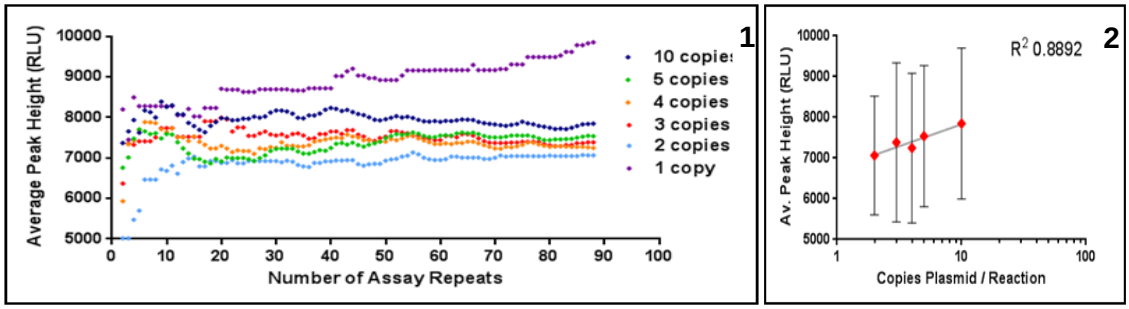


Figure 4.54: Average peak height (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3 and 2 copies per partition

The average peak height (Figure 4.54) for the one copy per partition was much higher than the other results and corresponded with a batch change for the Bst polymerase. The influence of minor changes in the LAMP-BART components on peak height was noted and where possible, as with these assays, the same aliquot of Thermopol buffer, ATP sulphurylase and Bst polymerase were used. For the other results, ten to two copies per partition, there is a slight general decrease in average peak height with reducing copy number but this is insufficient for the separation of copy numbers. The order of four and three copies per partition is incorrect after 88 replicates. The variability in average peak heights is high and this method is unsuitable for low copy number quantification and discrimination of individual copy numbers.

4.8.7 Average FWHM of T_{max} peaks

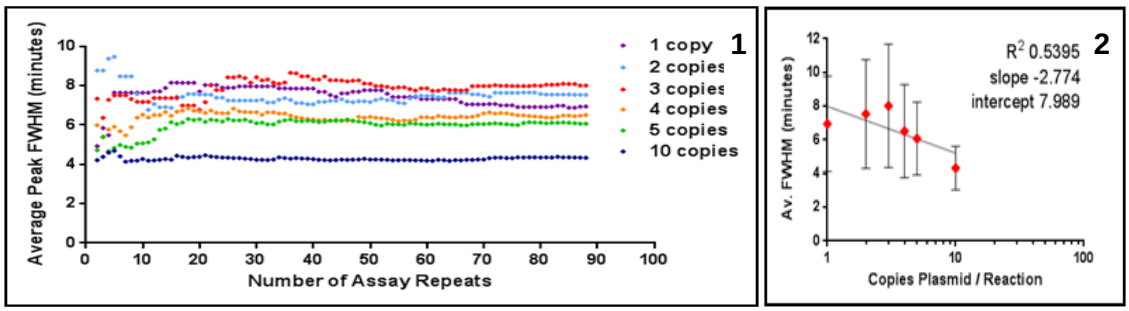


Figure 4.55: Average full width half maximum of T_{max} peaks (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

The average full width half maximum (Figure 4.55) for the ten copies per partition is very consistent from less than ten repeats onwards and at five copies per partition approximately twenty repeats are required. The separation between these two is approximately 2 minutes. There is poor separation however between the four and five

copies and three, two and one copy FWHM results are in the wrong order if increasing average FWHM with reducing copy number is the trend. The graph of average FWHM against copy number is similar to that seen with the standard deviation against copy number when the total assay time was reduced. A problem with those results was the number of positive results to analyse at the lowest copy numbers and this may be the case here. This method may yet be useful for separating single copy numbers at low copy number concentrations, but on this evidence average FWHM is unsuitable.

4.8.8 Percentage modal T_{max} frequency

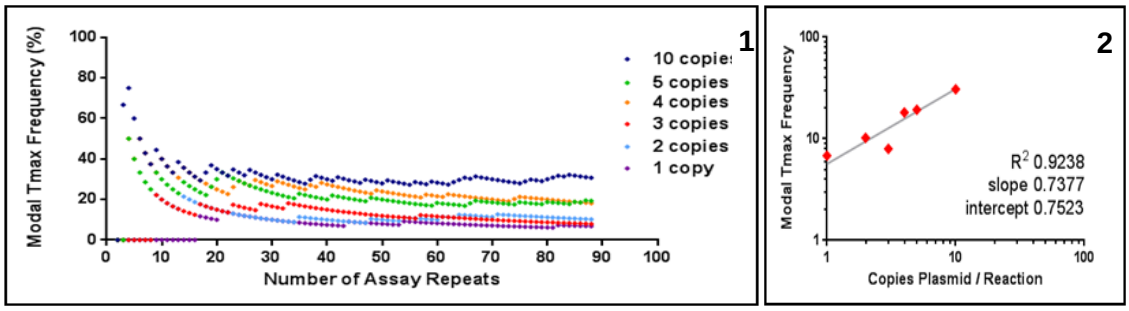


Figure 4.56: Percentage modal T_{max} frequency (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

The frequency of the modal T_{max} as a percentage of the total number of assay partitions (Figure 4.56) can separate ten copies from one copy per partition from less than 20 assay repeats of each, but the separation of the other copy numbers becomes increasing muddled with increasing assay repeats. The frequency for a particular copy number can be divided between two adjacent T_{max} values when the modal T_{max} is on the cusp of both. As a consequence the frequency may be lower than expected and this may be the case with the three copies per partition data.

4.8.9 FWHM of the moving average of T_{max} frequencies

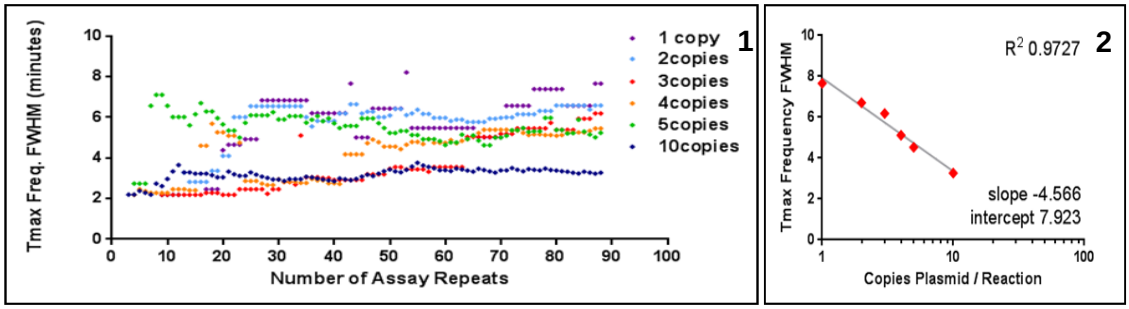


Figure 4.57: Full width half maximum of the moving average of T_{max} frequencies (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 10, 5, 4, 3, 2 and 1 copy per partition

The FWHM from the two point moving average of T_{max} frequencies from 88 repeats showed a close relationship with copy numbers per partition (Figure 4.57). With fewer repeats it is only the ten copies per partition that maintains a stable level from twenty repeats onwards. Five copies per partition has a high frequency FWHM value initially but this gradually comes down towards the ten. Four starts low and gradually increase to end higher than ten and five. The lowest copy numbers with fewer positive results are highly variable but are in the correct order at 88 repeats. The data from the first of the single copy 384 partition assays (described in the digital BART chapter) gives a frequency FWHM of 8.87 from 127 positive results. In this single copy 96 partition assay the frequency FWHM is 7.66 from 35 positive results. A higher number of positive results would be beneficial at the lowest copy numbers.

The data from 60, 30, 6 and 3 in the order of loading and using the random number generation described in chapter 2 to randomise the position on the 96 well plate is in the Appendix (Figures: App4.4 to 4.18).

4.9 Ultra-quantification methods

A number of quantification methods show potential from the 60, 30, 6 and 3 copies per partition data, and these are highlighted in the table below (Figure 4.58).

method	60, 30, 6 and 3 copies per partition						
	R^2	slope	60↔30	30↔6	6↔3	replicates required	minimum assay time
average Tmax	0.86	-9.08	yes	yes	no	not known	100 minutes
average Tmax <50 minutes	0.87	-7.23	yes	yes	yes	25 replicates	50 minutes
average Tmax <40 minutes	0.87	-7.07	yes	yes	yes	25 replicates	40 minutes
average Tmax +negatives	0.98	-31.09	yes	yes	yes	5 replicates	100 minutes
fastest Tmax	0.28	-1.64	yes	yes	no	not known	25 minutes
modal Tmax	0.84	-5.88	yes	yes	yes	40 replicates	30 minutes
median Tmax	0.89	-6.89	yes	yes	yes	18 replicates	100 minutes
median Tmax <40 minutes	0.88	-6.50	yes	yes	yes	18 replicates	40 minutes
standard deviation (normal)	0.39	-5.98	yes	yes	no	not known	100 minutes
standard deviation <50 minutes	0.96	-3.11	yes	yes	yes	87 replicates	50 minutes
standard deviation <40 minutes	0.99	-2.80	yes	yes	yes	28 replicates	40 minutes
average peak height	0.85	1177	yes	yes	yes	78 replicates	100 minutes
average peak FWHM	0.58	-3.10	yes	no	yes	not known	100 minutes
percentage modal Tmax frequency	1.00	4.60	yes	yes	yes	10 replicates	30 minutes
FWHM Tmax frequency	0.98	-3.47	yes	yes	yes	35 replicates	35 minutes

Figure 4.58: The various methods for quantification of the 60, 30, 6 and 3 copies per partition linearised pART7 template assayed with 35Sp LAMP-BART. The value of R^2 is the correlation to a semi-logarithmic model. The slope is the gradient of the semi-logarithmic trend line. ↔ denotes the differentiation in the correct order between two copy numbers. The number of replicates required is derived from the analysis of assay replicates and the minimum assay time denotes the time at which the required data has been collected.

The average T_{max} and the standard deviation methods were greatly affected by outlying values for the 6 copies per partition assay and the removal of these values showed the possibilities of these methods to quantitate with a low number of replicates in a short assay time. Fastest T_{max} would have the fastest minimum assay time, but the discrimination was poor. The other methods showed great potential with the exception of the average peak FWHM.

Some of the highlighted methods from Figure 4.58 remain highlighted under the more demanding requirements of the quantification of 10, 5, 4, 3, 2 and 1 copy per partition (Figure 4.59).

method	10, 5, 4, 3, 2 and 1 copies per partition								
	R^2	slope	10↔5	5↔4	4↔3	3↔2	2↔1	replicates required	minimum assay time
average Tmax	0.94	-11.93	yes	yes	yes	no	yes	not known	100 minutes
average Tmax <50 minutes	0.91	-7.88	yes	no	yes	no	yes	not known	50 minutes
average Tmax <40 minutes	0.90	-6.96	yes	yes	yes	no	yes	not known	40 minutes
average Tmax +negatives	0.91	-57.12	yes	yes	yes	yes	yes	30 replicates	100 minutes
fastest Tmax	0.80	-3.47	yes	yes	no	no	no	not known	25 minutes
modal Tmax	0.97	-8.15	yes	yes	yes	yes	yes	85 replicates	30 minutes
median Tmax	0.90	-7.35	yes	yes	yes	yes	yes	86 replicates	100 minutes
median Tmax <40 minutes	0.87	-6.36	yes	yes	yes	no	yes	not known	40 minutes
standard deviation (normal)	0.88	-11.15	yes	yes	yes	yes	yes	60 replicates	100 minutes
standard deviation <50 minutes	0.66	-3.41	yes	yes	yes	yes	no	not known	50 minutes
standard deviation <40 minutes	0.70	-2.11	yes	no	yes	no	no	not known	40 minutes
average peak height	0.89	1075	yes	yes	no	yes	n/a	not known	100 minutes
average peak FWHM	0.54	-2.77	yes	yes	yes	no	no	not known	100 minutes
percentage modal Tmax frequency	0.92	0.74	yes	yes	yes	no	yes	not known	30 minutes
FWHM Tmax frequency	0.97	-4.57	yes	yes	yes	yes	yes	86 replicates	35 minutes

Figure 4.59: The various methods for quantification of the 60, 30, 6 and 3 copies per partition linearised pART7 template assayed with 35Sp LAMP-BART. The value of R^2 is the correlation to a semi-logarithmic model. The slope is the gradient of the semi-logarithmic trend line. ↔ denotes the differentiation in the correct order between two copy numbers. The number of replicates required is derived from the analysis of assay replicates and the minimum assay time denotes the time at which the required data has been collected.

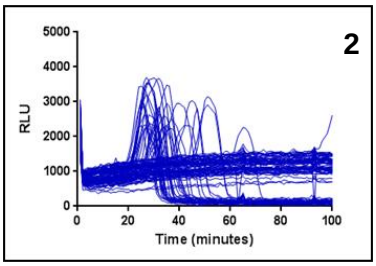
In Figure 4.58 the standard deviation method for the full assay time was affected by the high variance for one of the assays. In Figure 4.59 it is the standard deviation from all the data that shows the best quantification and not only the standard deviation with reduced assay time data. Modal and median T_{max} and the FWHM T_{max} frequency continue to discriminate copy numbers appropriately but require more replicates to achieve this.

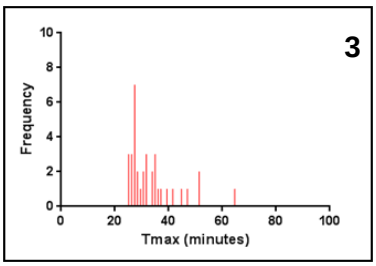
4.10 Robustness of ultra-quantification methods

To test the robustness of the methods summarised in section 4.9 for sensitivity to specific alterations to assay components and conditions, the total assay volume was reduced to 5µl and the freshly prepared BART mix was replaced by Lumora's BARTmaster and NEB Isothermal Buffer. The 35Sp LAMP-BART assay tested six, five, four, three, two and one copy per partition of the linearised pART7 plasmid template in 96 partitions at 60°C (Figures 4.60 to 4.65). No calculations of peak height were made due to the large difference with the previous data from section 4.4.4. Average peak FWHM and the fastest T_{max} were not analysed due to the poor results shown in the previous section.

1 copy per partition

32						25		27	35	32	28
		34	39								27
26						32	65				42
27	37	31	51	27		27	30				
		51			35	25					27
47						27			26	26	
								45		36	
25	31	28			35			34			





1 copy per partition	current values	previous results
amplification frequency	36%	40%
average Tmax	33.55	33.14
average Tmax <50 minutes	31.46	29.13
average Tmax <40 minutes	30.12	28.61
average Tmax +negatives	75.77	73.41
modal Tmax	27.34	27.33
median Tmax	30.61	28.43
median Tmax <40 minutes	28.43	27.33
standard deviation (normal)	9.01	12.80
standard deviation <50 minutes	5.76	5.42
standard deviation <40 minutes	4.04	4.65
percentage modal Tmax frequency	7.29	6.82
FWHM Tmax frequency	9.09	7.66

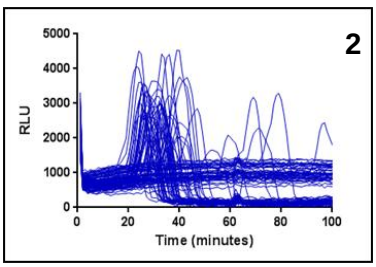
4

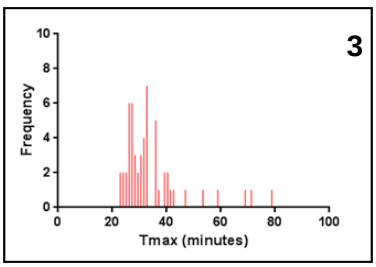
Figure 4.60: (L to R) 1 copy per partition pART7 BARTmaster reduced volume assay (1) position and T_{max} value in green, negative partitions in white (2) BART curves for all partitions (3) frequency distribution of T_{max} results (4) table of results for 1 copy per partition compared to previous results from section 4.4.4.6

A number of these measurements are similar to the previous results from section 4.4.4.6 with the 20µl total assay volume and freshly prepared BART mix. This includes the average and modal T_{max}, the highest T_{max} frequency and the frequency FWHM. The variance is higher and this may be caused by the reduced assay volume; although the concentration stays the same, the copy number per partition has a reduced quantity of reagents, primers and enzymes which may also lead to slower T_{max} peaks. If the combination of template with the LAMP-BART mix is insufficiently homogenous, the 5µl partitions will give rise to a greater variation in T_{max} values through variation in copy number and by sub-optimal reaction conditions.

2 copies per partition

	36		31			33		47			27
28		32		25	23	79		71	27	37	
	33	27	25	39	40		69				
			43	36	29	29	39	36	27		24
41	26	26	31			28			33		
28	26		32			27		32	31	36	
53	26	33				40	27	33	23		33
59	32		26	26		24	36		33		





2 copies per partition	current values	previous results
amplification frequency	57%	63%
average Tmax	34.44	29.67
average Tmax <50 minutes	31.25	27.92
average Tmax <40 minutes	30.02	26.17
average Tmax +negatives	62.44	56.05
modal Tmax	32.72	26.19
median Tmax	31.63	26.19
median Tmax <40 minutes	29.44	26.19
standard deviation (normal)	11.80	11.71
standard deviation <50 minutes	5.60	6.63
standard deviation <40 minutes	4.34	3.74
percentage modal Tmax frequency	7.29	10.23
FWHM Tmax frequency	8.35	6.58

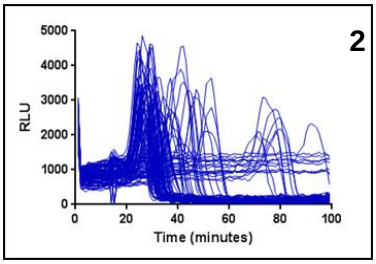
4

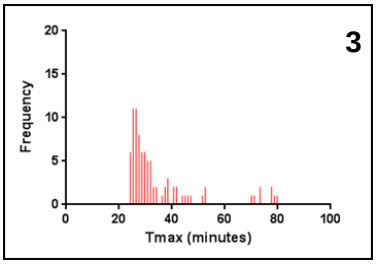
Figure 4.61: (L to R) 2 copies per partition pART7 BARTmaster reduced volume assay (1) position and T_{max} value in green, negative partitions in white (2) BART curves for all partitions (3) frequency distribution of T_{max} results (4) table of results for 1 copy per partition compared to previous results from section 4.4.4.5

The frequency distribution has an unusual shape with the highest frequency next to two zero frequencies rather than a gradual decline in frequencies. The modal T_{max} from the first T_{max} with a frequency of 6 would have been 26.17 minutes and would be a good comparison to the modal T_{max} from the previous results from section 4.4.4.5 which has a modal T_{max} value of 26.19 minutes. The amplification frequency for both of the assays are similar and therefore so are the values from average T_{max}, with negative results given a value of 100 minutes. The standard deviation values are closely correlated.

3 copies per partition

38	73	78	53	30	26	30	25	26	26		24
28	51	26	53	42	29	31	25	44	31	30	70
45	28	80	26	29	42		26	32	25	36	25
32	32		29	26	24	41		25	41		38
24	31	30	34	26	73		28		26	24	37
26	28	32	31	24	28	25	71	29	47	33	30
26	25	33	79	46	25	28		31	25	38	32
34	25	37	29	78		29	25	28	28	30	24





3 copies per partition	current values	previous results
amplification frequency	91%	68%
average Tmax	35.21	30.13
average Tmax <50 minutes	30.33	27.63
average Tmax <40 minutes	28.80	27.04
average Tmax +negatives	41.28	52.36
modal Tmax	26.42	24.00
median Tmax	29.70	26.73
median Tmax <40 minutes	27.51	26.19
standard deviation (normal)	14.51	11.43
standard deviation <50 minutes	5.81	5.56
standard deviation <40 minutes	3.81	4.69
percentage modal Tmax frequency	11.46	7.95
FWHM Tmax frequency	6.06	6.19

4

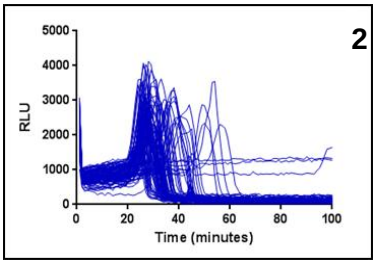
Figure 4.62: (L to R) 3 copies per partition pART7 BARTmaster reduced volume assay (1) position and T_{max} value in green, negative partitions in white (2) BART curves for all partitions (3) frequency distribution of T_{max} results (4) table of results for 1 copy per partition compared to previous results from section 4.4.4.4

There was a much higher amplification frequency for this assay compared to the previous result from section 4.4.4.4. The only closely matching values are for the FWHM T_{max} frequency calculations and the standard deviation measurements with outlying values removed.

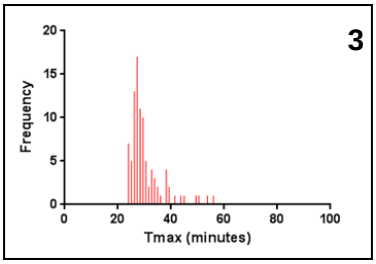
4 copies per partition

35	29	29	25	28	25	24	38	44	26	28	29
27	27	45	26	27	31	28	26	27	29	29	32
25	50	24	33	28	27	28	54		24	26	34
29	31	28	27	26		28	29	29	34	27	26
	29	49	56	38	28	33	38	25	27	39	35
26	28	27	26	24	31	27	24	27	41	27	31
26	24	27	25	39	29	33	38	36	27	26	32
33	34	27	26	28	26	27	28	24	31	27	26

2



3



4 copies per partition	current values	previous results
amplification frequency	97%	93%
average Tmax	30.41	26.31
average Tmax <50 minutes	29.65	25.62
average Tmax <40 minutes	28.94	25.13
average Tmax +negatives	32.59	31.33
modal Tmax	27.27	22.91
median Tmax	28.36	25.10
median Tmax <40 minutes	28.36	25.10
standard deviation (normal)	6.49	7.72
standard deviation <50 minutes	5.01	4.65
standard deviation <40 minutes	3.81	3.42
percentage modal Tmax frequency	17.71	18.18
FWHM Tmax frequency	5.55	5.45

4

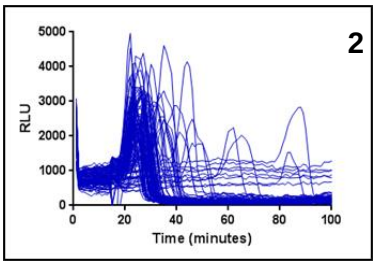
Figure 4.63: (L to R) 4 copies per partition pART7 BARTmaster reduced volume assay (1) position and T_{max} value in green, negative partitions in white (2) BART curves for all partitions (3) frequency distribution of T_{max} results (4) table of results for 1 copy per partition compared to previous results from section 4.4.4.3

Frequency of amplification and the average T_{max} with negatives are closely matched but other T_{max} results are dissimilar. The standard deviation values, percentage modal T_{max} frequency and FWHM T_{max} frequency are all well matched.

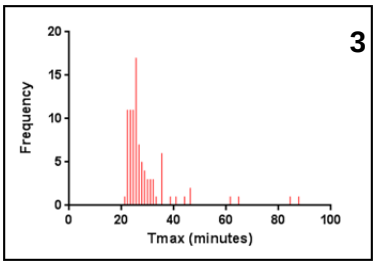
5 copies per partition

24	23	23		23	23	29		24	29	28	84
26	65	35		24	22	30	28	24	46	35	22
26	26	26	31	22	35	35	27	26	23	46	27
26	21	23	26	27	23	22	41	27	28	22	22
26	22	26	26	24	24	26	26	32	26		26
29	32	27	23		24	23	31	23	24	26	35
24	27	26	33	26	44	23	31	32	24	62	28
22	28	22	24	29	88	27	22	39		35	22

2



3



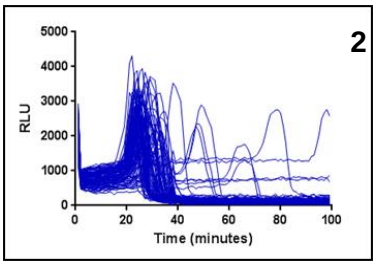
5 copies per partition	current values	previous results
amplification frequency	94%	100%
average Tmax	29.40	25.16
average Tmax <50 minutes	27.29	24.77
average Tmax <40 minutes	26.46	24.36
average Tmax +negatives	33.81	25.16
modal Tmax	25.55	21.82
median Tmax	25.55	24.01
median Tmax <40 minutes	25.55	24.01
standard deviation (normal)	11.41	5.63
standard deviation <50 minutes	5.42	4.33
standard deviation <40 minutes	3.94	3.45
percentage modal Tmax frequency	17.71	19.32
FWHM Tmax frequency	5.06	5.24

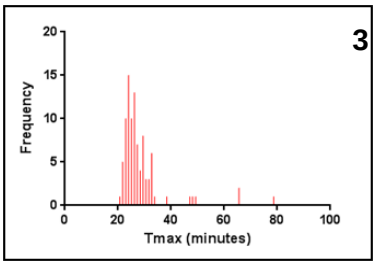
Figure 4.64: (L to R) 5 copies per partition pART7 BARTmaster reduced volume assay (1) position and T_{max} value in green, negative partitions in white (2) BART curves for all partitions (3) frequency distribution of T_{max} results (4) table of results for 1 copy per partition compared to previous results from section 4.4.4.2

All the T_{max} values are slower for this assay of 5 copies per partition and the amplification frequency is less than the previous assay from section 4.4.4.2. The best match comes from the FWHM T_{max} frequency values.

6 copies per partition

22	25	66	24	23	25	27	24	24	33	25	27
25	29	24	34	24	23	27	33	26	29	23	31
23	23	25	26	23	27	27	32	26	23	26	66
25	25	24	49	28	26	26	22	26	33	22	32
29	47	33	33	22	29	26	29	29	24	31	28
31	26	24	33	25	24	26	32	26	24		27
27	23	24	24	24	25	38	22	23	29	28	28
	48	25	24		24	29	21	79	26	26	23





6 copies per partition	current values	previous results
amplification frequency	97%	81%
average Tmax	28.59	32.40
average Tmax <50 minutes	27.21	28.48
average Tmax <40 minutes	26.49	27.97
average Tmax +negatives	30.82	45.46
modal Tmax	23.99	27.23
median Tmax	26.18	28.32
median Tmax <40 minutes	26.18	27.23
standard deviation (normal)	9.21	13.50
standard deviation <50 minutes	5.19	4.56
standard deviation <40 minutes	3.46	3.60
percentage modal Tmax frequency	15.63	12.50
FWHM Tmax frequency	5.36	5.84

Figure 4.65: (L to R) 6 copies per partition pART7 BARTmaster reduced volume assay (1) position and T_{max} value in green, negative partitions in white (2) BART curves for all partitions (3) frequency distribution of T_{max} results (4) table of results for 1 copy per partition compared to previous results from section 4.4.3.3

The comparison with the previous assay of 6 copies per partition from section 4.4.3.3 shows that all the T_{max} results are faster for this assay. None of the measurements are closely matched.

The six assays were plotted with the different approaches for low copy number quantification (Figure 4.66).

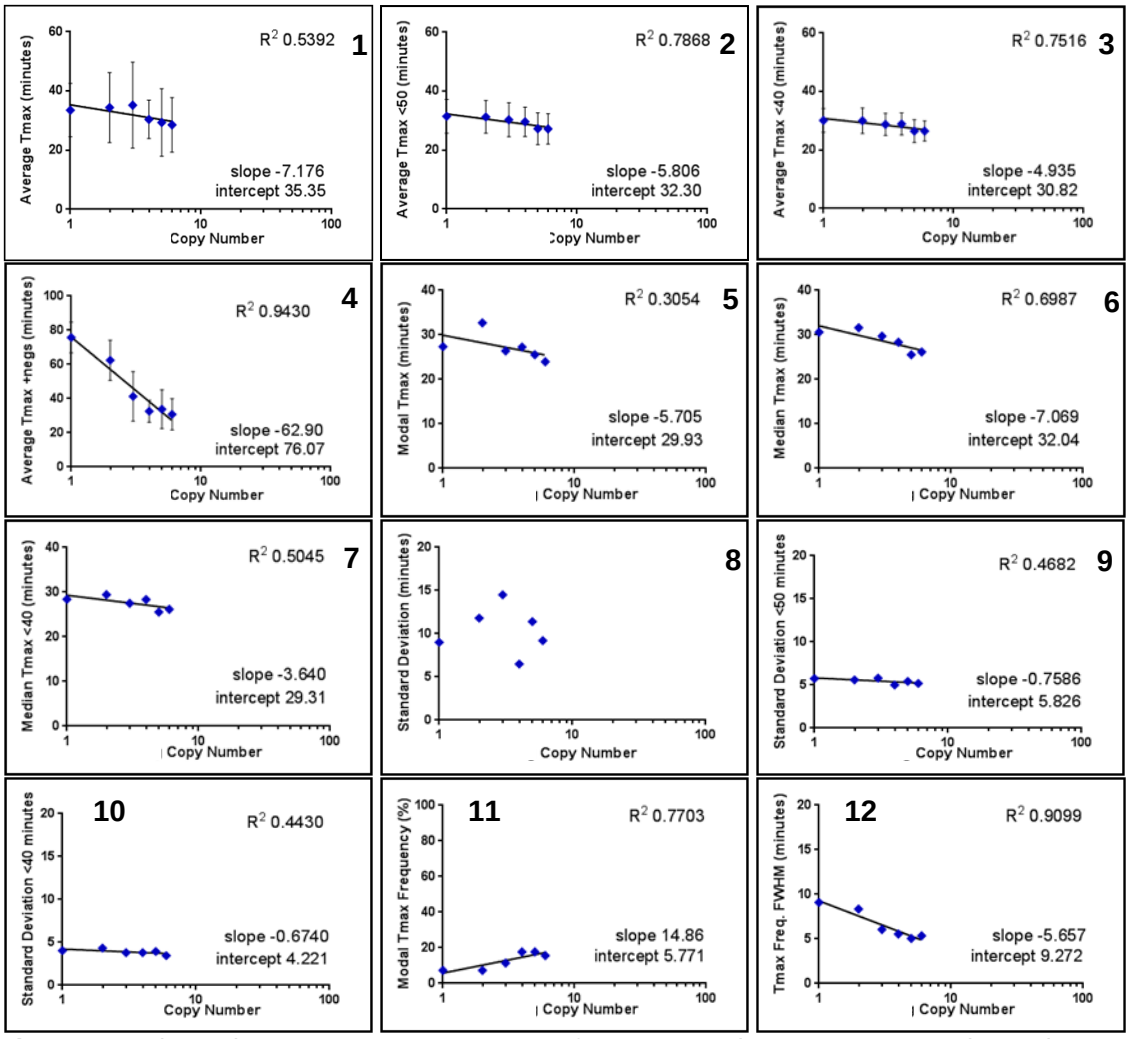


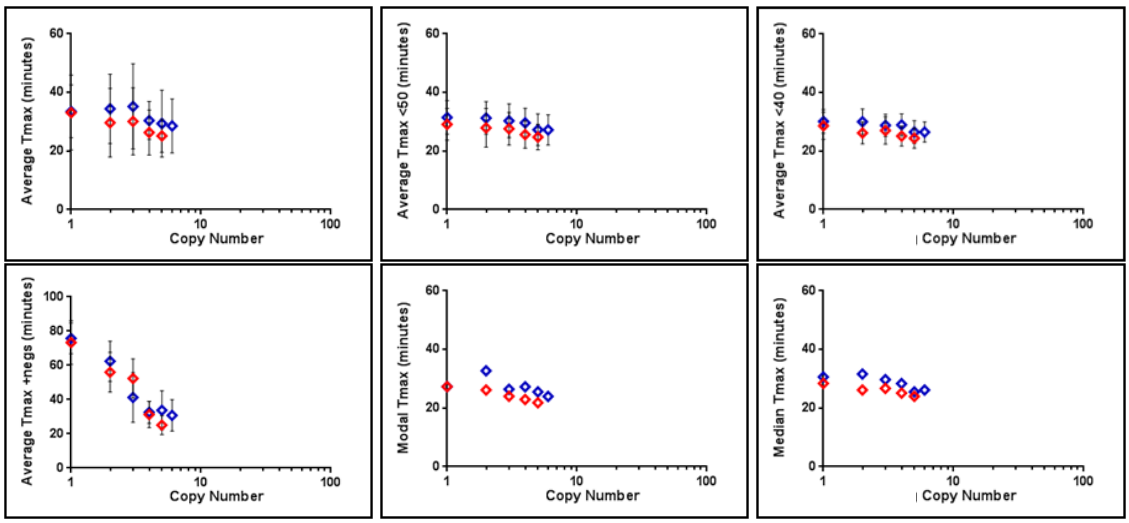
Figure 4.66: (L to R) 35Sp LAMP-BART assay of pART7 template 6, 5, 4, 3, 2 and 1 copies per partition, BARTmaster reaction mix, total assay volume 5µl per partition (1) average T_{max} (2) average T_{max} from <50 minutes T_{max} results only (3) average T_{max} from <40 minutes T_{max} results only (4) average $_{Tmax}$ from all results with negative results assigned 100 minutes (5) modal T_{max} (6) median $_{Tmax}$ (7) median $_{Tmax}$ from <40 minutes $_{Tmax}$ results only (8) standard deviation assuming normal distribution (9) standard deviation from <50 minutes $_{Tmax}$ results only (10) standard deviation from <40 minutes $_{Tmax}$ results only (11) percentage modal $_{Tmax}$ frequency (12) FWHM of moving average of $_{Tmax}$ frequency distribution

All the approaches to quantification display the expected relationship between the measurement and the increasing copy number with the exception of the standard deviation from all positive partitions; which was poorly correlated to the semi-logarithmic model. In terms of the correlation to the model, the best methods were the average T_{max} utilising the decreasing amplification frequency and the FWHM of T_{max} frequencies, but both of these methods fail to differentiate single copy numbers at the higher end of the range. The results are summarised in the table below (Figure 4.67):

method	10, 5, 4, 3, 2 and 1 copies per partition								
	R^2	slope	6↔5	5↔4	4↔3	3↔2	2↔1	replicates required	minimum assay time
average Tmax	0.54	-7.18	yes	yes	yes	no	no	not known	100 minutes
average Tmax <50 minutes	0.79	-5.81	yes	yes	yes	yes	yes	94 replicates	50 minutes
average Tmax <40 minutes	0.75	-4.94	no	yes	no	yes	yes	not known	40 minutes
average Tmax +negatives	0.94	-62.90	yes	no	yes	yes	yes	not known	100 minutes
modal Tmax	0.31	-5.71	yes	yes	no	yes	no	not known	30 minutes
median Tmax	0.70	-7.07	no	yes	yes	yes	no	not known	100 minutes
median Tmax <40 minutes	0.50	-3.64	no	yes	no	yes	no	not known	40 minutes
standard deviation (normal)	0.00	-0.50	yes	no	yes	no	no	not known	100 minutes
standard deviation <50 minutes	0.47	-0.76	yes	no	yes	no	yes	not known	50 minutes
standard deviation <40 minutes	0.44	-0.67	yes	no	yes	yes	no	not known	40 minutes
percentage modal Tmax frequency	0.77	14.86	no	yes	yes	yes	yes	not known	30 minutes
FWHM Tmax frequency	0.91	-5.66	no	yes	yes	yes	yes	not known	35 minutes

Figure 4.67: The various methods for quantification of the 6, 5, 4, 3, 2 and 1 copy per partition linearised pART7 template assayed with 35Sp LAMP-BART. The value of R^2 is the correlation to a semi-logarithmic model. The slope is the gradient of the semi-logarithmic trend line. ↔ denotes the differentiation in the correct order between two copy numbers. The number of replicates required is derived from the analysis of assay replicates and the minimum assay time denotes the time at which the required data has been collected.

Only one of the methods successfully discriminated the individual copy number concentrations, and that was the average T_{max} with outlying T_{max} values above 50 minutes removed. The summary of 10, 5, 4, 3, 2 and 1 copy results in section 4.9 showed five methods that differentiated single copies successfully. The average T_{max} with negative results was one of those and in this dataset 5 to 4 copies could not be determined. The modal T_{max} was another and in this experiment 4 to 3 and 2 to 1 copies could not be determined. The median T_{max} was the third and in this case although the correlation was good and the gradient sufficiently steep, 6 to 5 and 2 to 1 copies could not be determined. The standard deviation quantification approach was not as successful as before. The FWHM T_{max} frequency only failed to differentiate between 6 to 5 copies to match the previous success.



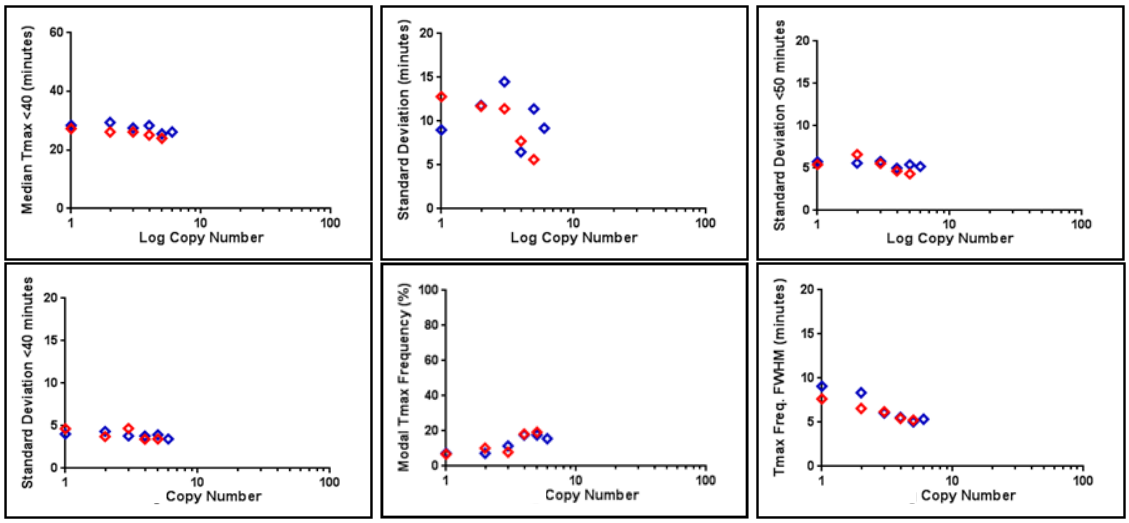


Figure 4.67: (L to R) 35Sp LAMP-BART assay of pART7 template combined results of current assays in blue with previous results from section 4.4.4 in red (1) average T_{max} (2) average T_{max} from <50 minutes T_{max} results only (3) average T_{max} from <40 minutes T_{max} results only (4) average T_{max} from all results with negative results assigned 100 minutes (5) modal T_{max} (6) median T_{max} (7) median T_{max} from <40 minutes T_{max} results only (8) standard deviation assuming normal distribution (9) standard deviation from <50 minutes T_{max} results only (10) standard deviation from <40 minutes T_{max} results only (11) percentage modal T_{max} frequency (12) FWHM of moving average of T_{max} frequency distribution

The combined data (Figure 4.67) indicates an almost parallel gradient between the T_{max} results that are approximately two minutes slower. Previous investigations into assay volume (Gandelman 2010) have shown that time to peak is independent of the assay volume and therefore this increase in time to peak is likely to be from the changes made to the LAMP-BART reaction mix.

4.11 Discussion

With the linearised plasmid target at 60 copies per partition, the reproducibility of time to peak (T_{max}) measurements was very high with a variation of only 0.8 minutes from 88 replicates. Above this copy number the reproducibility becomes extremely high and quantification of target molecules can therefore be achieved using the average T_{max} approach with a low number of repeats. Below 60 copies per partition the variability of the time to peak increases.

Before the pART7 linearised plasmid 35Sp LAMP-BART assay was optimised with improved, specific primers of higher purity, reagent batch control and the incorporation of the optimal concentration of carrier DNA, the variability at 1000 copies per partition was high in Chapter 3. Therefore improvements to the assay have improved the limit of quantification (LOQ). Optimisation of the assay has also improved the template detection to a single copy with approximately 35-40% of partitions showing amplification. The amplification frequencies for 4 and 5 copies per partition were 93%

and 100% respectively, the limit of detection (LOD) based on 95% probability approach is therefore 5 copies per partition. The LOD for the LAMP-BART assay is near to the theoretical minimum LOD for qPCR of 3 copies per partition (Bustin et al. 2009) and implies a lower amplification efficiency for the LAMP-BART assay.

The frequency distribution patterns of time to peak measurements from sixty copies per partition down to one copy per partition showed that there is a fastest time to peak value followed by a cluster of peak values of slightly slower time-to-peak values. As the copy number reduced to below thirty copies per partition a number of higher outlying T_{max} values start to appear whose frequency reflects the copy number. This suggest that a LAMP-BART assay of a defined quantity of template therefore has a minimum optimal time-to-peak value that may be delayed by a time limiting step in the LAMP amplification which would therefore appear to be a stochastic process. The minimum time to peak is termed the fastest T_{max}, which fits a semi-logarithmic model of increasing fastest T_{max} with reducing copy number. The gradient of this line is however shallow and quantification is uncertain. The fastest T_{max} value for an assay may also not be amongst the pool of T_{max} results from a number of replicates and from very low copy numbers it may represent a larger concentration of template in a particular partition due to higher stochastic variation.

The increasing variability of time to peak values with decreasing copy number on a log scale had an excellent relationship with a steep gradient for the assays between ten and one copy per partition. Therefore the standard deviation has the potential for discrimination between individual copy numbers at this level. A drawback with the standard deviation quantification approach is the requirement for the full 100 minutes assay to give a greater spread of standard deviation values. Also at low copy numbers the measurement appears to be less robust due to the reducing amplification frequency and therefore more replicates are required to give sufficient positive results.

Again from observations of the frequency distribution it was apparent that a T_{max} value was particularly favourable especially at the higher copy numbers. At low copy numbers the frequency distribution is more variable and this is coupled with reducing amplification frequency to give a less defined frequency distribution with possibly more than one highest frequency. The value of this highest frequency and its associated T_{max} time, both show interesting characteristics when correlated with copy number. The first of these, the highest frequency value, was related to the number of total partitions and expressed as a percentage. The measurement showed an increase with increasing copy number that had a good fit to the semi-logarithmic model. There was however poor discrimination between copy numbers at the lowest copy numbers and there were some anomalies from the frequency distributions. The second of these is the modal

T_{max} which is the highest frequency T_{max} value. This value represents the most favourable time to peak and is found within the main cluster of T_{max} values close to or equalling the fastest T_{max}. From the ten copies to one copy per partition data the relationship between modal T_{max} and copy number was particularly strong providing discrimination between individual copy numbers in this range. This approach to quantification does not require time to peak values after the modal T_{max} has been reached and a shorter assay can therefore be run.
Another approach that uses the main cluster of time to peak values is the median T_{max}, which takes the median time to peak value between the lowest and highest half of the positive results. This method does require the full assay time of 100 minutes because it is more reliant on the variation at low copy number to provide a sufficiently wide range of median T_{max} values to discriminate copy numbers. The quantification of one and two copy numbers per partition was undoubtedly hampered by the low number of positive results for these two; only 35 positive results for one copy and 50 positives for two.
A final investigation of the frequency distribution data looked at the morphology of the main cluster which appears to get shorter and wider as the copy numbers reduce. I used a two point averaging method to convert the column data into a curved peak and then calculated the full width half maximum (FWHM) to give a value for the changing shape. This approach only requires the main cluster of positive results and can therefore have a reduced assay time; with this 35Sp linearised pART7 plasmid assay for one copy per partition, a total assay time of only 35 minutes would be acceptable. Another benefit of this method is the independence from inter-assay variation of T_{max} times. A slower assay will not affect the frequency FWHM unless the range of the main cluster of T_{max} results is increased. As with the other methods more positive results are required at the lowest copy numbers and this can be by improving the amplification efficiency of the assay or by increasing the number of replicates.
I also investigated two aspects of the BART peaks themselves. The first of these is the average peak height which showed a downward trend with reducing copy number but was highly susceptible to a batch change of the Bst polymerase. The gradient was shallow and accompanied by high variability at each copy number. The second approach is the full width half maximum of the BART peaks. Although this method showed an increase in FWHM with reducing copy number the correlation was weak.

In the robustness testing with changes to components and conditions using the 35Sp pART7 LAMP-BART assay, the trends were apparent with the majority of techniques. The methods based on time to peak were slower and this may be a difference between using BARTmaster and preparing the BART reagent from its components fresh. The

method which produced the best fit to the previous results with 20µl total assay volume is the frequency FWHM which is less dependent on variations between assays. Poor results were recorded from the standard deviation which showed no relationship with the copy number assays. Decreasing the assay volume may require tighter control on assay variability for the ultra-quantification methods to remain valid.

Chapter 5
Digital BART

5.1 Introduction

Previous results chapters have aimed to quantify target DNA in terms of whole copy numbers per partition. The aim of this chapter was to dilute the target DNA below this level to reduce the average below one copy per partition. The number of positive results from such a dilution will give an indication of the actual number of target molecules in the partitioned sample. This approach moves away from quantifying concentrations to quantifying the number of target copies without the requirement for a calibration curve. This absolute quantification method has been successfully developed with PCR and this chapter investigates the linearised plasmid pART7 35Sp assay and genomic DNA 35Sp and ADH1 assays for the potential of this absolute quantification for LAMP amplification with the BART reporter.

5.1.1 Digital PCR

Digital PCR (dPCR) was first pioneered in 1999 by Vogelstein and Kinzler (Vogelstein and Kinzler 1999) and describes the transformation of the exponential analogue nature of the polymerase chain reaction into a linear digital approach. Through limiting dilution of the target to one copy for every two chambers, their work quantified the proportion of variants within a DNA sample and could therefore show the relative abundance of the target of interest. This highlighted the dual requirements of reliable target amplification of a single target and the limiting dilution of a DNA target.
Digital PCR is achieved by partitioning a sample prior to amplification such that each reaction chamber contains either zero copies of the target DNA or greater or equal to one copy. A Poisson correction can be factored into the results to account for chambers that contain more than one target molecule and an absolute target sequence quantity can be estimated (Dube et al. 2008). This is described in the equation:

Copies per partition = –ln ((total partition - positives) / total partitions)

5.1.2 Applications and Limitations

The detection of rare genetic variants in clinical medicine was the objective of Vogelstein and Kinzler. There have been a number of other applications for single-molecule genomic (SMG) techniques for example determining viral load for tailored point of care (PoC) antiviral medication for HIV (De Spiegelaere et al. 2013). Absolute quantitation through digital PCR has led to many studies into copy number variations (CNVs). These are abnormalities in the number of copies of a DNA sequence caused by deletions, duplications or structural arrangements of the genome. Examples of CNVs include Down's syndrome (trisomy 21) and many cancers. Microfluidics have been used to study CNVs using digital PCR (Whale et al. 2012) to a greater precision than the benchmark qPCR quantitation.

There have been developments in the platforms and partitioning of the template. The use of 96 and 384 well microtitre plates is described in this chapter. This format is ideally suited to robotics and automation. Other platforms have been developed that utilise emulsion PCR (Shao et al. 2011) to produce partitioned droplets which has been commercialised (Hindson et al. 2011). Droplet digital PCR can provide precise target quantification from a low sample volume over many partitions and has been used to detect infections such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Kelley et al. 2013) and ocular *Chlamydia trachomatis* (Roberts et al. 2013).

The requirement for the large sample dilution that is essential for digital PCR approaches has the benefit of reducing the amplification inhibition caused by some samples. However the technique is limited by the requirement for an initial assessment of target concentration to make appropriate dilutions from the sample of unknown template concentration. Digital PCR also needs every target molecule to be accurately and reliably amplified and this precise single copy number detection is difficult to achieve.

5.1.3 Digital Isothermal Amplification

There are numerous isothermal amplifications which have already been described in Chapter 1. Of these only a few have been investigated for their potential for digital amplification. These include digital multiple strand displacement (MDA) amplification (Blainey and Quake 2011)and loop-mediated amplification (LAMP). Some early success with digital LAMP has been observed (Gansen et al. 2012) with a droplet digital chip design with λ phage DNA template, and in another study using β-actin DNA

template (Zhu et al. 2012). Attempts to replicate the results of the λ-DNA assay failed to generate positive digital results (Sun et al. 2013) and there was uncertainty as to whether the DNA was preheated before LAMP amplification.

5.2 Total LAMP-BART assay volume

In the previous two chapters, the total assay volume was composed of 15µl LAMP-BART reagents and 5µl of the template in molecular grade water. This total assay volume is appropriate for the partitions of a 96 well plates but too large for 384 well digital plates. Reducing the overall volume to 5µl per partition has the benefit of reduced sample and reagent usage. Although for a digital assay the maintenance of amplification efficiency is the most important requirement from a reduction in assay volume, it is important to maintain the ultra-quantification measurements to dovetail the two quantification approaches. Therefore in these experiments the aim was to ensure amplification efficiency is not reduced by the reduced assay volume, but also to gain an insight into the variability of the time to peak results with this different assay set-up when compared with those previously recorded at low copy number. The following experiments were all conducted with the optimised 35Sp LAMP-BART assay of the linearised plasmid pART7.

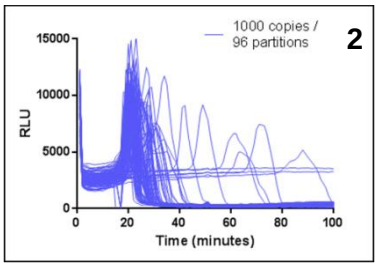
5.2.1 1000 Copies into 96 Partitions

The proportion of LAMP-BART reagents and primers was kept constant and a dilution was prepared to add the number of copies of the linearised plasmid target divided across the partitions. In the first two experiments, this quantity was 1000 copies across a 96 well plate with either 20µl or 5µl loaded to each partition. 1000 copies in 96 partitions is slightly more than ten copies per partition and is therefore in the range for ultra-quantification but may have too many positive results for digital analysis.

5.2.1.1 1000 copies 96 partitions 20µl total assay volume

1000 copies across 96 partitions, 20µl total assay volume

63	27	71	19	22	21	23	34	21	49	19	22
24	20	21	20	23	22	22	22	19	28	20	23
23	31	25	31	20	19	22	21	29	34	25	31
21	61	28	22	22	21	23	20	25	30	30	20
20	24	28	20	23	20	21	21	22	88	22	24
20	24	27	24	21	23	21	27	22	20	20	35
22	25	20	42	21	20	22	23	19	21	20	
20		22	27	28	21	27	24	21	22	21	19



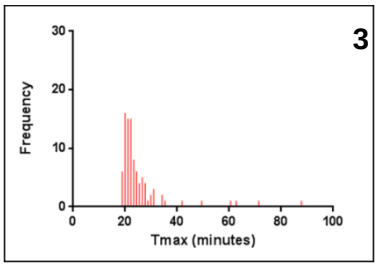


Figure 5.1: (L to R) 1000 copies pART7 template per 96 partitions, total assay volume 20µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

There were 94 positive results from a total of 96 partitions giving a success rate of 98% (Figure 5.1). The high amplification frequency puts the assay outside the range for digital quantification analysis. In Chapter 4 100% amplification frequency was observed for 10 copies per partition and this slightly higher concentration has a lower frequency. This may be due to the difference in the timing of addition of the template to the assay, insufficient mixing or clustering of template.

Using the two (Dube et al. 2008); (Huggett et al. 2013) digital PCR calculators of copy number described in section 2.8.3 gives:

1) copies per partition = –ln ((total partition - positives) / total partitions)
= –ln ((96 - 94) / 96)
= 3.87 copies per partition

2) uCount^{SM} mean: 3.87 copies per partition (95% confidence level)
(upper boundary 6.23, lower boundary 2.82)

The digital PCR calculations correct for the Poisson distribution of positive partitions; some partitions will contain one copy of the template, but others may contain more than one or no template. The calculations indicate a value of copies per partition greater than one and would imply that a further dilution of the sample is required. However for copy numbers per partition above one, ultra-quantification methods would be appropriate providing there was suitable calibration of the assay.
The time to peak (T_{max}) results are clustered after the fastest T_{max} of 18.87 minutes. The average T_{max} is 25.73 minutes with standard deviation 10.93 and coefficient of variation of 42%. From the frequency distribution data, the modal T_{max} is calculated to be 19.97 minutes with a percentage modal T_{max} frequency of 16.67. The T_{max} frequency moving average FWHM has a value of 4.67 minutes. These values are plotted in the graphs below (Figure 5.2).

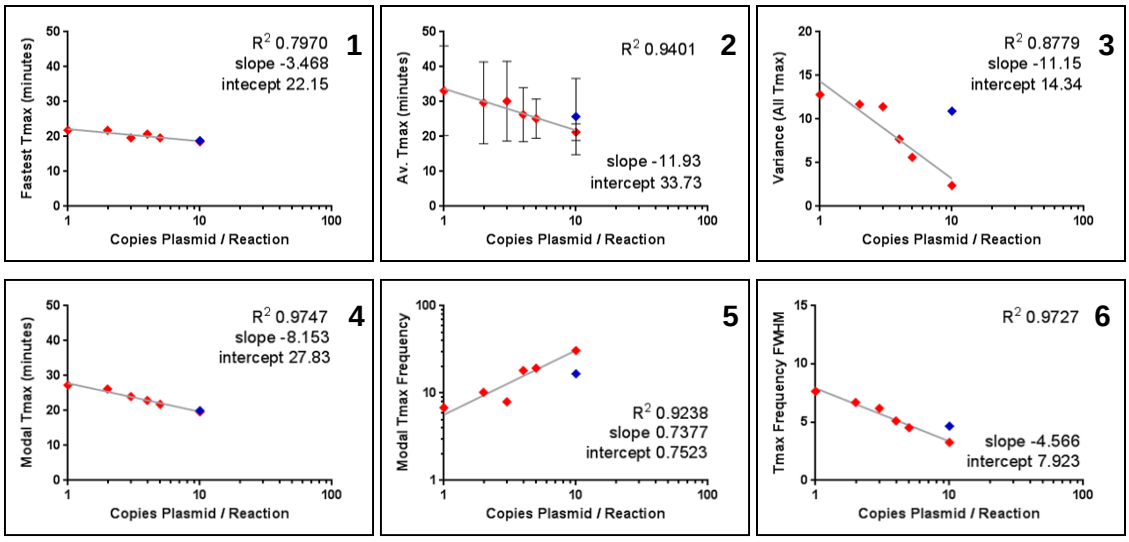


Figure 5.2: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

Some of these comparisons suggest that there are less than ten copies per partition for this assay. This is also reflected in the less than 100% amplification frequency observed and the result from the digital PCR calculations. However the fastest T_{max} and the modal T_{max} results fit closely with the previous data, and the other methods are influenced by the high variance of T_{max} results.

5.2.1.2 1000 copies 96 partitions 5µl total assay volume

1000 copies across 96 partitions, 5µl total assay volume

	22	24	25	24	20	20	19	17	34	19	24
36	20	20	24	17	17	17	17	20	19	20	17
19	21	17	16	21	19	19	21	19	17	17	17
19	16	17	19	17	17	16	17	21		17	17
23	21	15	17	19		16	17	23	19	20	
17	17	22	19	16	19	19	38	19	19	20	16
20	16	20	26	19	19	19		17		17	22
16	20	17	37	21	20	17	16	17	17	20	23

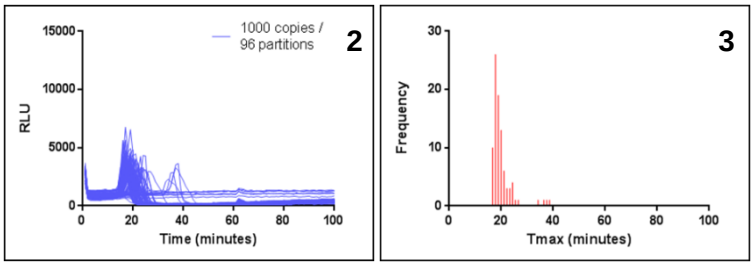


Figure 5.3: (L to R) 1000 copies per partition 96 wells, total assay volume 5µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

There were 90 positive results from a total of 96 partitions giving a success rate of 94% (Figure 5.3). This high amplification frequency puts the assay outside the range for accurate statistical analysis for digital quantification. As with the previous assay the

frequency of amplification was below 100% and may be the result of poor mixing of the template. Using a digital PCR calculation of copy number gives:

uCount^{SM} mean: 2.77 copies per partition (95% confidence level)
(upper boundary 3.76, lower boundary 2.11)

This suggests that amplification efficiency was less than 100%.
The digital PCR analysis indicates a value of copies per partition greater than one and it would be appropriate to dilute the sample further for the binary approach.
The time to peak (T_{max}) results are clustered after the fastest T_{max} of 15.28 minutes. The average T_{max} is 19.73 minutes with standard deviation 4.21 and coefficient of variation of 21%. From the frequency distribution data, the modal T_{max} is 17.46 minutes with a percentage modal T_{max} frequency of 27.08. The T_{max} frequency moving average FWHM has a value of 3.67 minutes. These values are plotted in the graphs below (Figure 5.4).

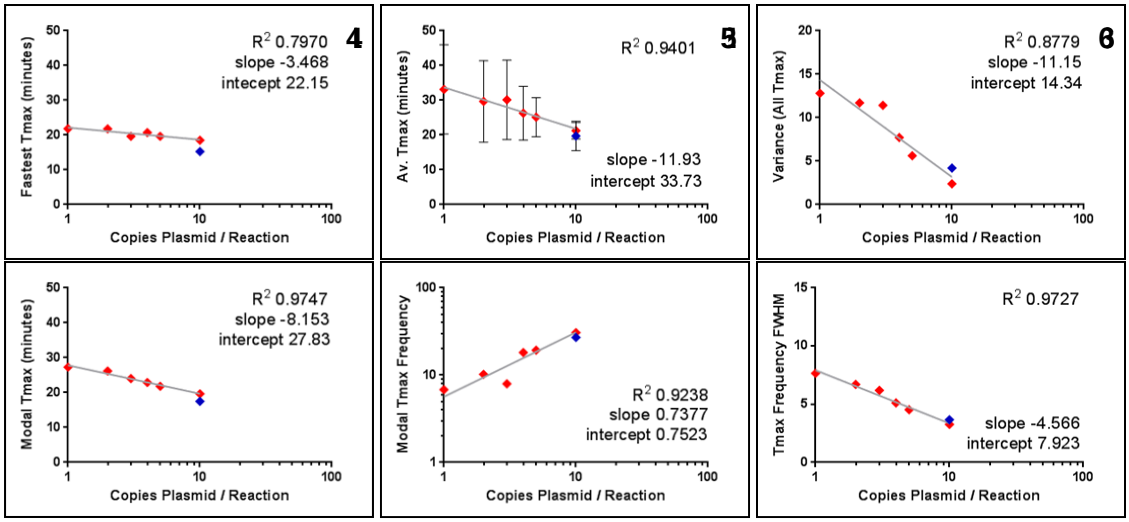


Figure 5.4: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The variance was much lower for this assay when compared to the previous one and all the correlation to the previous data is improved.
The reduction in total assay volume has caused the reduction in peak height from approximately15000 to 5000 relative luminescent units (RLU). The peaks remain easy to differentiate from the baseline, however lower total assay volumes may reduce this

difference and as a consequence BART peaks may become increasingly difficult to assign a Tmax value to.

5.2.2 100 Copies into 96 Partitions

Reducing the copy numbers to 100 into the 96 partitions which approximates to 1 copy per partition should reduce the number of positive results allowing for interpretation with digital PCR tools. From the previous assays in Chapter 4, this copy number level would reduce amplification frequency to around 35 positives from a total of 96 partitions. This indicates a below optimal amplification efficiency and this LAMP-BART assay is therefore not truly digital. Statistically there should be more than 60 positive results from a distribution of 1 copy per partition across 96 partitions allowing for one or more copies in some wells and no template in others.

5.2.2.1 100 copies 96 partitions 20µl total assay volume

100 copies across 96 partitions, 20µl total assay volume

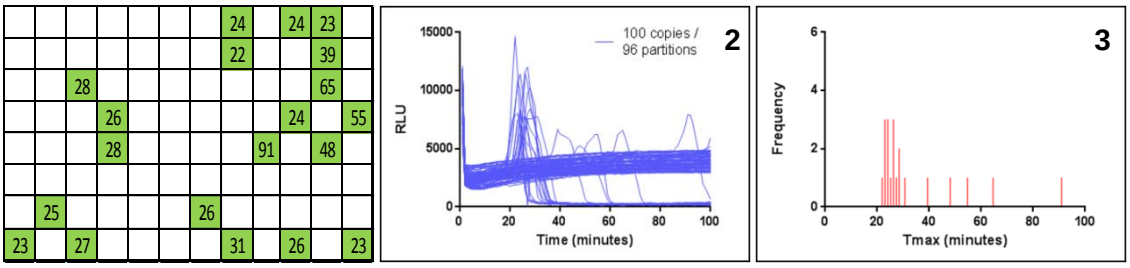


Figure 5.5: (L to R) 100 copies per partition 96 wells, total assay volume 20µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

There were 20 positive results from a total of 96 partitions giving a success rate of 21% (Figure 5.5). The low amplification frequency puts the assay in the range for statistical analysis for digital quantification and absolute quantification would be possible if the amplification efficiency was 100%. Using a digital PCR calculation of copy number gives:

uCount^{SM} mean: 0.23 copies per partition (95% confidence level)
(upper boundary 0.34, lower boundary 0.13)

The digital PCR analysis indicates a value of copies per partition less than the dilution and this is a strong indication that the amplification efficiency is below 100%. The time

to peak (T_{max}) results are clustered after the fastest T_{max} of 21.89 minutes. The average T_{max} is 33.99 minutes with standard deviation 17.79 and coefficient of variation of 52%. From the frequency distribution data, the modal T_{max} is 26.27 minutes (slowest time of three possible modes) with a percentage modal T_{max} frequency of 3.13. The T_{max} frequency moving average FWHM has a value of 5.84 minutes. These values are plotted in the graphs below (Figure 5.6).

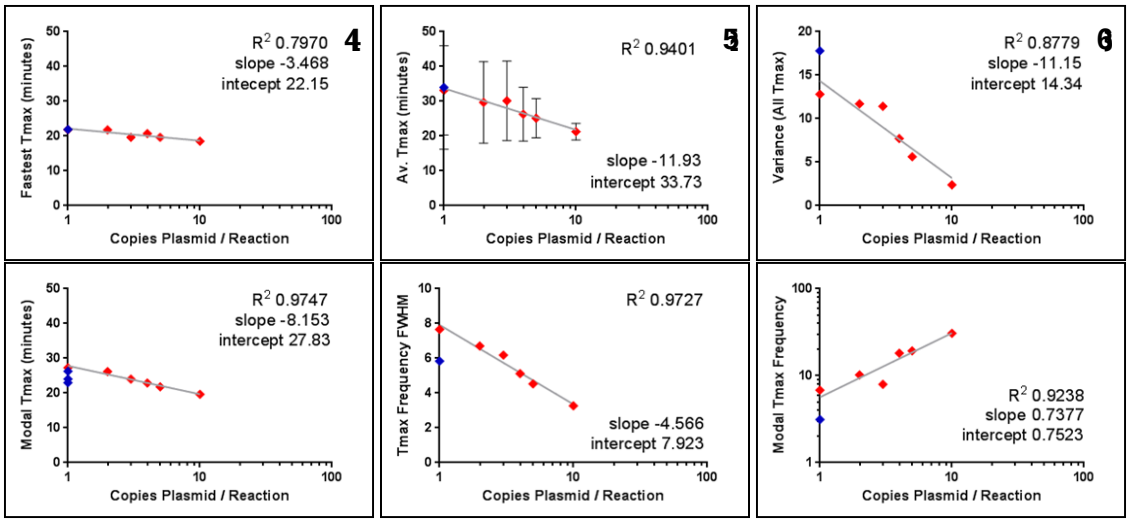


Figure 5.6: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The low number of positive results from the 96 partitions has increased the variability for some of the methods and there is insufficient data for the modal T_{max}, modal T_{max} frequency and the T_{max} frequency FWHM measurements.

5.2.2.2 100 copies 96 partitions 5µl total assay volume

100 copies across 96 partitions, 5µl total assay volume

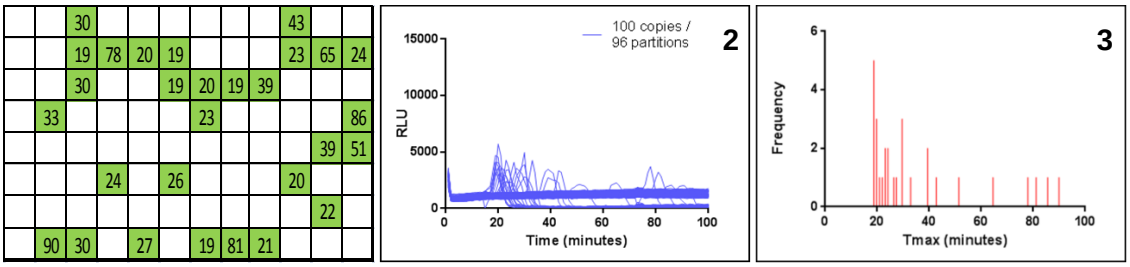


Figure 5.7: (L to R) 100 copies per partition 96 wells, total assay volume 5µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

There were 29 positive results from a total of 96 partitions giving a success rate of 30% (Figure 5.7). Therefore at this limiting dilution of the template the reduced assay volume hasn't reduced the amplification frequency. The number of positive results was too low for ultra-quantification approaches and therefore 384 partitions would improve on this. Using a digital PCR calculator of copy number gives:

uCount^{SM} mean: 0.36 copies per partition (95% confidence level)
(upper boundary 0.50, lower boundary 0.23)

The digital PCR analysis indicates a value of copies per partition less than one. Either the amplification efficiency needs to be increased or kept constant and factored into the calculations.

The time to peak (T_{max}) results are clustered after the fastest T_{max} of 18.70 minutes. The average T_{max} is 35.79 minutes with standard deviation 22.29 and coefficient of variation of 52%. From the frequency distribution data, the modal T_{max} is 18.70 minutes with a percentage modal T_{max} frequency of 5.21. The T_{max} frequency moving average FWHM has a value of 5.67 minutes. These values are plotted in the graphs below (Figure 5.8).

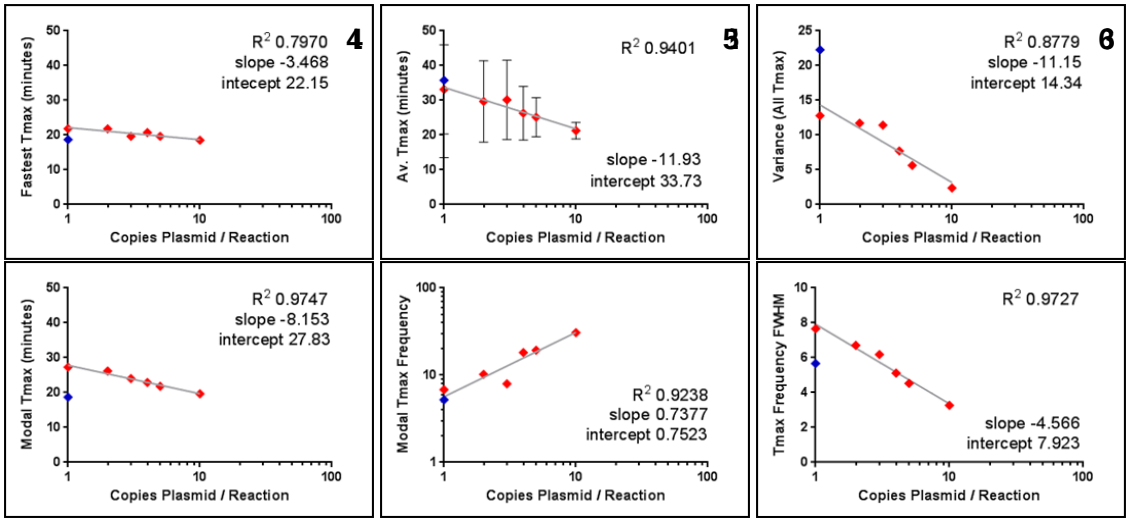


Figure 5.8: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

Twenty nine positive results is an improvement on the previous assay but remains indicative of a sub-optimal assay. The time to peak data is again faster than the data from the higher total assay volume but there are insufficient positive results at this copy number.

The amplification frequency for these two assays is between 33% and 50% of expected for single copy detection per partition.

5.2.3 10 Copies into 96 Partitions

In these two assays there were ten copies spread across the 96 partitions. This created a large number of negative partitions and any positive results would be expected to tend towards single copies of the template. The uCount^{SM} software predicts between 9 and 10 positive results indicating that one partition may have one or two copies of the template and the others will have one copy. As the number of positive partitions decreases so does the likelihood of two copies in a partition.

5.2.3.1 10 copies, 96 partitions, 20µl total assay volume

10 copies across 96 partitions, 20µl total assay volume

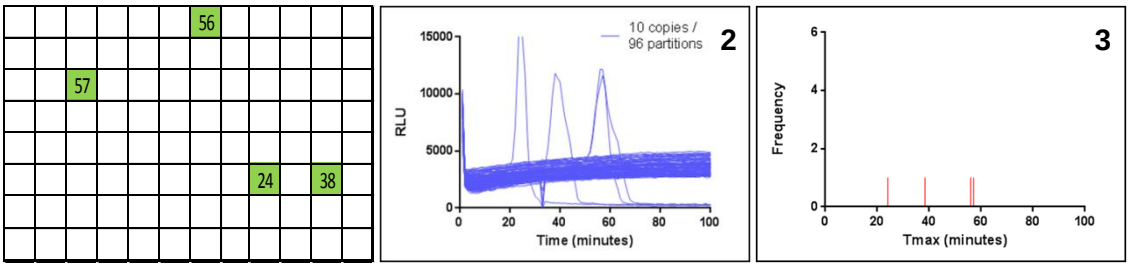


Figure 5.9: (L to R) 10 copies per partition 96 wells, total assay volume 20µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the spread of T_{max} values after the fastest T_{max}

There are 4 positive results from a total of 96 partitions giving a success rate of 4% (Figure 5.9). The low amplification frequency puts the assay in the range for digital analysis only; there is insufficient data to make inferences from the time-to-peak results. Using a digital PCR calculator of copy number gives:

uCount^{SM} mean: 0.043 copies per partition (95% confidence level)
(upper boundary 0.085, lower boundary 0.001)

The result of the digital calculation is four copies in the dilution volume which was prepared to contain ten copies. The upper boundary of the 95% confidence is eight copies in the dilution. The discrepancy may be due to sub-optimal amplification efficiency, loss of copy numbers during the dilution steps, slow amplification initiation

leading to a positive result beyond the scope of the assay time or perhaps from the unavailability of the target for amplification. The digital calculation does suggest that each of the positive LAMP-BART peaks represents a single copy in that partition. It is interesting how the time to peak varies for these single copies and shows that variance at low copy number is a feature of LAMP-BART assays.

The time to peak (T_{max}) results are clustered after the fastest T_{max} of 24.07 minutes. The average T_{max} is 43.88 minutes with standard deviation 15.72 and coefficient of variation of 36%. The dataset of positive results is too small to calculate modal T_{max}, percentage modal T_{max} or T_{max} frequency moving average FWHM values.

The calculated time to peak values are plotted in the graphs below (Figure 5.10).

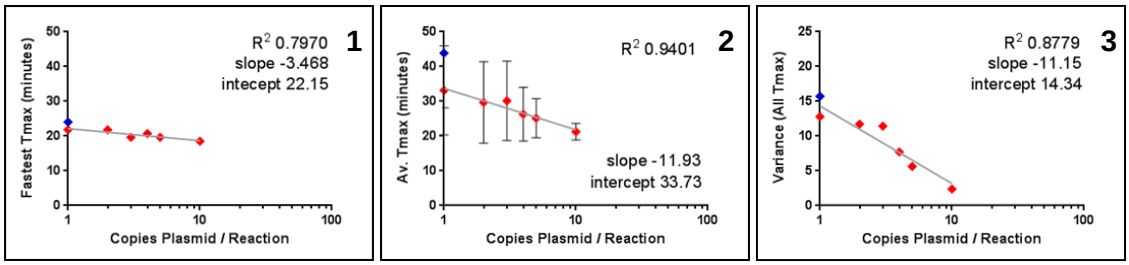


Figure 5.10: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation

From this small pool of data the fastest T_{max} result is unlikely to represent the optimum initiation, amplification and detection by BART. The average T_{max} is high due to the lack of clustered T_{max} results after the fastest T_{max}.

5.2.3.2 10 copies, 96 partitions, 5µl total assay volume

10 copies across 96 partitions, 5µl total assay volume

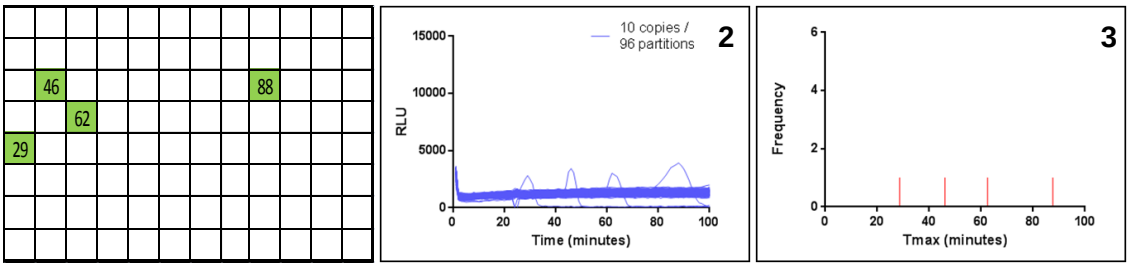


Figure 5.11: (L to R) 10 copies per partition 96 wells, total assay volume 5µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the spread of T_{max} values after the fastest T_{max}

There were again 4 positive results from a total of 96 partitions giving a success rate of 4% (Figure 5.11). As with the previous assay the low amplification frequency puts the assay in the range for digital analysis only; there is insufficient data to make inferences from the time to peak results. Using a digital PCR calculator of copy number gives:

uCount^{SM} mean: 0.043 copies per partition (95% confidence level)
(upper boundary 0.085, lower boundary 0.001)

Again, each of the positive LAMP-BART peaks represents a single copy in that partition and the peaks were spread from 29 to 88 minutes.
The time to peak (T_{max}) results are clustered after the fastest T_{max} of 28.63 minutes. The average T_{max} is 56.20 minutes with standard deviation 25.09 and coefficient of variation of 45% (Figure 5.12). Again the dataset of positive results is too small to calculate modal T_{max}, percentage modal T_{max} or T_{max} frequency moving average FWHM values.

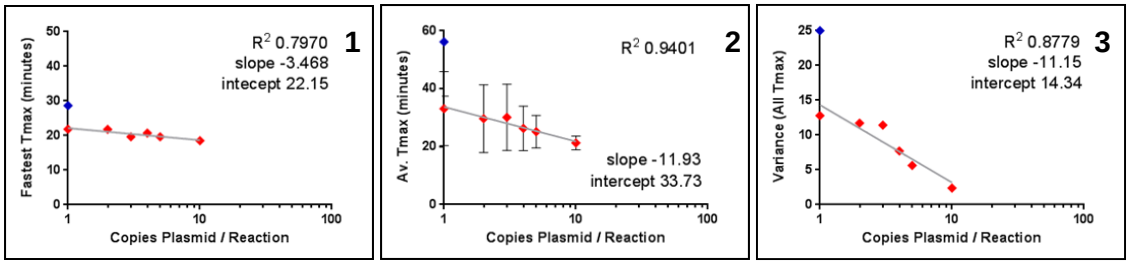


Figure 5.12: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation

These assays with ten and a hundred copies across 96 wells show that there is no loss of amplification frequency with reduced assay volume at dilutions appropriate for digital analysis. The high variance observed for these assays is a concern if ultra-quantification is to be used for concentrations greater or equal to 1 copy per partition and more efficient mixing will require investigation.

5.3 Primer Optimisation

The optimised primer set used for the CaMV 35S promoter target sequence in the pART7 template has a redesigned B3 displacement primer (version 3). The 35Sp target sequence in the maize MON810 event genomic DNA does not have the mismatches to the reference sequences and can therefore use the original version of

the B3 primer. However the version 3 primer was also designed to match the length and melting temperature of the F3 version 3 displacement primer and therefore there is a requirement for a B3 version 4.

5.3.1 Redesign of CaMV 35Sp B3 displacement primer for genomic template

A comparison was made of the two versions of the B3 displacement primer appropriate to the 35Sp target sequence in the maize genomic DNA from event MON810 (Figure 5.13). The aim of the experiment was to show that the new version did not cause primer dimer products in the NTCs, achieves low copy number sensitivity and maximises amplification frequency at low copy number. For the digital assay, single copy detection would be advantageous.

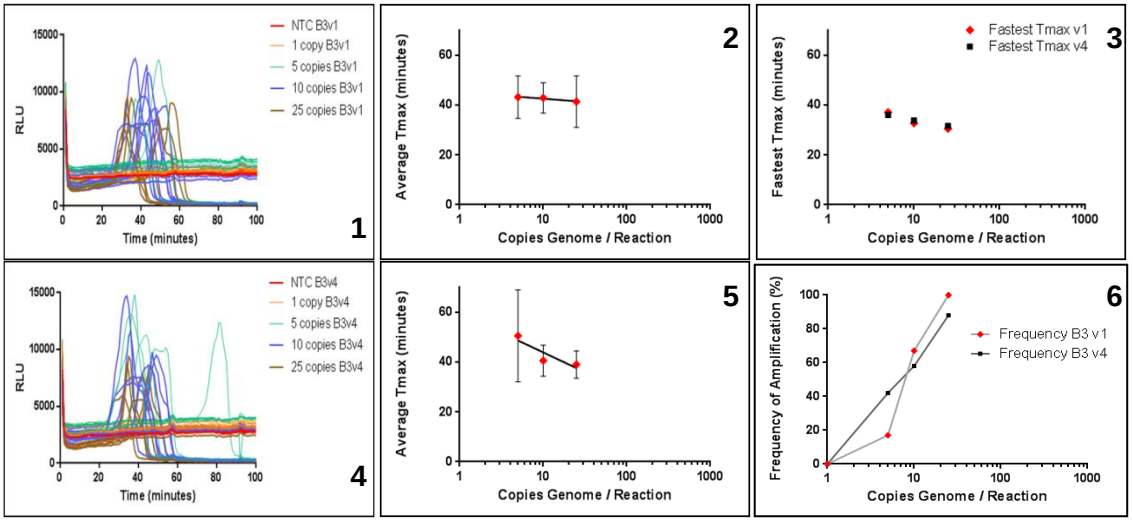


Figure 5.13: (L to R) CaMV 35Sp LAMP displacement primer B3 versions 1 and 4 (1) BART peaks for 25 copies in brown, 10 copies in blue, 5 copies in green and 1 copy of genomic DNA in orange for B3 version 1. NTC in red (2) average T_{max} for B3 version 1 (3) comparison of fastest T_{max} (4) BART peaks for 25 copies in brown, 10 copies in blue, 5 copies in green and 1 copy of genomic DNA in orange for B3 version 4. NTCs in red (5) average T_{max} for B3 version 4 (6) comparison of amplification frequency with B3 version 1 in red and B3 version 4 in black

The assays with the new B3 displacement primer and the old version both had NTCs free from evidence of primer dimer. Both assays successfully detected some of the partitions containing 5 copies of the genomic template but none of the 1 copy partitions. The amplification frequency for the 5 copies per partition assay which included the B3 displacement primer version 4 was over twice that of the old version. Therefore B3 version 4 was selected for subsequent 35Sp LAMP-BART genomic template assays.

5.3.2 Mismatching of displacement primer to target sequence

The LAMP displacement primer B3 for the 35Sp assay was redesigned due to the mismatches with the pART7 sequences. The aim of the experiments in this section was to investigate the effect of these mismatches on amplification frequency at very low copy number by using the new displacement primer B3 version 4 in the assay of the linearised pART7 plasmid.

5.3.2.1 1000 copies, 96 partitions, 5µl total assay volume

In the first experiment, 1000 copies were spread across a 96 well plate at 5µl total assay volume using the 35Sp primer set with mismatching displacement primer B3 version 4 (Figure 5.14). The presence of this number of target molecules in each partition should ensure a high amplification frequency. A reduction in the number of positive results would imply that the mismatched primer was interfering with amplification. It has been shown before that the BIP LAMP primer can displace at amplification initiation and therefore amplification would be able to progress albeit with slower time to peak times.

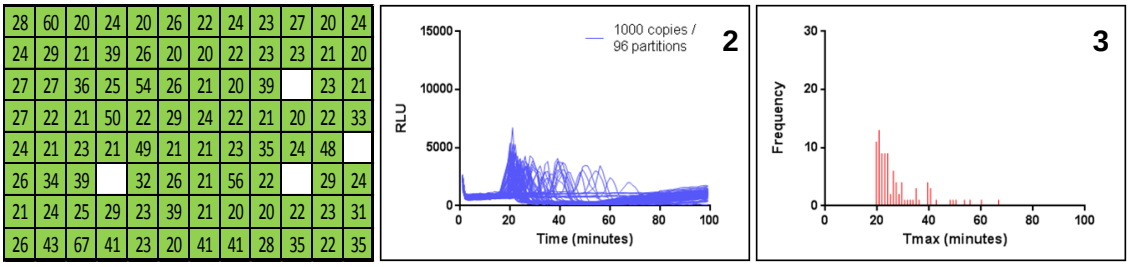


Figure 5.14: (L to R) 1000 copies pART7 template per 96 partitions, total assay volume 5µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

There are 92 positive results from a total of 96 partitions giving a success rate of 96%. This is slightly higher than the success rate achieved with the optimised primer set at this total assay volume.

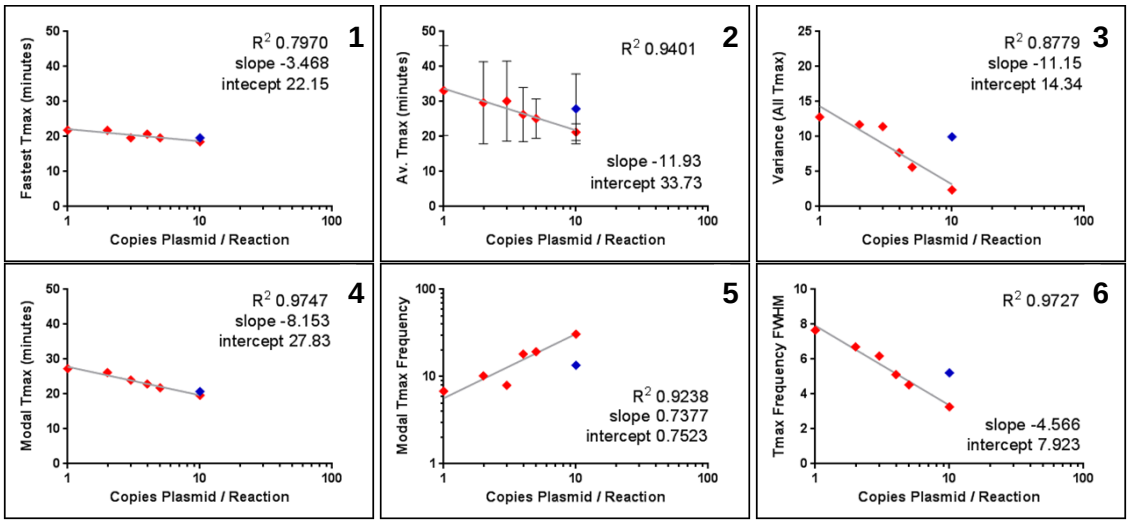


Figure 5.15: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The light output from positive results against time indicates an average T_{max} of 27.94 minutes with standard deviation 9.96 and coefficient of variation of 36%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 19.63 minutes with the modal T_{max} at 20.72 minutes.

The time to peak measurements are all slower than those recorded at the same total assay volume with the optimised set of primers (Figure 5.15). The function of the B3 displacement primers in the initiation of amplification is therefore compromised with greater reliance on the BIP LAMP primer for displacement.

5.3.2.2 100 copies, 96 partitions, 5µl total assay volume

The second experiment assayed 100 copies across a 96 well plate at the 5ul total assay volume using the 35Sp primer set with mismatching displacement primer B3 version 4 (Figure 5.16). At this reduced copy number per partition the impact of the mismatched displacement primer would be expected to be more pronounced in its effect on the initiation of amplification and the subsequent time to peak values. This is because slow initiation of one or two copies at this level is not masked by the rapid initiation of amplification of others in that partition which may be the case at higher copy numbers.

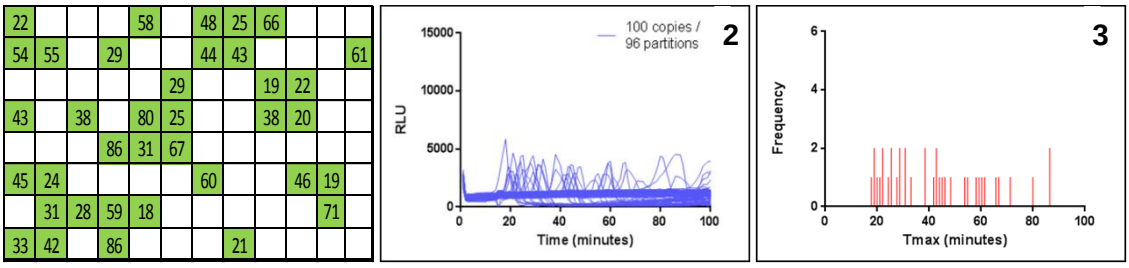


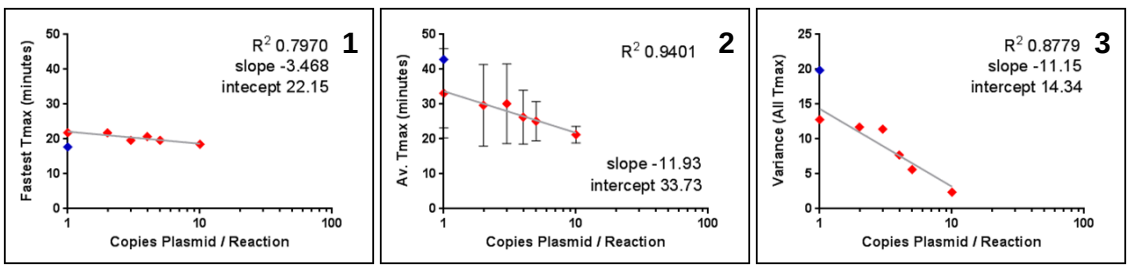
Figure 5.16: (L to R) 100 copies per partition 96 wells, total assay volume 5µl (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

There were 37 positive results from a total of 96 partitions giving a success rate of 39%. This is higher than previously seen with the optimised set of primers at this total assay volume. One explanation may be an increased number of non-specific interactions due to the mismatched B3 primer. However there did seem to be a high degree of variability in amplification frequency that requires further investigation to control. It is uncertain whether this is an issue with mixing, variability from the sample dilution or from stochastic variation. A stable amplification frequency will be crucial for digital BART.

The light output from positive results against time indicates an average T_{max} of 42.87 minutes with standard deviation 19.86 and coefficient of variation of 46%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 17.73 minutes with a modal T_{max} at 18.82 minutes (fastest time of eight possible modes).

The frequency distribution data lacks the clustering of T_{max} values after the fastest T_{max} that is a familiar feature of these LAMP-BART assays. The highest frequency is only two results and as a consequence there are eight possible modal T_{max} values. With the increased number of positive results observed with this assay over the previous optimised primer set assay, a higher frequency for one of the times would be expected. Therefore the mismatched primer is causing a wide spread of time to peak values due to a more variable initiation of amplification.

<u>Compatibility with previous data from the ultra-quantification chapter</u>



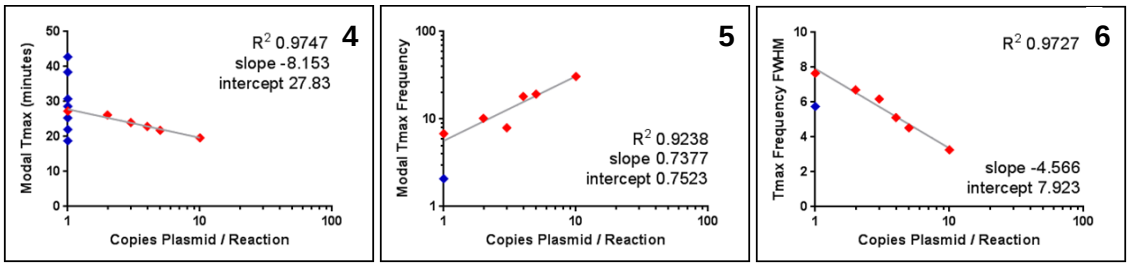


Figure 5.17: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The time to peak values are slower with the exception of the fastest T_{max} which is one time integral faster than the previous optimal primer set assay at this total assay volume (Figure 5.17).

5.3.2.3 10 copies, 96 partitions, 5µl total assay volume

The final experiment used 10 copies of the pART7 linearised plasmid template across 96 partitions at 5ul total volume per partition with the 35Sp primer set with mismatching displacement primer B3 version 4 (Figure 5.18).

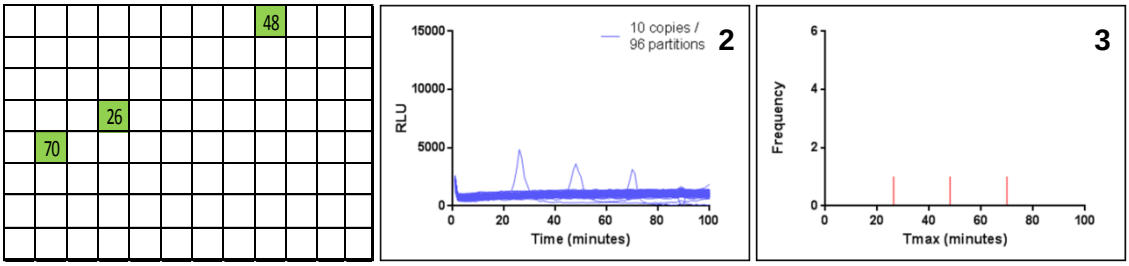


Figure 5.18: (L to R) 10 copies per partition 96 wells, total assay volume 5µl, mismatched displacement primer. The graphic shows the spread of positive results in green and the corresponding T_{max} values.

There were 3 positive results from a total of 96 partitions giving a success rate of 3%. The previous assay with the optimised set of primers showed 4 positive results across the 96 well plate. The assay shows that the mismatches did not prevent the detection of single copies of the template. The reduced frequency of amplification was minor and may well be stochastic.

The light output from positive results against time indicates an average T_{max} of 48.11 minutes with standard deviation 21.82 and coefficient of variation of 45%. The

frequency distribution of T_{max} results shows the fastest T_{max} of 26.29 minutes and the absence of a clear modal T_{max} (Figure 5.19).

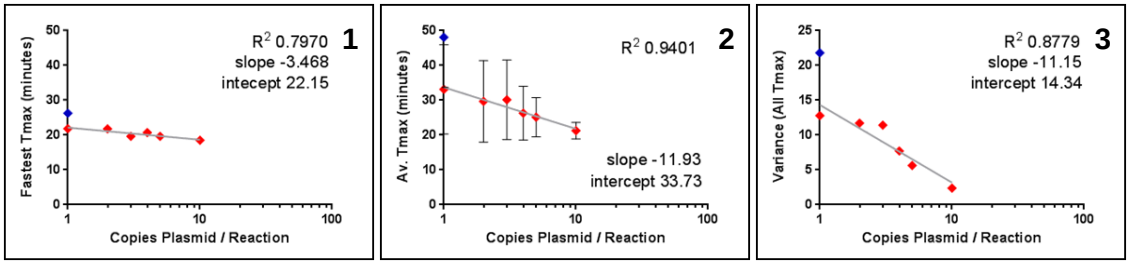


Figure 5.19: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation

The fastest T_{max} result is unlikely to represent the optimum initiation, amplification and detection by BART. The average T_{max} is high due to the lack of clustered T_{max} results after the fastest T_{max}. The low number of positive results prevents comparison with previous results.

5.3.3 Redesign of maize ADH1 reference gene primers

In the previous section (5.3.2) the effect of primer mismatches was investigated with 35Sp primers and pART7 template. In this section the maize ADH1 target sequence was assessed for mismatches with the LAMP primers.

A pair of PCR primers to produce an amplicon of the ADH1 gene in maize event Mon810 was designed. The sequenced amplicon was compared to the GenBank accession: NM_001111939.1 reference sequence and there were no mismatches.

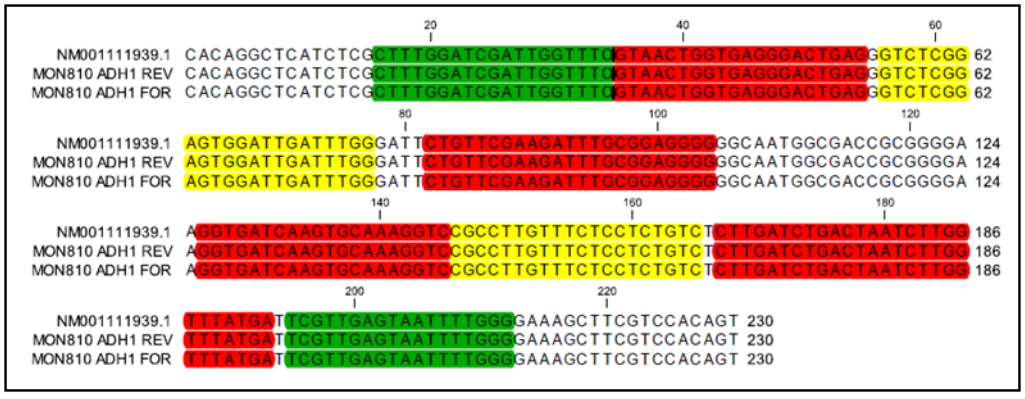


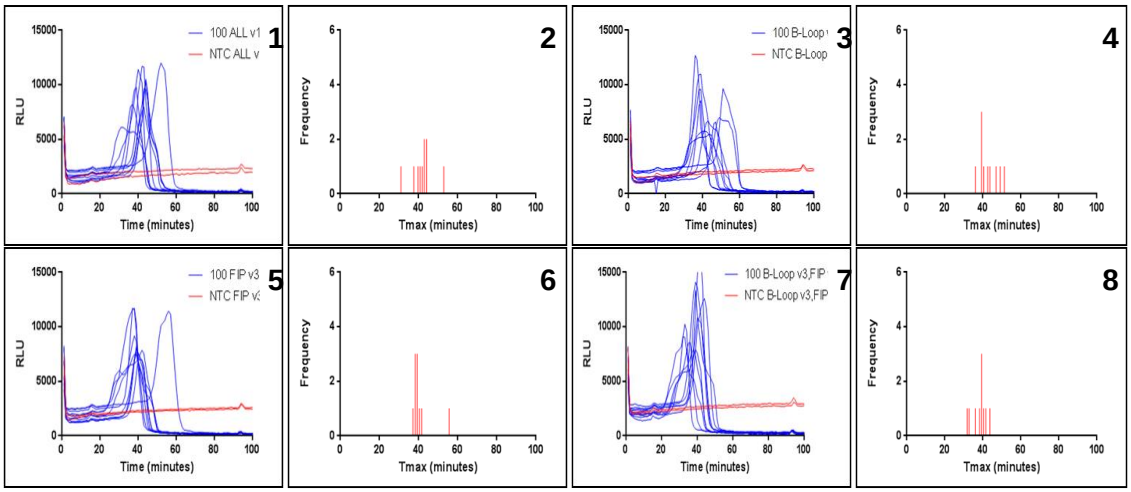
Figure 5.20: Schematic of the position of LAMP primers for the ADH1 maize reference gene (displacement primers in green, LAMP primers in red and LOOP primers in yellow).

However the ADH1 original set of primers has mismatches in the primers FIP and B-Loop (Figure 5.20). The position of these single mismatched nucleotides is at least five nucleotides away from the 3' end, but any mismatch may have an effect on the amplification efficiency which is important for digital assays.

Corrections for the mismatches and alterations to the length, GC content and melt temperature were made for the FIP and B-Loop (known as version 3). The new primers were combined with the original primers in combinations to assay 100 copies per partition of the ADH1 reference gene in the maize event MON810.

5.3.3.1 ADH1 LAMP-BART assay reproducibility

The aim of this first experiment was to firstly ensure that the NTCs remain clear of non-specific primer interactions and contamination, but also to maintain amplification frequency at this copy number and to assess the variation between replicates for the primer combinations. All the assays were run on a single plate to control inter-assay variation (Figure 5.21).



copies	v3 primers	mean	SD	cv	s/rate
100	None	41.40	5.52	13%	100%
100	Bloop	42.82	4.92	11%	100%
100	FIP	40.74	5.39	13%	100%
100	Bloop FIP	38.24	3.74	10%	100%

9

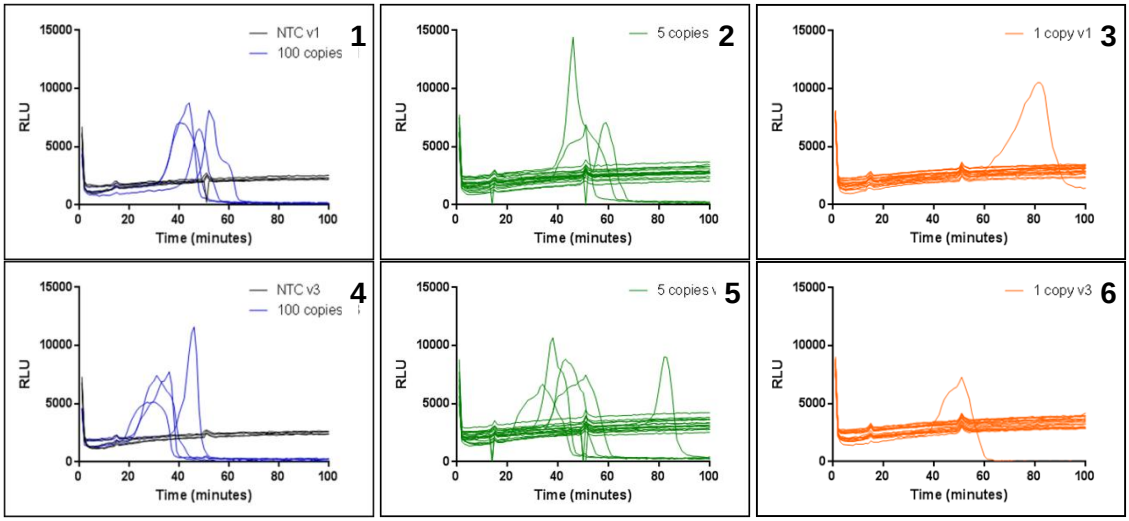
Figure 5.21: ADH1 LAMP-BART assay of 100 copies per partition maize genomic DNA from Mon810 seeds extracted with Promega Wizard kit and rehydrated with TE buffer (1) ADH1 primers version 1, time to peak BART curves (2) frequency distribution of T_{max} results (3) ADH1 B-Loop version 3, time to peak BART curves (4) frequency distribution of T_{max} results (5) ADH1 FIP version 3, time to peak BART curves (6) frequency distribution of T_{max} results (7) B-Loop and FIP version 3, time to peak BART curves (8) frequency distribution of T_{max} results (9) summary table of results

All assays with the combination of ADH1 primers successfully amplified from all ten partitions containing a calculated 100 copies of target sequence in maize genomic DNA. The NTCs were free from contamination and non-specific primer interactions.

The two primers, redesigned for the ADH1 sequence mismatches, resulted in similar results for average T_{max} and variation as the original primers. However the combination of the two new primers showed improved average T_{max} by over three minutes, and the standard deviation was the lowest of these assays.

5.3.3.2 ADH1 LAMP-BART assay sensitivity

The combination of ADH1 primers with FIP and B-Loop version 3, was tested further against the original set to determine amplification frequency at 5 and 1 copies per partition of the genomic template (Figure 5.22).



copies	v3 primers	mean	SD	cv	s/rate
100	None	46.41	5.34	12%	100%
5	None	52.44	6.72	13%	15%
1	None	82.46			5%
100	Bloop FIP	35.68	7.50	21%	100%
5	Bloop FIP	49.90	19.35	39%	25%
1	Bloop FIP	51.78			5%

7

Figure 5.22: ADH1 LAMP-BART assay of 100 copies per partition maize genomic DNA from MON810 seeds extracted with Promega Wizard kit and rehydrated with TE buffer, ADH1 primers version 1 (1) 4 from 4 at 100 copies per partition (2) 3 from 12 for 5 copies per partition (3) 1 from 12 for 1 copy per partition, ADH1 B-Loop and FIP version 3 (4) 4 from 4 at 100 copies per partition (5) 5 from 12 for 5 copies per partition (6) 1 from 12 for 1 copy per partition (7) summary table of results

The amplification frequency for 100 copies is 100% and the NTCs remain clear. Both primer combination assays achieved single copy detection of the maize genomic ADH1

reference gene target. The average T_{max} values for all the copy numbers assays using the version 1 primers alone were slower than those seen with the two ADH1 version 3 primers in the combination. There is a slightly increased amplification frequency for the 5 copies per partition associated with the new primer combination.

5.4 Digital Assays – 88 partitions

The experiments in section 5.2 (total LAMP-BART assay volume) used the optimised 35Sp primers and linearised pART7 template mixed with the LAMP-BART mastermix before addition to the plate. In Chapter 4 the LAMP-BART mastermix and primers were added to the plates followed by the addition of a volume of the target concentration. In this assay the method from Chapter 4 was used and 5µl of the dilution containing 10 copies was added to the partitions already containing 15µl of LAMP-BART mix (Figure 5.23).

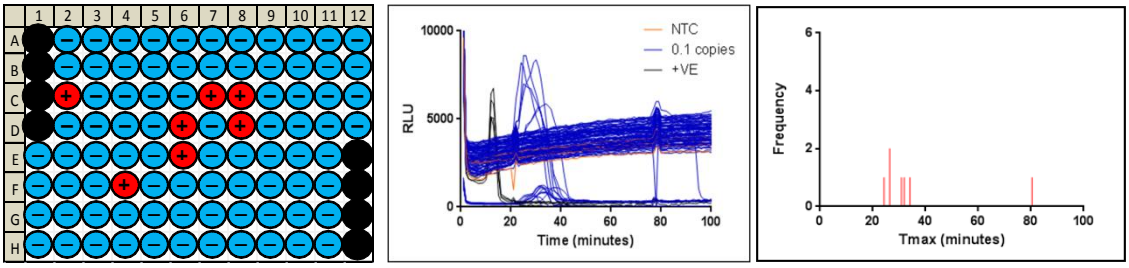


Figure 5.23: (L to R) 0.1 copies per partition 88 wells. The graphic shows the spread of positive results in red (negative results in blue). There are 7 positive results from a total of 88 partitions.

The number of positive results from this assay was greater than previously seen with the target/LAMP-BART mixing before loading. It implies near 100% amplification efficiency.

uCount^{SM} mean: 0.083 copies per partition (95% confidence level)
(upper boundary 0.145, lower boundary 0.023)

The BART peaks indicate an average T_{max} of 35.59 minutes with standard deviation 19.64 and coefficient of variation of 55%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 24.27 minutes with the modal T_{max} at 25.37 minutes. The T_{max} measurements are plotted in the graphs below (Figure 5.24):

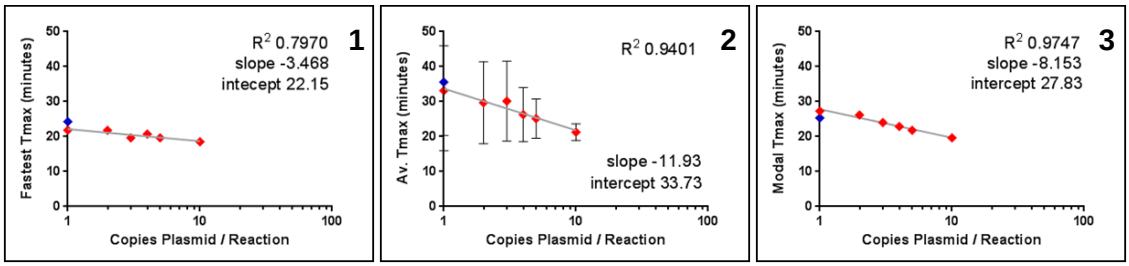


Figure 5.24: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) modal T_{max}

Although there are only seven positive results the time to peak data measurements closely correlate with the data from the ultra-quantification assays in Chapter 4 which were set up in a similar way. The interpretation assumes that the positive results are from single copies per partition.

The amplification frequency was higher than previously observed, and may indicate variability introduced by adding template to the LAMP-BART mix in the partition, since this requires a very dilute sample of 0.02 copies per µl. Adding template as part of a combined mix LAMP-BART preparation to achieve this copy number per partition requires much fewer dilution steps. Therefore for digital BART the method that used the fewest dilution steps will be used.

5.5 Digital Assays – 384 partitions

384 partition LAMP-BART experiments, as described Chapter 2, were investigated to assess the potential of digital BART using conventional digital PCR analysis techniques. The 35Sp LAMP-BART assay of linearised pART7 template and genomic template, and the ADH1 LAMP-BART assay of genomic template have previously shown in section 5.3, suitability for digital BART due to single copy number detection. The first of these experiments compares the low copy number assay results from Chapter 4 on 96 well plates in the Cardiff laboratory with 100 copies of the pART7 template using the 35Sp primers in Lumora with 384 well plates and robotic loading of 5µl per partition.

5.5.1 35Sp multiple copies per partition

One hundred copies per partition of the pART7 linearised plasmid with the 35Sp LAMP-BART assay was used to investigate the variance associated with robotic plate loading and to compare time to peak extrapolated results from Cardiff (Figure 5.25).

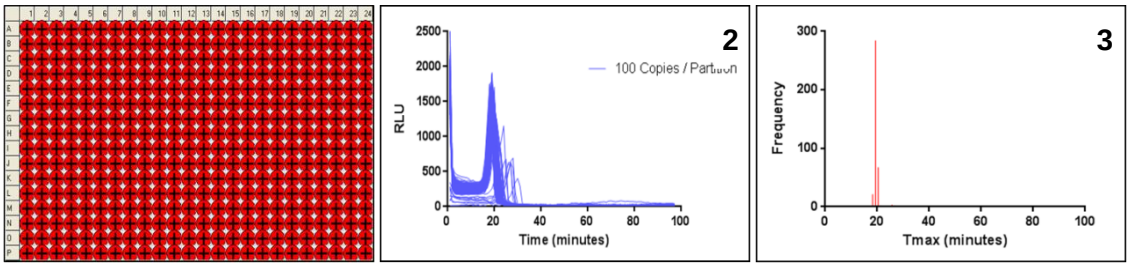


Figure 5.25: (L to R) 100 copies per partition 384 well plate, total assay volume 5µl (1) table to show the spread of positive results in red and negative results in blue (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

There were no loading errors from the robot across the 384 well plate and there is a remarkable reproducibility between partitions shown by the BART curves and the frequency distribution data.

The 384 positive partitions give a success rate of 100%. The average T_{max} of 19.62 minutes has a standard deviation of 1.21 and coefficient of variation of 6%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 18.23 minutes. The modal T_{max} is 19.31 minutes and the frequency for the mode is 284 giving a percentage modal frequency of 73.96. The frequency moving average FWHM is 2.24 minutes. These results are plotted on the graphs below (Figure 5.26) to show the comparison with the previous measurements in the Cardiff lab:

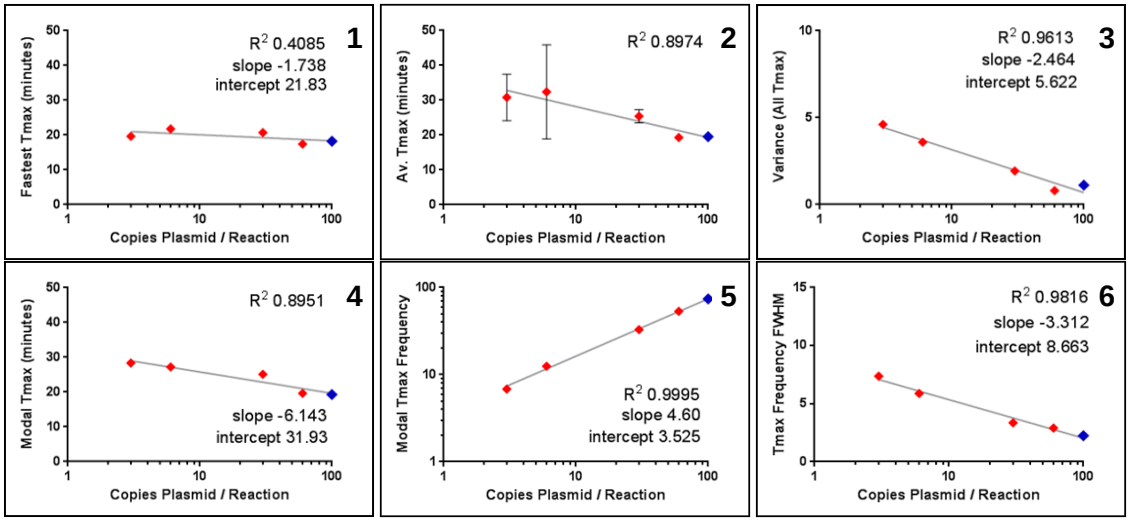


Figure 5.26: (L to R) previous data from Chapter 4 showing the results from 60, 30, 6 and 3 copies per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The new data from the 384 plates, in which other components were also different including BARTmaster, isothermal buffer, robotic loading and the BISON detection platform, shows remarkable correlation with the earlier 96 well ultra-quantification data

from 60, 30, 6 and 3 copies per partition. This suggests remarkable robustness of the assay.

5.5.2 35Sp single copy per partition

In the next set of experiments, one copy per partition duplicate 35Sp pART7 template assays were set up to firstly aim to achieve the 63% amplification frequency required to indicate 100% amplification efficiency statistically, secondly to establish a consistent amplification frequency and thirdly to compare the time to peak results with the results from Cardiff.

5.5.2.1 Linearised plasmid template

In this first assay the BART mix was composed of BARTmaster with the NEB Isothermal Buffer. The 35Sp primers were not denatured and were added at twice the standard concentration. A fresh aliquot of the pART7 linearised plasmid DNA was initially titrated to 10 copies/µl. The aim was to minimise the number of dilution steps and to add the template directly to the LAMP-BART mix.

5.5.2.1.1 Digital Assay 1

A strong correlation with the ultra-quantification data has already been observed using 100 copies per partition assay. This experiment aimed to determine if this was also observed at 1 copy per partition, providing more accurate data for ultra-quantification at this level (Figure 5.27).

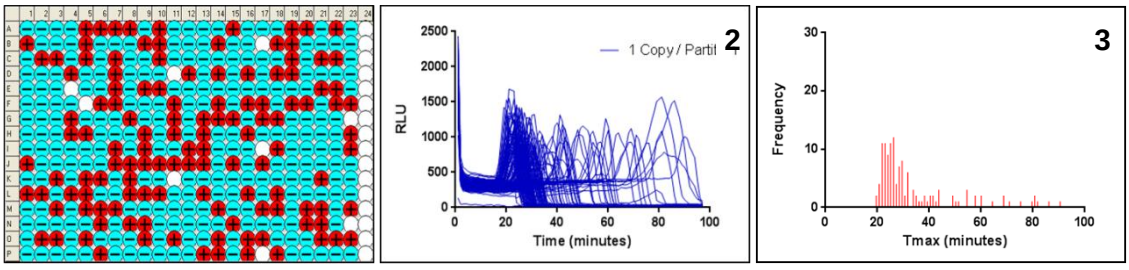


Figure 5.27: (L to R) 1 copy per partition 384 well plate, total assay volume 5µl (1) table to show the spread of positive results in red and negative results in blue (white circles indicate loading errors by the robotic injection system and plate misalignment) (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

The figure that shows the spread of positive results across the 384 wells plate also shows a number of white circles. These indicate wells without reagent and therefore the column on the right was the result of the misalignment of the detection software (which was subsequently corrected) and the other white circles were a loading error after every 41 partitions by the robotic injection system. Adjacent wells were unaffected by misalignment or loading errors.
There were 127 positive results from a total of 359 partitions giving a success rate of 35%. Using the digital PCR calculators of copy number gives:

uCount^{SM} mean: 0.437 copies per partition (95% confidence level)
(upper boundary 0.515, lower boundary 0.361)

The 35% frequency of amplification is in the range of previously calculated results for this LAMP-BART assay at single copy numbers. It does represent suboptimal amplification efficiency but if this efficiency is constant then digital calculations can be adjusted appropriately.

The peak of light output against time indicates an average T_{max} of 33.85 minutes with standard deviation 16.36 and coefficient of variation of 48%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 19.30 minutes. The modal T_{max} is 25.74 minutes and the frequency for the mode is 12 giving a percentage modal frequency of 3.34. The frequency moving average FWHM is 8.87 minutes. These results are plotted on the graphs below (Figure 5.28) to show the comparison with the previous measurements in the Cardiff lab:

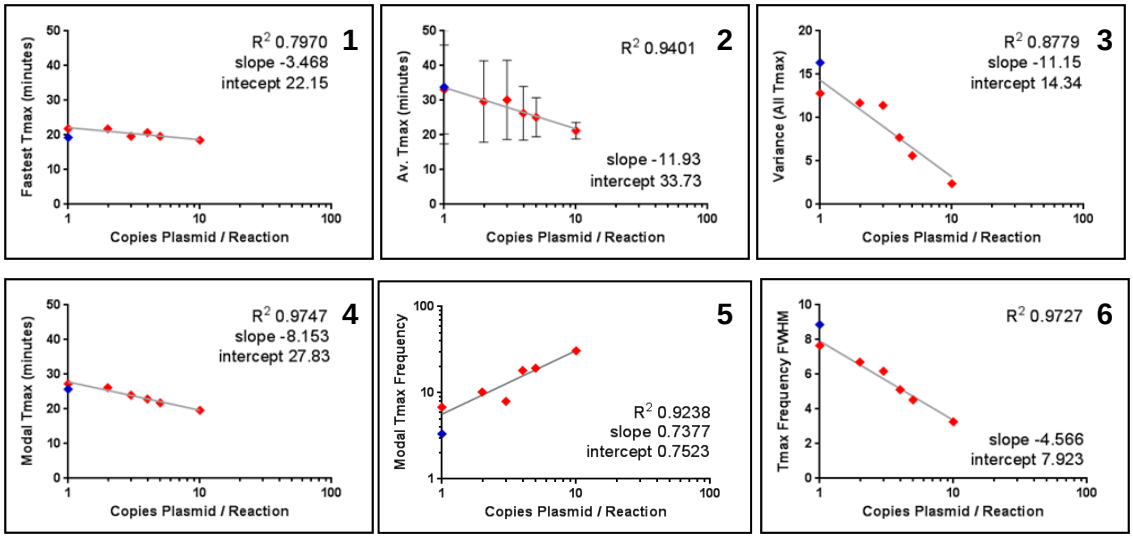


Figure 5.28: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The greater number of positive results (127 in comparison to approximately 35 from a 96 well plate) at one copy per partition has produced time to peak based values that fit well with discriminating one from two copies on the graphs above. The exception to that is the low value for the fastest T_{max} which may be a more accurate result from the larger dataset.

5.5.2.1.2 Digital Assay 2

This experiment is a repeat of the previous one with the continued aim of achieving consistent or maximum amplification efficiency for digital BART (Figure 5.29). Although the experiment was set up in the same way there was a delay of approximately 25 minutes from the addition of the mineral oil to the start of the assay at 60°C on the BISON.

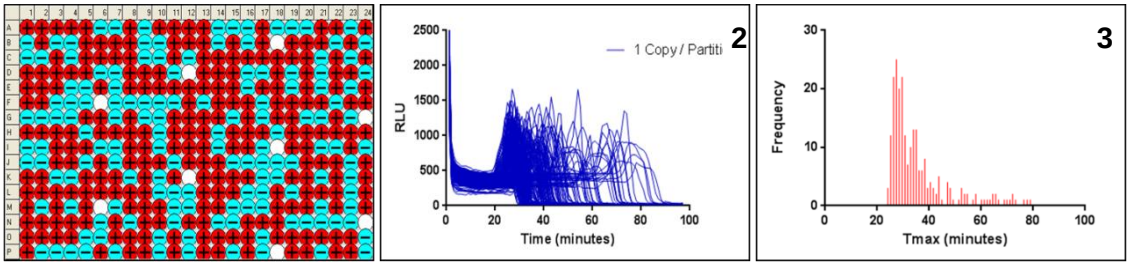


Figure 5.29: (L to R) 1 copy per partition 384 well plate, total assay volume 5µl (1) table to show the spread of positive results in red and negative results in blue (white circles indicate loading errors by the robotic injection system and plate misalignment) (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

Again the white circles spread across the graphical representation of the 384 wells plate is the result of a repeated error with the robotic injection system.
There are 234 positive results from a total of 375 partitions giving a success rate of 62%. This is the highest amplification frequency observed and would indicate an almost 100% amplification efficiency.

uCount^{SM} mean:	0.978 copies per partition (95% confidence level)
	(upper boundary 1.113, lower boundary 0.852)

The results from the digital analysis are very close to 1 copy per partition.

The light output from positive results against time indicates an average T_{max} of 35.22 minutes with standard deviation 12.01 and coefficient of variation of 34%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 23.62 minutes. The modal T_{max} is 26.84 minutes and the frequency for the mode is 25 giving a percentage modal frequency of 6.67. The frequency moving average FWHM is 9.72 minutes. These results are plotted on the graphs below (Figure 5.30) to show the comparison with the previous measurements in the Cardiff lab:

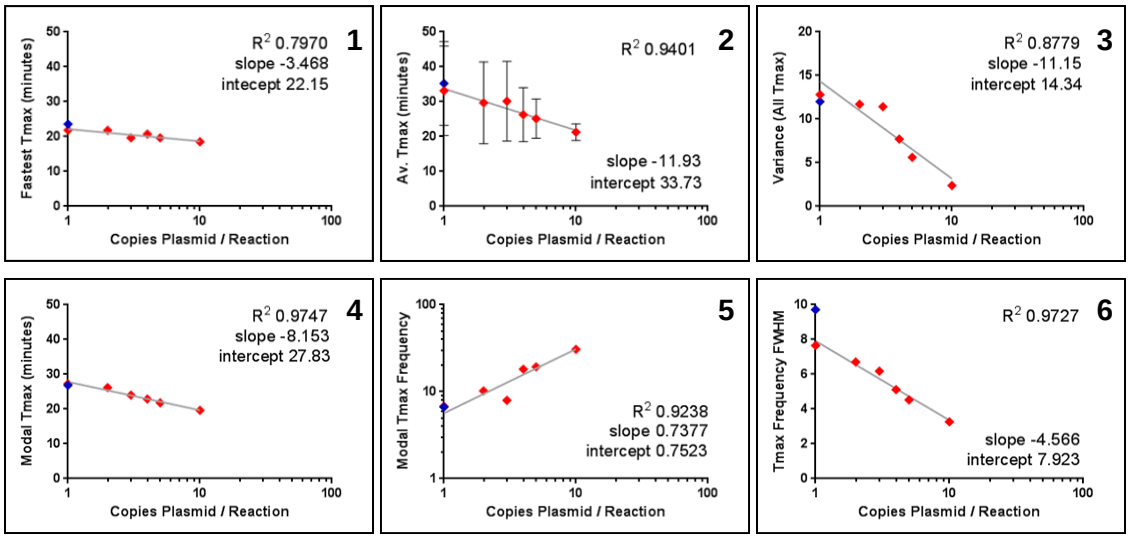


Figure 5.30: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The 234 positive results from 375 partitions suggests that if this could be consistently achieved then absolute quantification using digital BART would be possible. In comparison to the previous data from Chapter 4, all the quantification values correlate strongly and in all cases 1 copy is differentiated from 2 copies per partition.

5.5.2.1.3 Digital Assay 3

A repeat of the average one copy per partition of pART7 experiment was carried out to assess reproducibility (Figure 5.31). There were no changes to procedure or reagents used.

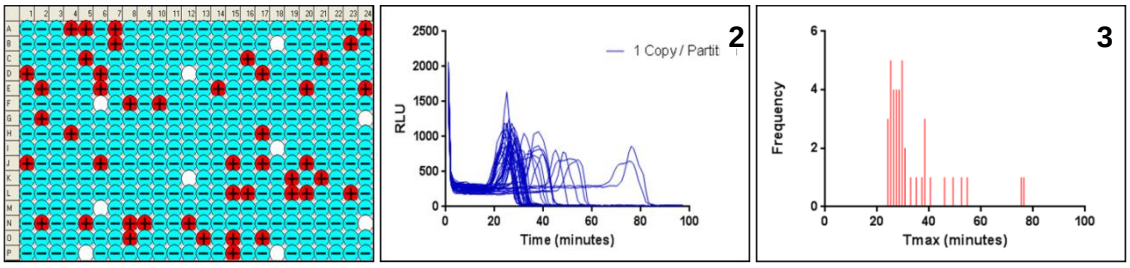


Figure 5.31: (L to R) 1 copy per partition 384 well plate, total assay volume 5µl (1) table to show the spread of positive results in red and negative results in blue (white circles indicate loading errors by the robotic injection system and plate misalignment) (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

The robotic loading of the reagent to the 384 wells plate continued to miss one in every forty one partitions. Unfortunately there were only 44 positive results from 374 partitions giving a success rate of 12%. It is unclear as to why the amplification frequency should drop from 62% to 12%, but such variation is unacceptable for digital analysis. A possible explanation could be the preparation of the dilution, but the native primers at twice the standard concentration could be the cause. One difference between the two assays was a time delay loading the plate to the BISON platform for the previous assay.

uCount^{SM} mean:	0.125 copies per partition (95% confidence level)
	(upper boundary 0.162, lower boundary 0.088)

The digital analysis based on the number of positive results calculates there to be 47 copies in the 374 partitions. This is almost an order of magnitude fewer copies than expected which suggests an error with dilution.

The average T_{max} from the data is 32.14 minutes with standard deviation 12.33 and coefficient of variation of 38%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 21.43 minutes. The modal T_{max} values are 24.65 and 28.94 minutes and the frequency for these modes is 5 giving a percentage modal frequency of 1.34. The frequency moving average FWHM is 6.77 minutes. These results are plotted on the graphs below (Figure 5.32) to show the comparison with the previous Cardiff results:

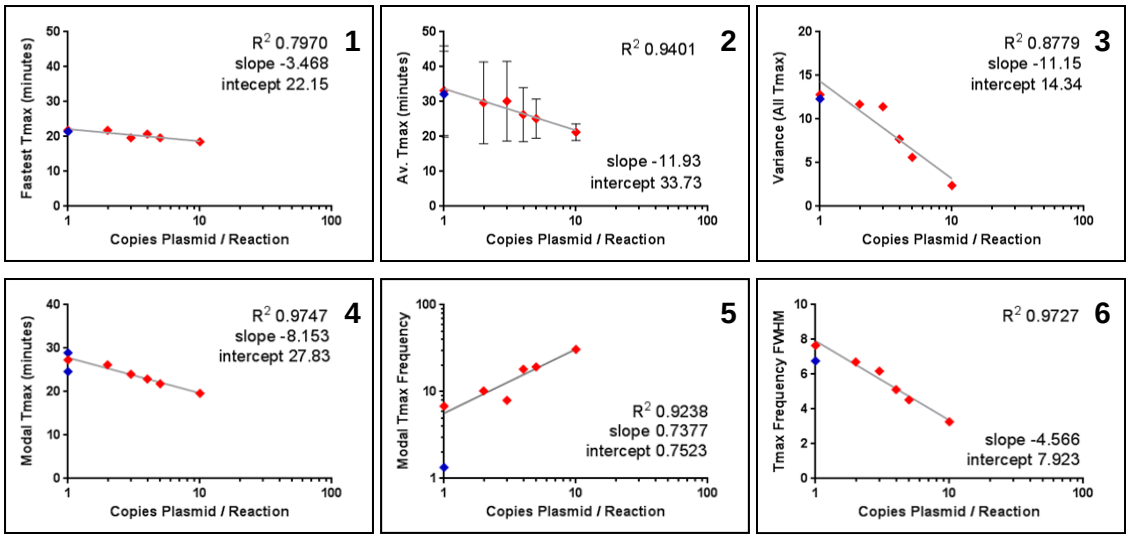


Figure 5.32: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The low number of positive results is only slightly more than previously observed for single copy assays on 96 well plates. Although the digital quantification calculations are affected by the low amplification frequency the ultra-quantification methods are concluding that those partitions that are positive mostly contain single copies of the template.

5.5.2.1.4 Digital Assay 4

A further one copy per partition linearised plasmid pART7 assay with the 35Sp primers aimed to address the poor success rate of the previous assay (Figure 5.33). To return to some of the conditions by which the ultra-quantification assays were performed, the 35Sp primers were denatured and the whole assay preparation was conducted at room temperature. Extra care was taken in the preparation of the template and the subsequent titration to 10 copies/µl.

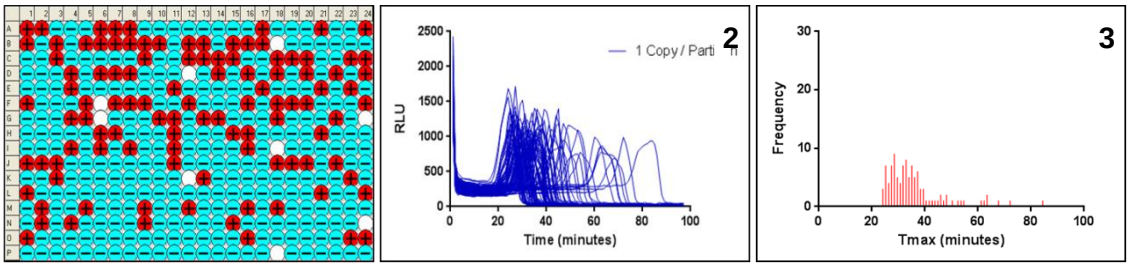


Figure 5.33: (L to R) 1 copy per partition 384 well plate, total assay volume 5µl (1) table to show the spread of positive results in red and negative results in blue (white circles indicate loading errors by the robotic injection system and plate misalignment) (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max}

The loading by the robot continued to miss one in every forty one partitions. There are 106 positive results from a total of 375 partitions giving a success rate of 28%. Although this is an improvement on the previous assay, it was below the 62% amplification frequency originally achieved.

uCount^{SM} mean: 0.125 copies per partition (95% confidence level)
(upper boundary 0.397, lower boundary 0.270)

From the digital calculations there are 125 copies spread across the 375 partitions which is a third of the number calculated to be loaded from the sample titration. The time to peak values generated give an average T_{max} of 35.11 minutes with standard deviation 11.14 and coefficient of variation of 32%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 23.58 minutes. The frequency distribution lacks the Poisson shape that has previously been typical of these assays. The wide spread of T_{max} results is similar to the distribution of one copy per partition with the mismatching displacement primer assay and suggests that there was an issue with the primers. The modal T_{max} is 27.88 minutes and the frequency for the mode is 9 giving a percentage modal frequency of 2.40. The frequency moving average FWHM is 13.56 minutes. These results are plotted on the graphs below (Figure 5.34) to show the comparison with the previous measurements in the Cardiff lab:

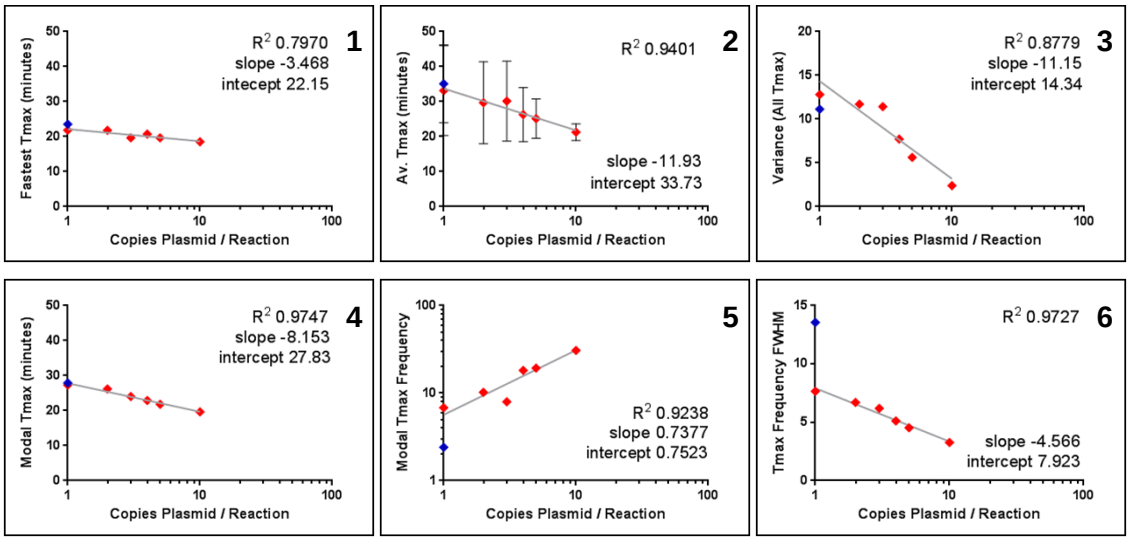


Figure 5.34: (L to R) previous data from Chapter 4 showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the value from this assay in blue (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The atypical frequency distribution with this last assay has an effect on those analysis methods that rely on a Poisson distribution of data. There is differentiation of the one copy per partition results from this assay from two copies per partition when the various T_{max} methods are employed.

This group of assays highlighted the potential for the assay for digital BART if the variation in amplification in frequency can be controlled. The primers need to be investigated for non-specific interactions when utilised without denaturing and at higher concentrations.

5.5.2.2 Genomic template

In a final experiment with the last remaining 35Sp primers, one copy of maize event Mon810 genomic DNA per partition was LAMP-BART assayed on a 384 well plate. In previous assays with the displacement primer B3 version 3, single copy detection was not achieved from a low number of replicates and therefore the aim of this experiment was to assess the potential for single copy genomic DNA detection (Figure 5.35).

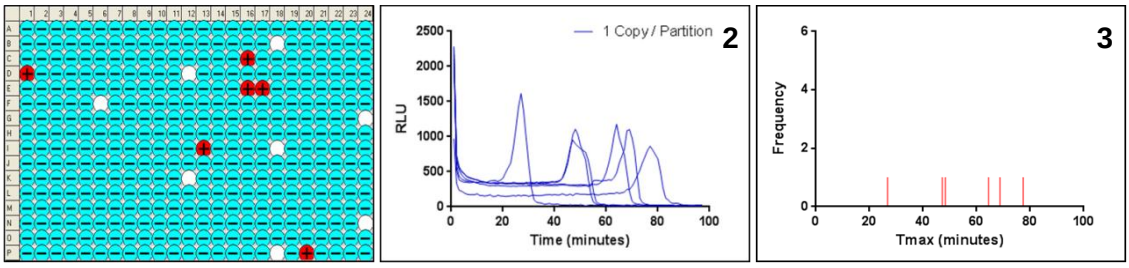


Figure 5.35: (L to R) 1 copy per partition 384 well plate, 35Sp LAMP-BART assay with genomic template (1) table to show the spread of positive results in red and negative results in blue (white circles indicate loading errors by the robotic injection system) (2) the light output from positive results against time (3) the frequency distribution of T_{max} results

The robotic assay plate loading continued to miss one in 41 partitions.
There are 6 positive results from a total of 376 partitions giving a success rate of 2%. The lack of single copy per partition detection from a low number of replicates is explained by this low amplification frequency.

From the six positive results there is an average T_{max} of 55.47 minutes with standard deviation 18.31 and coefficient of variation of 33%. The frequency distribution of T_{max} results shows a fastest T_{max} of 26.80 minutes but there is no clustering of values subsequently. Due to the low number of positive results, the modal T_{max} is not assigned. There is insufficient data here to compare to the single copy detection with linear plasmid template.

5.5.3 ADH1 genomic low copy number per partition

The same maize genomic template sample was used for low copy number detection with the improved ADH1 primers in a 384 partition LAMP-BART assay. A slightly different manifestation of BARTmaster was used; one container of LAMP-20 provides four times the reagent as the standard BARTmaster and provided sufficient reagent for 400 partitions. The ADH1 primers were denatured and used at twice the standard concentration.

5.5.3.1 One copy per partition

The maize genomic DNA extract from event MON810 was diluted to 10 copies/µl and loaded to the BARTmaster reagent mix to give one copy per partition across the 384 well plate (Figure 5.36).

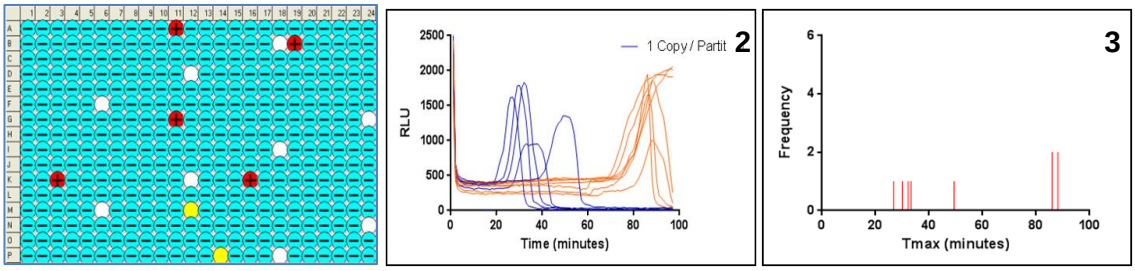


Figure 5.36: (L to R) 1 copy per partition 384 wells, ADH1 LAMP-BART assay with genomic template (1) The graphic shows the spread of positive results in red and negative results in blue (white circles indicate loading errors by the robotic injection system and yellow circles indicate result that fall outside the parameters of the algorithm and therefore require manual designation) (2) positive results are indicated in blue and the outliers are shown in orange (3) frequency distribution of all results

The robot continued to miss some wells in a regular pattern. The positive results appear to be in two main groups and the fastest group has been assumed to be true ADH1 LAMP products. The latter group is likely to be caused by primer interaction due to the doubled concentration of the primers. Excluding the results at the end of the assay time, there are five positive results from a total of 375 partitions giving a success rate of 1%. From these five results the average T_{max} is 34.32 minutes with standard deviation 8.76 and coefficient of variation of 26%. The frequency distribution of T_{max} results shows some signs of clustering of T_{max} values after the fastest T_{max} of 26.80 minutes. Due to the low number of positive results the modal T_{max} is not assigned. The amplification frequency is similar to that for one copy of the genomic template per partition with the 35Sp LAMP-BART assay, but that assay didn't have outlying T_{max} values.

uCount^{SM} mean: 0.01342 copies per partition (95% confidence level)
(upper boundary 0.02519, lower boundary 0.001494)

The digital software predicts that there were five copies spread across the 375 partitions and this shows that the five positive results contain single copies of the template.

5.5.3.2 Two copies per partition

With the low frequency of amplification from the previous assay, target concentration was increased to two copies of the maize event MON810 genomic DNA with the maize gene ADH1 sequence per partition (Figure 5.37). The aim here is to see if the digital

calculations could be used to quantify with single copy number differentiation for assays with low amplification efficiency.

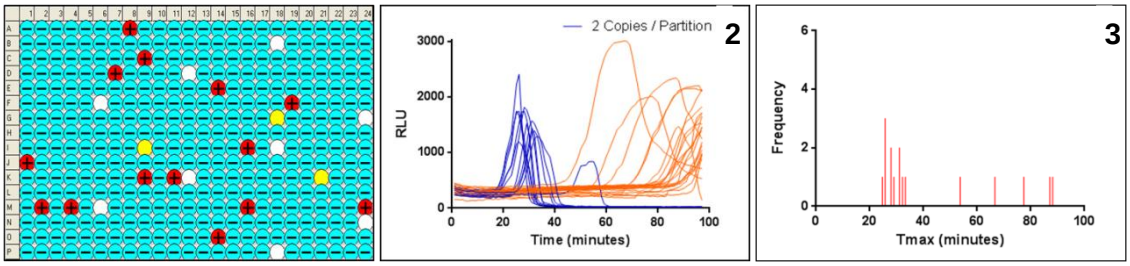


Figure 5.37: (L to R) 2 copies per partition 384 wells, ADH1 LAMP-BART assay with genomic template (1) the graphic shows the spread of positive results in red and negative results in blue (white circles indicate loading errors by the robotic injection system and yellow circles indicate results that fall outside the parameters of the algorithm and therefore require manual designation) (2) positive results are indicated in blue and the outliers are shown in orange (3) frequency distribution of all results

From this assay there were 12 positive results indicated in blue which excludes the outliers shown in orange, from a total of 375 partitions giving a success rate of 3%. The average T_{max} from these twelve positive results is 30.66 minutes with standard deviation 7.78 and coefficient of variation of 25%. The frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} of 24.67 minutes in a typical Poisson shape. The modal T_{max} is 25.74 minutes.

uCount^{SM} mean: 0.03252 copies per partition (95% confidence level)
(upper boundary 0.05099, lower boundary 0.01409)

The digital software predicts that there were twelve copies spread across the 375 partitions and this shows that the twelve positive results contain single copies of the template. This is approximately double the number of copies present in the last assay with half the amount of copies by dilution.

The lower frequency of amplification of genomic DNA could be because of an error in quantification or a lower probability of amplification.

5.6 Optimisation of Assay Amplification Frequency

The 384 partition assays raised many questions about the requirements for a digital BART assay. The optimisation of amplification frequency is the key to all these assays and this may be template or primer related or the assay conditions. If 100%

amplification efficiency cannot be achieved, then a predictable efficiency would nevertheless enable digital quantification.

5.6.1 Template

Linearised DNA of the pART7 plasmid was used for CaMV 35S promoter detection assays. A further plasmid containing the CaMV 35S promoter, the NOS terminator sequence and an insert of the maize alcohol dehydrogenase gene ADH1, which is referred to as pUC35S ADH1, was constructed. This linearised plasmid template was not lyophilised with carrier DNA and could therefore be used for denatured template experiments. The genomic template used was derived from *Zea mays* event MON810 seeds which are ground to a powder and extracted with a commercial kit; Promega Wizard. Although this extraction method was found to be the most appropriate when compared to a number of other techniques (Kiddle et al. 2012) for LAMP-BART assays, the kit is designed with PCR in mind. Therefore in this section the aim is to improve the suitability of the templates for LAMP-BART assays.

5.6.1.1 Conformation of plasmid DNA template

The 35Sp LAMP-BART assays of the linearised pART7 target achieved amplification frequencies for single copy detection in the range 12% to 62% of partitions. As discussed previously, the non-linear forms of the plasmid are amplified by PCR with less efficiency than the linear form; therefore an experiment was designed to investigate the difference in sensitivity and amplification frequency for these target conformations using LAMP (Figure 5.38).

No denaturing step is used with LAMP amplification and the LAMP and displacement primers require the fluctuating breaking and reforming of bonds between the A-T and G-C base pairs at the 60°C isothermal assay temperature. Once the target strand has been invaded by a LAMP primer, the initiation of amplification can begin. The complexity of the target may influence the initiation of amplification in LAMP and circular plasmid DNA is likely to ‘breathe’ to a lesser degree than the linearised plasmid DNA. This would manifest itself as increased time to peak values and a potential decrease in amplification frequency particularly at low copy number. The templates were also assayed with qPCR as the benchmark technology for comparison. Due to the low variation in Ct values four replicates were employed. For LAMP-BART the

number of replicates was set at twenty due to the higher variation of time to peak values at low copy numbers.

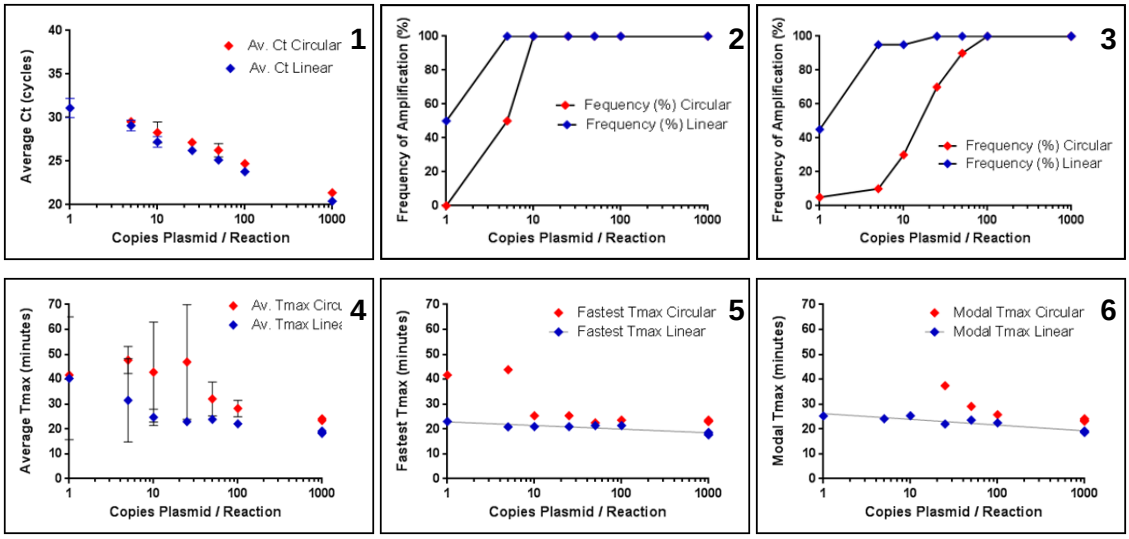


Figure 5.38: (L to R) Linear plasmid and circular plasmid LAMP-BART and qPCR assays (1) qPCR cycle threshold results (2) qPCR amplification frequency (3) LAMP-BART amplification frequency (4) LAMP-BART average T_{max} (5) LAMP-BART fastest T_{max} (6) LAMP-BART modal T_{max}

Fig. 5.38 shows that there is a full cycle difference between the circular and linear plasmid when assayed with qPCR. The amplification efficiency for both declines from near 100% for both template forms with the circular form falling below 90% efficiency. The frequency of amplification diverges at 5 copies per partition, when the circular form drops to 50% of replicates whereas the linear form remains at 100% of replicates positive. For the linear template 50% of the partitions are positive at one copy per partition; there are only four replicates and therefore this value is lower than what would be expected for digital PCR with 100% amplification efficiency. This might also indicate quantitation error in the original sample.

LAMP is affected by the conformation of the plasmid template to a greater degree than qPCR and this could be due in part to the template denaturation at the start of the qPCR program. The linear plasmid amplification frequency is close to the values set by the benchmark 35Sp qPCR assay. However the amplification frequency for the circular form drops rapidly from 100% at 100 copies per partition to 10% of positive results for 5 copies per partition. The problem with this template is also evident in the time to peak values which are slower for the circular template. The reduced amplification frequency and slower time to peak values are evidence that the initiation of amplification by the LAMP primers is less likely to occur. This becomes more acute as template copy numbers reduce.

5.6.1.2 Denatured plasmid DNA template

The previous experiment confirmed that linearised plasmid template has a higher amplification frequency at low copy number and is more appropriate for digital BART. The amplification frequency results from qPCR were similar, potentially because of the initial denaturing step used. Denaturing the target for LAMP amplification could also increase amplification frequency at low copy number further. To assess this, the results of native and denatured linear plasmid templates were compared (Figure 5.39).
The pART7 linearised plasmid aliquots used for most experiments were prepared with carrier DNA and can therefore not be denatured due to the known deleterious effect of denatured carrier on the LAMP assay. The recently constructed and linearised plasmid pUC35S ADH1 was quantified using the NanoDrop spectrophotometer and assayed with the 35Sp primer set which included the B3 displacement primer version 4. The target was either denatured or native before dilution. The spectrophotometer result was taken as a guideline only because of the low value and lack of a clear peak at 260nm.

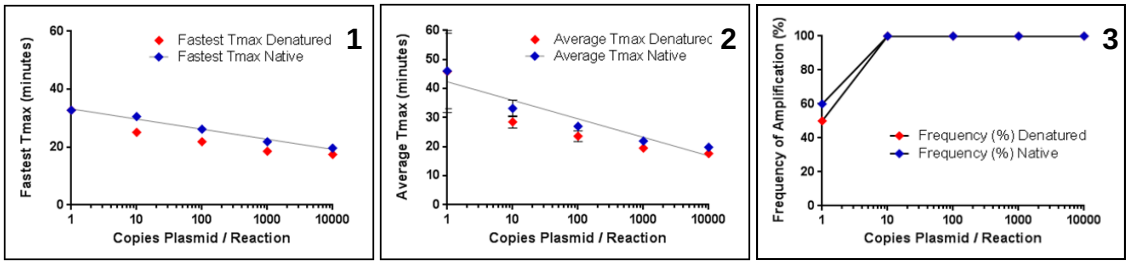


Figure 5.39: (L to R) 35Sp LAMP-BART assay of linearised plasmid DNA (1) fastest T_{max} of native and denatured template (2) average T_{max} of native and denatured template (3) frequency of amplification of native and denatured linearised plasmid DNA

The denatured target enabled faster time to peak results. This may be the result of increasing the likelihood of LAMP amplification initiation by removing the requirement for strand invasion of the LAMP primers. However, denaturing the target did not improve the amplification frequency for the linearised plasmid target.

5.6.1.3 Denatured genomic DNA template

The time to peak for denatured linearised plasmid template were faster as were those for double stranded linearised plasmid template compared to circular plasmid template. Therefore the complexity of the template influences the time to peak, potentially caused by a time limiting step in LAMP amplification initiation. Template complexity was increased further with the assaying of genomic DNA in native and denatured form. The

aim was to improve on the low amplification frequency associated with previous attempts at low copy assaying of genomic template.
Genomic DNA from the maize event Mon810 at 10 copies per partition were assayed with the 35S primers at reduced total assay volume of 5µl (Figure 5.40).

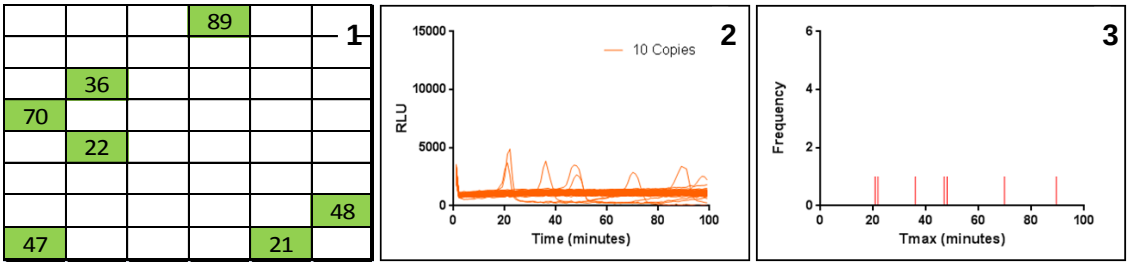


Figure 5.40: (L to R) 10 copies per reaction native genomic DNA target (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the time to peak values from the 48 partitions

The seven positive results from a total of 48 partitions give an amplification frequency of 15% at 10 copies per partition. The average T_{max} for this assay of native genomic template is 47.53 minutes with standard deviation 25.07 and coefficient of variation of 53%. The frequency distribution of T_{max} results lacks the clustering of T_{max} values typically observed after the fastest T_{max} which is 20.72 minutes. There are no T_{max} frequencies greater than one and the modal T_{max} is therefore not assigned. The 48 no template controls (NTCs) also run with this assay were all free from contamination.

Denatured maize event Mon810 genomic DNA target was assayed at 10 copies per reaction with 35S primers at the reduced total assay volume of 5µl (Figure 5.41).

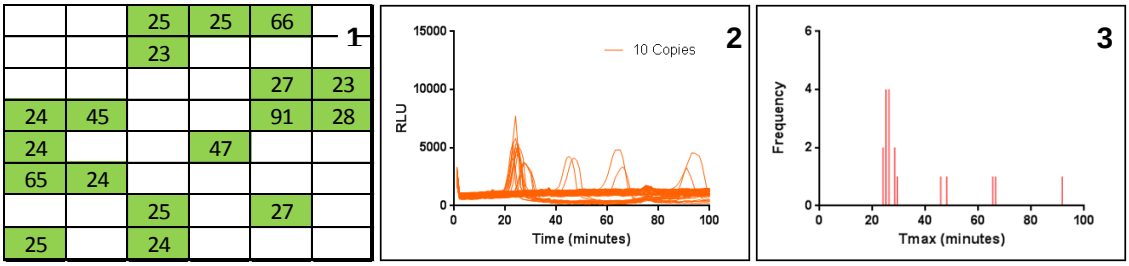


Figure 5.41: (L to R) 10 copies per reaction denatured genomic DNA target (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the time to peak values from the 48 partitions

Denatured the genomic template increased the number of positive results to 18 from a total of 48 partitions give an amplification frequency of 38% at 10 copies per partition. The average T_{max} for this assay of native genomic template is 35.56 minutes with

standard deviation 19.57 and coefficient of variation of 55%. The frequency distribution of T_{max} results shows the clustering of T_{max} values typically observed after the fastest T_{max} which is 23.03 minutes. There are two modal T_{max} values directly after the fastest T_{max} and they are 24.12 and 25.21 minutes both with a frequency of 4.

Denaturation of the genomic DNA compared to native genomic DNA showed a marked (approximately two fold) increase in observed amplification frequency which was not observed in the case of denatured compared to native linearised plasmid DNA. The complexity of the target is possibly a major factor in the limitations to amplification efficiency observed. The absence of improvement to the amplification frequency by denaturing the linearised plasmid suggests that the denaturation state of the template is not the source of the suboptimal amplification efficiency in this case.

5.6.1.4 Genomic DNA template quality

Linear plasmid DNA template was used as the comparative benchmark for experiments to improve the assays of genomic template. In this first experiment seeds from the maize event MON810 were ground and extracted with the Promega Wizard extraction kit. The quality of the genomic DNA was assessed visually on an agarose gel and gel quantified using ImageJ software. The extract was then assayed with qPCR and LAMP-BART using 35Sp primers (Figure 5.42).

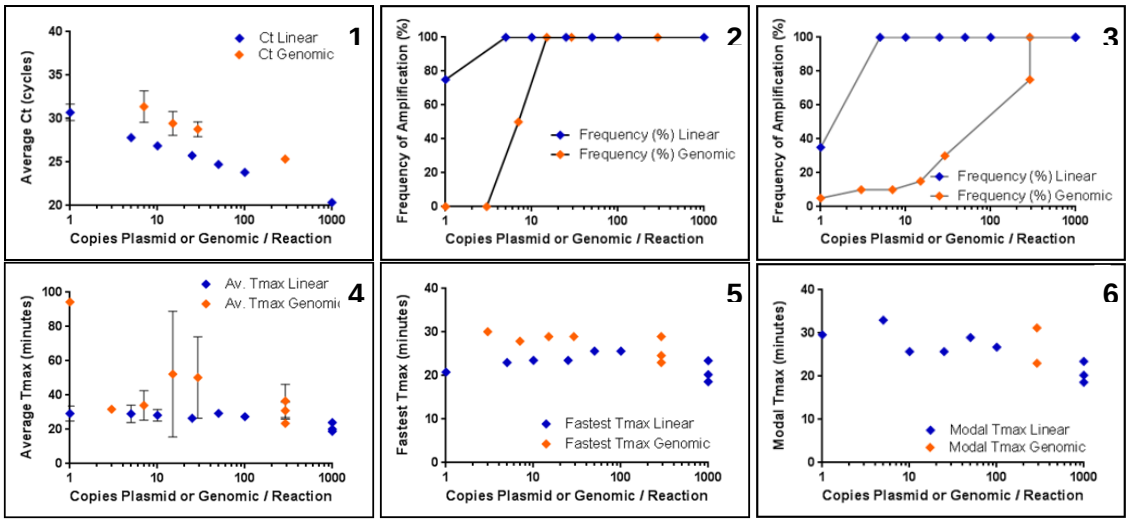


Figure 5.42: (L to R) Linear plasmid and genomic template LAMP-BART and qPCR assays (1) qPCR cycle threshold results (2) qPCR amplification frequency (3) LAMP-BART amplification frequency (4) LAMP-BART average T_{max} (5) LAMP-BART fastest T_{max} (6) LAMP-BART modal T_{max}

There was a wide cycle difference observed between the genomic and linear plasmid templates when assayed with qPCR (Fig. 5.42 Panel 1). The amplification efficiency for the linear plasmid template is 98% and only 90% for the genomic template. The frequency of amplification separates at 8 copies per partition when the genomic template drops to 50% of replicates whereas the linear plasmid template remains at 100% positive replicates. For the linear template 75% of the partitions are positive at one copy per partition. The low number of replicates is likely to be responsible for this high value in terms of digital PCR. There were no positive results for one or three copies of the genomic template per partition with qPCR.

LAMP is greatly affected by the complexity of the genomic template and the lack of a denaturing step to initiate amplification. The linear plasmid amplification frequency was close to the values set by the benchmark 35Sp qPCR assay. However the amplification frequency for the genomic template dropped rapidly from 100% at 300 copies per partition to 30% of positive results for 30 copies per partition. The lower copy numbers have very low amplification frequencies but continue to generate positive results. Where there is time to peak data available for genomic template, then the times are slower than the linear plasmid template.

5.6.1.4.1 Purified genomic DNA template

In a second experiment, the genomic DNA from Promega Wizard kit extraction of the maize event MON810 was purified with phenol chloroform and ethanol precipitation. The quality of the genomic DNA was again assessed visually on an agarose gel and gel quantified using ImageJ software. This purified extract was assayed with qPCR and LAMP-BART using 35Sp primers (Figure 5.43).

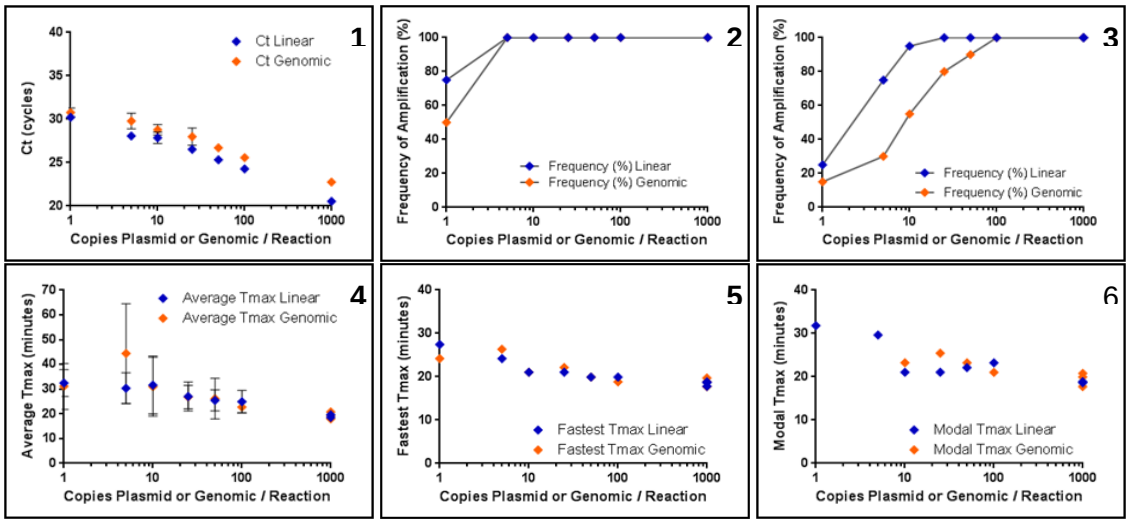


Figure 5.43: (L to R) Linear plasmid and refined genomic template LAMP-BART and qPCR assays (1) qPCR cycle threshold results (2) qPCR amplification frequency (3) LAMP-BART amplification frequency (4) LAMP-BART average T_{max} (5) LAMP-BART fastest T_{max} (6) LAMP-BART modal T_{max}

The difference in cycles between the genomic and linear plasmid templates when assayed with qPCR has narrowed with the further purification of the genomic template. The amplification efficiency for the linear plasmid template is 105% and the purified genomic template assay is compromised with a calculated value of 128%. These values drop to 96% and 111% with the removal of the Ct values for one copy per partition. The frequency of amplification for both templates is 100% at 5 copies per partition reducing to 75% for linear plasmid and 50% for purified genomic at one copy per partition.

The amplification frequency for the LAMP-BART assay of the refined genomic template is 100% at 100 copies per partition and gradually declines to 80% at 25 copies per partition. This compares to approximately 20% at 25 copies per partition of the unrefined genomic DNA extract. At one copy per partition there are three positive partitions from a total of twenty replicates. There is a clear improvement to amplification frequency from more highly purified genomic DNA.

The measurements of T_{max} show the gap between times has been reduced to almost parity for the two templates.

The same purified genomic DNA extract from maize event MON810 was assayed with the ADH1 primers version 1 with FIP and B-Loop version 3 (Figure 5.44).

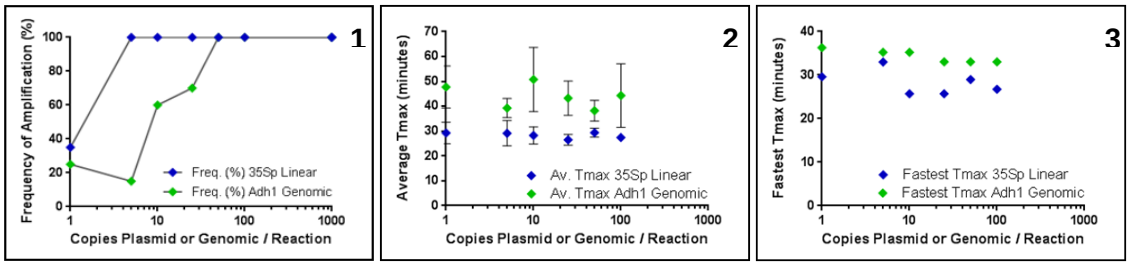


Figure 5.44: (L to R) Promega Wizard extracted maize event Mon810 genomic DNA refined with phenol chloroform and ethanol precipitation assayed with ADH1 primers with B-Loop and FIP version 3, in green with previous linear plasmid 35Sp LAMP-BART results in blue (1) LAMP-BART amplification frequency (2) LAMP-BART average T_{max} (5) LAMP-BART fastest T_{max} (3) LAMP-BART modal T_{max}

The improved ADH1 primer set also performed well with the refined genomic template. The amplification frequency at 25 copies per partition is 70% which is similar to the 80% achieved with the 35Sp primer LAMP-BART assay. The single copy frequency at

25% far exceeds the 2% frequency achieved with the unrefined genomic template on the 384 partition plates at one copy per partition. The time to peak measurements are slower at each copy number for the LAMP-BART ADH1 refined genomic template assay when compared to the linear plasmid template 35Sp assay, suggesting an effect of primers or the nature of the amplified sequence.

5.6.1.4.2 Double purified genomic DNA template

Improvement in the amplification frequencies was observed with more purified genomic DNA. In this experiment, the purified Promega wizard extract was put through a further round of purification with phenol chloroform and ethanol precipitation with the aim of assessing whether this further enhanced the template quality for qPCR and LAMP-BART assays (Figure 5.45). The quality of the genomic DNA was again assessed visually on an agarose gel and subsequently gel quantified using ImageJ software.

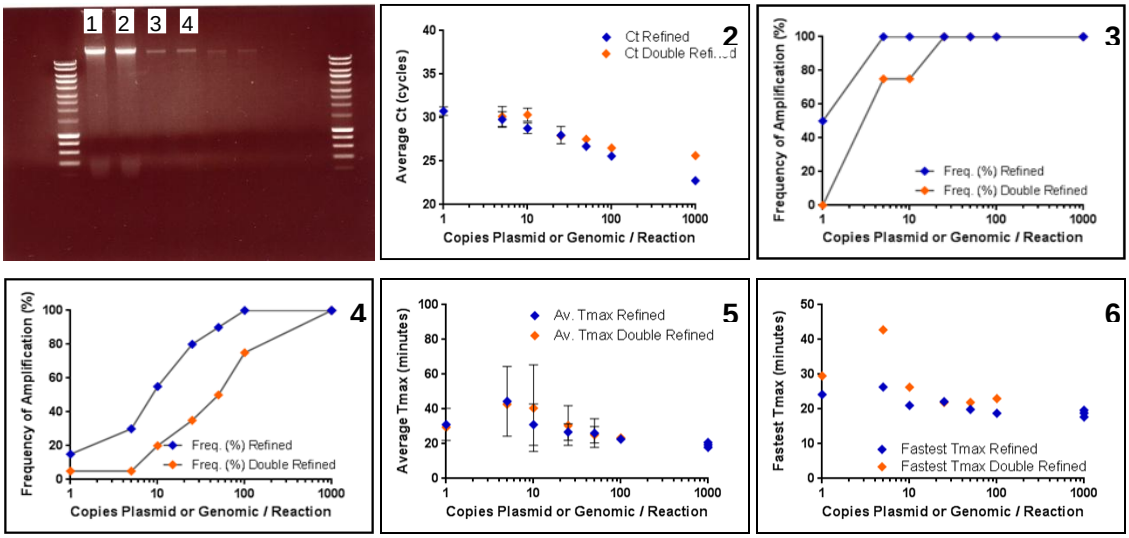


Figure 5.45: (L to R) Refine genomic and double refined genomic template LAMP-BART and qPCR assays (1) agarose gel to show double refined genomic DNA in left lanes 10^0 (marked as 1 and 2 on the gel image) and 10^{-1} dilution (marked as lanes 3 and 4) (2) qPCR cycle threshold results (3) qPCR amplification frequency (4) LAMP-BART amplification frequency (5) LAMP-BART average T_{max} (6) LAMP-BART fastest T_{max}

The comparison with the results from the LAMP-BART 35Sp assay of the refined genomic template shows a reduction in amplification frequency for both qPCR and LAMP-BART with the double refined template. The over-manipulation of the genomic extract has possibly increased residual phenol in the sample which is inhibiting the amplification. The template may also have lost some integrity.

5.6.1.4.3 Gel purified genomic DNA template

The Promega Wizard double refined extract used in the previous experiment was run on agarose gel by electrophoresis. The gel band was extracted and purified before agarose gel quantification. The resultant extract was assayed with qPCR and LAMP-BART using 35Sp primers (Figure 5.46).

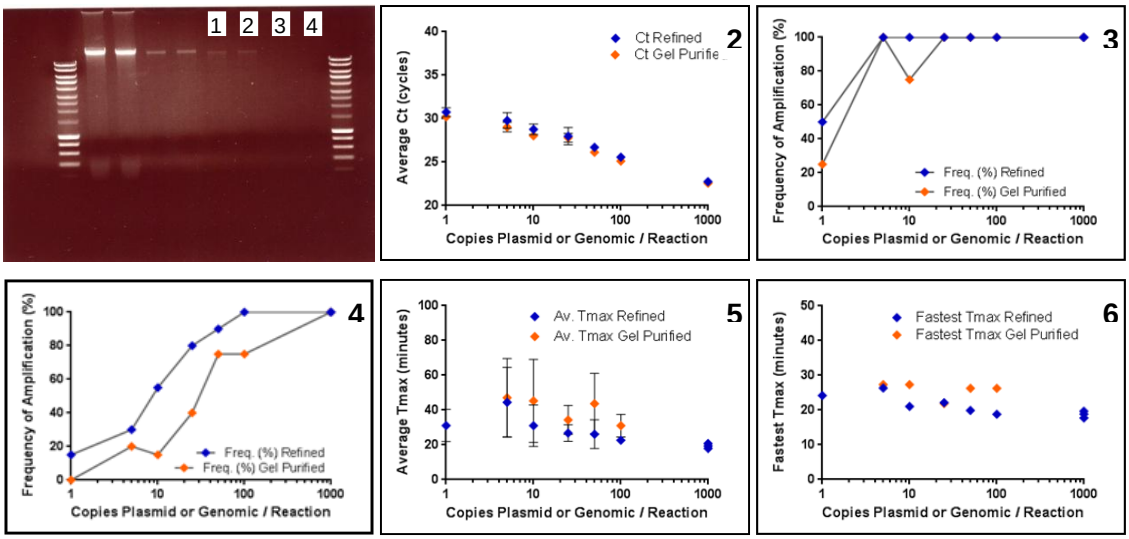


Figure 5.46: (L to R) Refine genomic and double refined genomic template LAMP-BART and qPCR assays (1) agarose gel to show double refined genomic DNA in right lanes 10^0 (marked as 1 and 2 on the gel image) and 10^{-1} dilution (marked as lanes 3 and 4) (2) qPCR cycle threshold results (3) qPCR amplification frequency (4) LAMP-BART amplification frequency (5) LAMP-BART average T_{max} (6) LAMP-BART fastest T_{max}

The amplification efficiency from the qPCR data shows that for both templates the efficiency breaks down below 25 copies per partition. The gel purification of the extract does improve the amplification frequency for qPCR almost to the level of the purified template. This may be due to the removal of residual phenol in the purification which would inhibit the amplification. The LAMP-BART amplification frequency recovers at some of the copy numbers but remains compromised.

5.6.2 Primers

While working with the 384 well plates, double the standard concentration of primers was used without denaturing them. It would be appropriate for an isothermal test not to require the denaturing of the primers therefore in this section multiple replicates containing carrier DNA but no template were performed with the CaMV 35S promoter primers at standard concentration and twice the concentration (as used for the 384 partition assays), in order to assess potential non-specific priming events.

5.6.2.1 Denatured and non-denatured primers

The first primer set to be investigated was the 35Sp with B3 version 3 displacement primer set, at standard and double the concentration. For each LAMP-BART assay with non-denatured or denatured primer sets and both concentrations of primers, 48 partitions of no template controls were used. The aim was to investigate the impact of increased concentrations of LAMP primers on the assay and whether the non-denatured state of the primers would increase non-specific priming events (Figure 5.47).

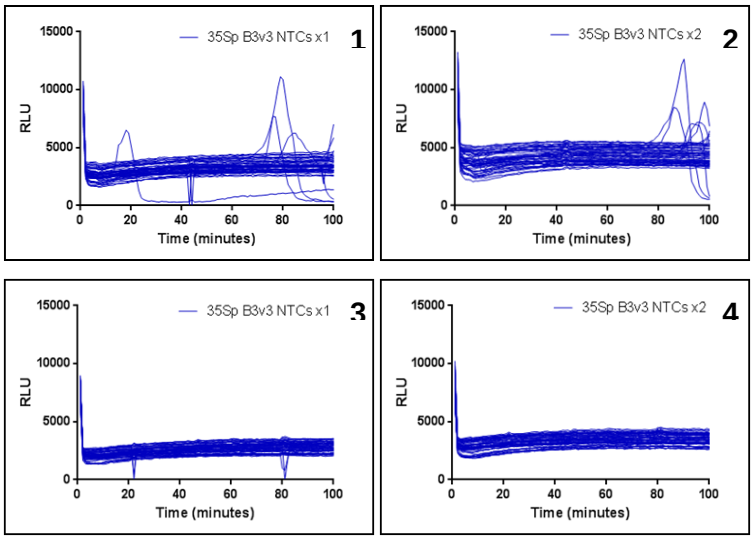


Figure 5.47: 35Sp LAMP-BART assays with B3 version 3 of NTCs with 20µl total volume per partition, non-denatured primers (1) standard concentration of primers (2) twice the concentration of primers, denatured primers (3) standard concentration of primers (4) twice the concentration of primers

The assays at both concentrations of non-denatured primers showed peaks which were not observed with denatured peaks. The products of amplification from the peaks in the non-denatured assays were analysed on an agarose gel for evidence of the 35Sp LAMP ladder pattern. The peak at approximately 20 minutes was the result of contamination. The other peaks towards the end of the assay time appeared to be the result of non-specific primer amplification. The denaturing of the primer set reduced the non-specific primer interactions at both primer concentrations.

In a second experiment, non-denatured and denatured primer set containing B3 version 4 displacement primer were compared at both concentrations of primers (Figure 5.48).

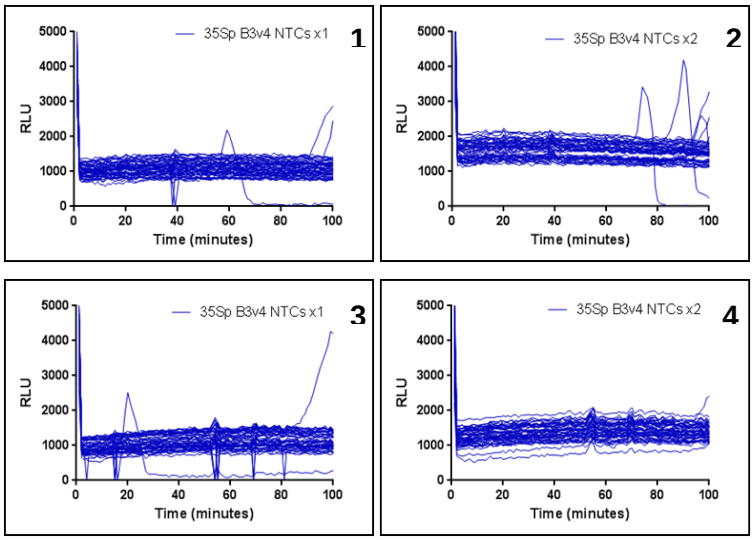


Figure 5.48: 35Sp LAMP-BART assays with B3 version 4 of NTCs with 5µl total volume per partition, non-denatured primers (1) standard concentration of primers (2) twice the concentration of primers, denatured primers (3) standard concentration of primers (4) twice the concentration of primers

The assays at both concentrations of non-denatured primers showed peaks. The peaks in the non-denatured assays were analysed on an agarose gel for evidence of the 35Sp LAMP ladder pattern and appeared to be the result of non-specific primer amplification and not contamination. The assays with denatured primers have no peaks associated with non-specific primer interactions; however, there is a peak at approximately 20 minutes associated with 35Sp target contamination. The denaturing of the primer set reduced the non-specific primer interactions at both primer concentrations. The 35Sp LAMP primers require redesigning to eliminate non-specific primer interactions under non-denaturing conditions for the LAMP-BART assay to be entirely isothermal. The non-target peaks were present towards the end of the assay after 70 minutes; reducing assay time would avoid these peaks occurring.

5.6.2.2 Primer concentration

For the assays on the 384 well plates the concentration of primers used was twice that of the standard 96 well plate LAMP-BART assays. Theoretically an increase in primer concentration should result in faster T_{max} times for low copy number and an increase in positive results within the assay timeframe, but alternatively the primer concentration may already be optimal and an increase could interfere with the amplification. In the following experiments with one copy of the linearised pART7 plasmid per partition, the effect of using different concentration of the 35Sp primer set was assessed with the aim of increasing amplification frequency. A fresh aliquot of pART7 was used for each

assay and the total assay volume per partition was maintained at 5µl for the 96 well plates.

5.6.2.2.1 1 x Primer concentration

The first assay was at the standard primer concentration and was duplicated to investigate inter-assay variation of amplification frequency which was previously apparent with the 384 partition assays (Figure 5.49).

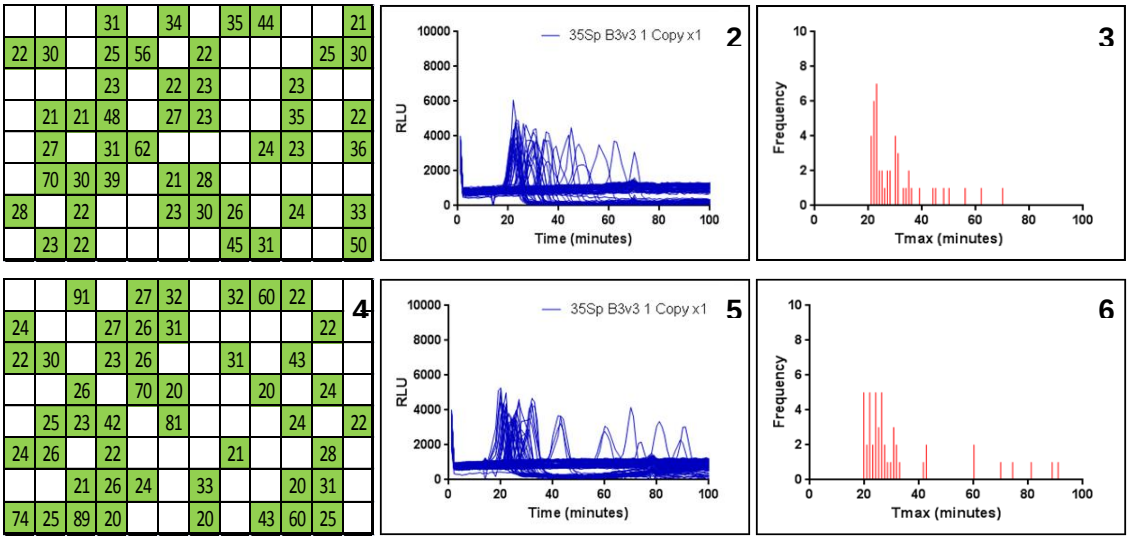


Figure 5.49: (L to R) 35Sp LAMP-BART assay of 1 copy pART7 template per partition 96 well plate, total assay volume 5µl, 1 x concentration denatured primers (1) table to show the spread of positive results in green with associated T_{max} value (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} (4)(5)(6) repeat of the assay

The amplification frequency for the first assay at the standard primer concentration was 48% and was very reproducible for the duplicate (49%). The time to peak data was analysed to compare with the single copy data and the low copy number trends from ultra-quantification methods from the experiments with the higher total assay volume per partition of 20µl (Figure 5.50).

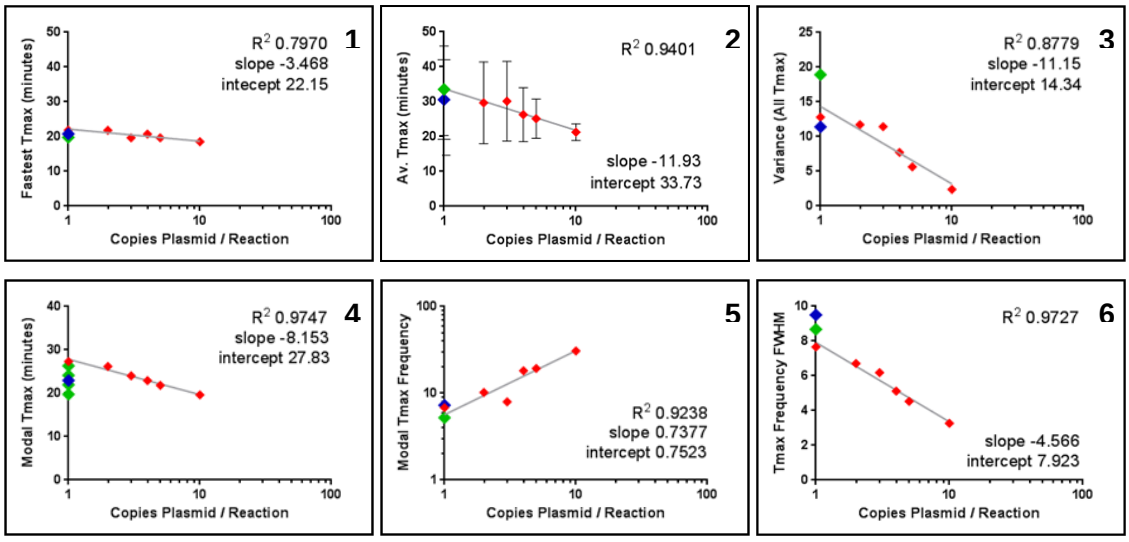
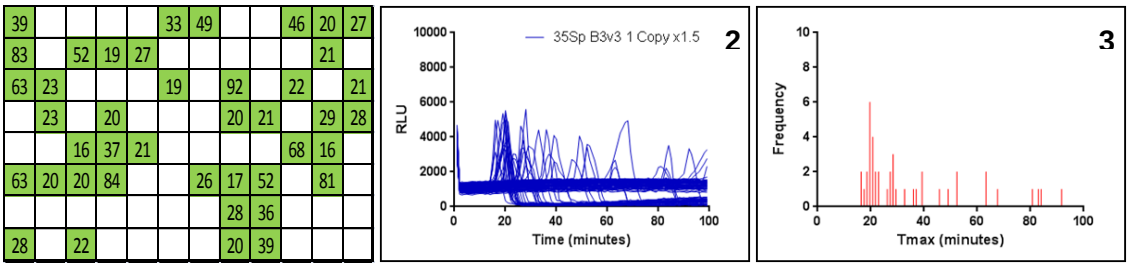


Figure 5.50: (L to R) previous data from the ultra-quantification chapter showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the values from these assay in (a) blue and (b) green (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

For the first assay the fastest T_{max} was 22.96 minutes with average T_{max} at 30.60, standard deviation 11.41 and modal T_{max} 22.96 minutes. The results from the duplicate assay were fastest T_{max} at 19.76 minutes with average T_{max} at 33.57, standard deviation 18.93 and modal T_{max} at 21.94. The fastest T_{max} and modal T_{max} results were faster than the previous results at one copy per partition, but the other measurements distinguished these results from those of two copies per partition.

5.6.2.2.2 1.5 x Primer concentration

In the second duplicate set of 35Sp LAMP-BART pART7 linearised plasmid assays the concentration of the primers was increased by 50% (Figure 5.51).



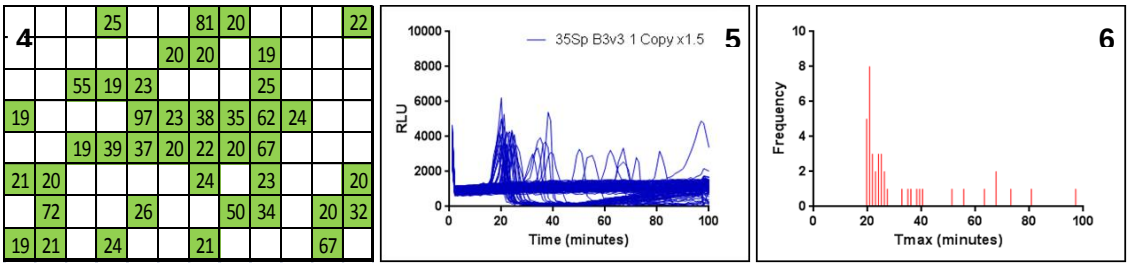


Figure 5.51: (L to R) 35Sp LAMP-BART assay of 1 copy pART7 template per partition 96 well plate, total assay volume 5µl, 1.5 x concentration denatured primers (1) table to show the spread of positive results in green with associated T_{max} value (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} (4)(5)(6) repeat of the assay

The amplification frequency for the first assay at the 50% increased primer concentration is 44% and for the duplicate 43%. This represents a small reduction in amplification frequency with the increased primer concentration. As with the previous duplicate assays the inter-assay variation between amplification frequencies of the duplicates is very low. Again, the time to peak data was analysed to compare with the single copy data and the low copy number trends from ultra-quantification methods from the experiments with the higher total assay volume per partition of 20µl (Figure 5.52).

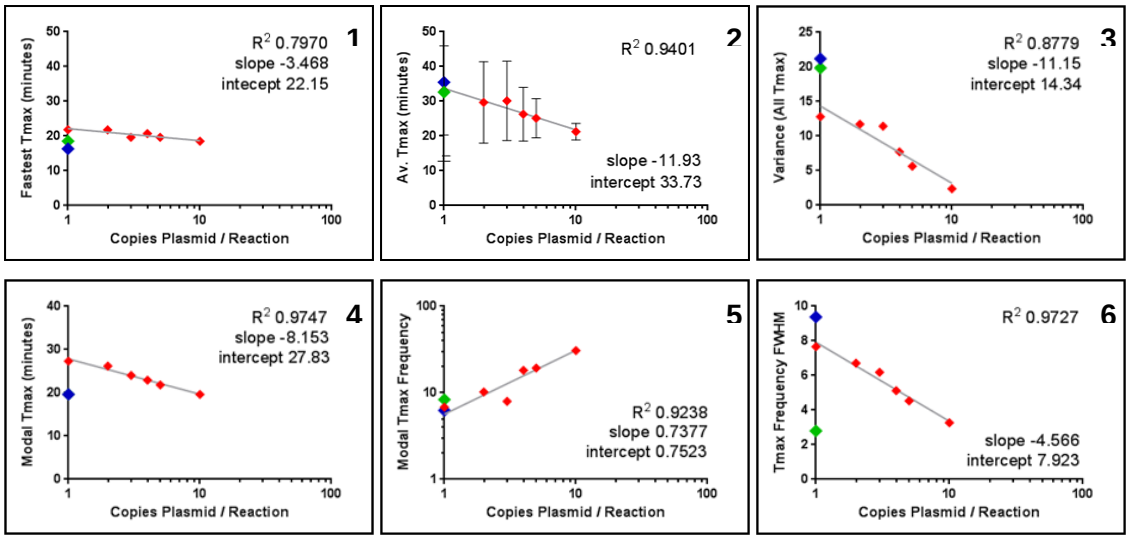


Figure 5.52: (L to R) previous data from the ultra-quantification chapter showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the values from these assay in (a) blue and (b) green (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The fastest T_{max} for these two assays are 16.37 and 18.56 minutes which are faster than the values from the standard primer concentration assays as are the modal T_{max} values of 19.64 and 19.65 minutes. The average T_{max} and standard deviations of 35.49

and 32.64 minutes with 21.19 and 19.85 minutes respectively, were similar to the previous results for single copy per partition.

5.6.2.2.3 2 x Primer concentration

The third duplicate set of 35Sp LAMP-BART pART7 linearised plasmid assays had twice the concentration of the primers (Figure 5.53).

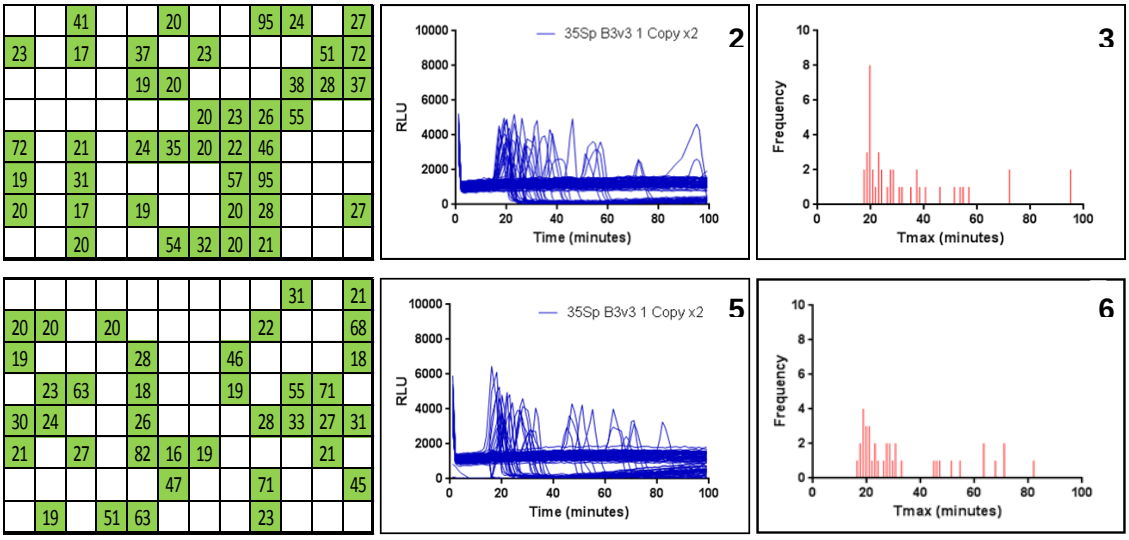


Figure 5.53: (L to R) 35Sp LAMP-BART assay of 1 copy pART7 template per partition 96 well plate, total assay volume 5µl, 2 x concentration denatured primers (1) table to show the spread of positive results in green with associated T_{max} value (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the clustering of T_{max} values after the fastest T_{max} (4)(5)(6) repeat of the assay

The amplification frequency for the first assay at twice the standard primer concentration is 44% and for the duplicate 39%. There is more variation in these amplification frequencies than previously observed, but both represent lower values than calculated for the standard concentration of primers. Therefore with this assay increasing primer concentration did not increase amplification frequency (Figure 5.54).

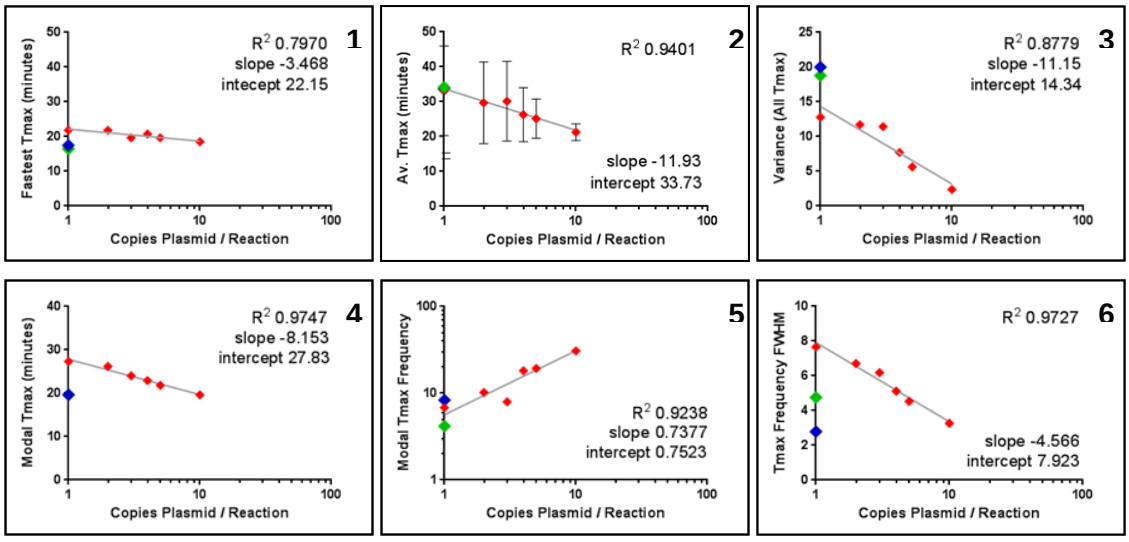


Figure 5.54: (L to R) previous data from the ultra-quantification chapter showing the results from 10, 5, 4, 3, 2 and 1 copy per partition with various quantification methods and the values from these assay in (a) blue and (b) green (1) fastest T_{max} (2) average T_{max} (3) standard deviation (4) modal T_{max} (5) percentage modal T_{max} frequency (6) T_{max} frequency FWHM of the moving average

The fastest T_{max} for these two assays are 1747 and 16.42 minutes which are faster than the values from the standard primer concentration assays as are the modal T_{max} values of 19.65 and 18.65 minutes. The average T_{max} values are 33.65 and 34.16 minutes with standard deviations of 20.02 and 18.80. These are similar results to the previous results for single copy per partition. The time to peak values do appear to have reduced but the high variance is again maintained keeping the average T_{max} in the range between 30 and 36 minutes. The increased primer concentration does appear to provide very consistent results for the T_{max} results between the duplicates, but has altered the shape of the T_{max} frequency distribution to reduce the utility of the T_{max} frequency FWHM method.

In summary, increasing the primer concentration produced faster times for the first time to peak, consistent with the increased concentration having a positive influence on amplification. However, the variance remained high and the frequency of amplification did not increase suggesting that amplification from some of the partitions is presumably slow to initiate regardless of the concentration of the primers. Perhaps somewhat surprisingly, this time limiting step does not appear to be reduced by raising primer concentration, whereas once amplification starts it may proceed more rapidly with higher primer concentration.

5.6.3 Assay conditions

A number of changes to the assay conditions were tested in an attempt to improve the amplification frequency of the 355p LAMP-BART pART7 assay. The first of these was based on the conditions that led to the 62% amplification frequency calculated from one of the 384 partition assays in Lumora (section 5.5.2.1.2). This was delaying the assay start.

5.6.3.1 Delayed start

The near ideal amplification frequency of 62% was achieved from the assay that was delayed from the point of mineral oil loading to the start of the 60°C assay run on the BISON by approximately 25 minutes. One possibility for this increase could be the presence of non-specific primer interactions being counted as positive results. Therefore this first experiment assays 96 no template controls with the standard concentration of native 35Sp primers with a delayed start of 25 minutes (Figure 5.55).

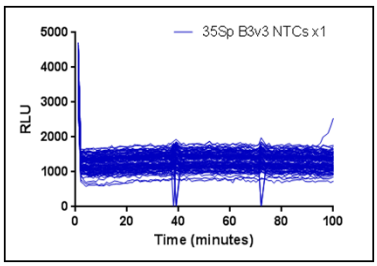
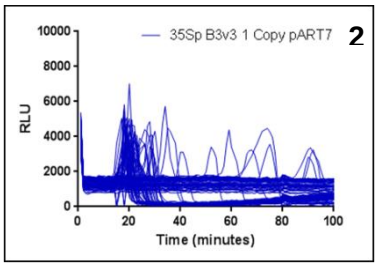


Figure 5.55: Native standard concentration 35Sp primers LAMP-BART assay of no template control samples, total assay volume 5µl, 96 replicates, delayed start of 25 minutes (1) the light output against time

Previously under these assay conditions with the exception of the delayed assay start, there was one peak observed late on in the assay after 80 minutes. In this assay with twice the number of NTCs there are no peaks and therefore no contamination or incidence of non-specific amplification.

In the following experiment all 96 wells were used with template at one copy per partition with the knowledge that the NTCs showed no non-specific amplification. The repeat assay with template was again delayed for 25 minutes with the aim of assessing whether this increased amplification frequency (Figure 5.56).

	22		22	21		21	18	24		19	19
22		26		19		21			52		91
	22				20		59	68			28
92			28	19	35	20	34	26	17		18
	29		29	19	18				18	41	
	23	26	22		19			18	18	18	22
	19	21	18	21		74			20	90	
			22	20		19	75	22		30	



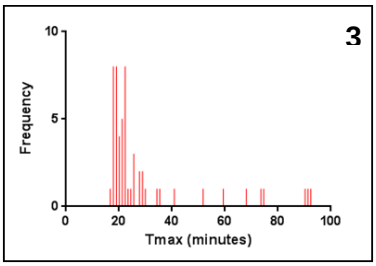
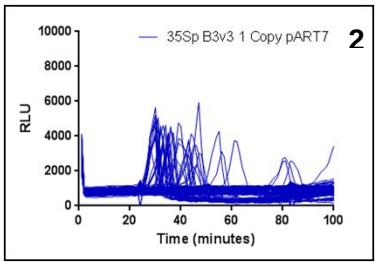


Figure 5.56: (L to R) 1 copy per reaction linearised pART7 DNA target LAMP-BART assay, total assay volume 5µl, 96 replicates, delayed start of 25 minutes (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the time to peak values from the 48 partitions

The amplification frequency of this delayed assay is 57% which is near the predicted level assuming 100% efficient amplification. The assay was also fast which may account for more positive results within the 100 minute assay time frame. Achieving this increased amplification frequency needs to be balanced with increasing the overall assay time by extending the assay preparation time.

The one copy per partition delayed start assay was repeated but the amplification frequency returned to the baseline figure for these conditions of 49% (Figure 5.57).

45	33		46		44	39	55			83	33
28					31			81		47	
81				30	33				30	30	56
		32			47	36	30		30	35	
	38	33	32		61		36	43	30		
31						39	30	35	37		
	46	30		32		31			43	35	35
43			34		39					35	



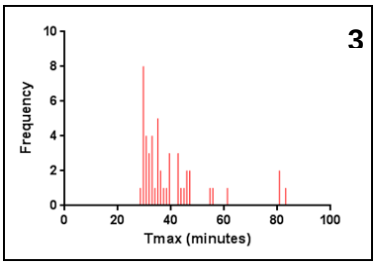


Figure 5.57: (L to R) 1 copy per reaction linearised pART7 DNA target LAMP-BART assay, total assay volume 5µl, 96 replicates, delayed start of 25 minutes (1) table to show the spread of positive results in green and the corresponding T_{max} values (2) the light output from positive results against time (3) the frequency distribution of T_{max} results shows the time to peak values from the 48 partitions

The time to peak values are slower in this assay than the previous one and may account for the reduction in amplification frequency within the 100 minutes assay timeframe. The fastest T_{max} is 28.48 minutes compared to 16.64 for the previous one. It appears that the delayed assay start may increase the amplification frequency to the expected level for single copy per partition but there is also inter-assay variation resulting in slower time-to-peak values and reduced amplification frequency.

5.6.3.2 Assay temperature

Increased assay temperature allows the potential introduction of Taq polymerase to the reaction mix as a possible way to increase amplification speed. Increasing the temperature from 60°C to 65°C would be expected to have impact on many of the components of the LAMP-BART mix including the primers and enzyme kinetics (still optimal range for Bst polymerase, but sub-optimal for luciferase), and also on the target DNA which should show increased 'breathing'.

5µl total assay volume per partition and 1x denatured primer concentration were used in a 35Sp LAMP-BART assay of the pART7 template at 65°C (Figure 5.58). A fresh aliquot of the pART7 was used.

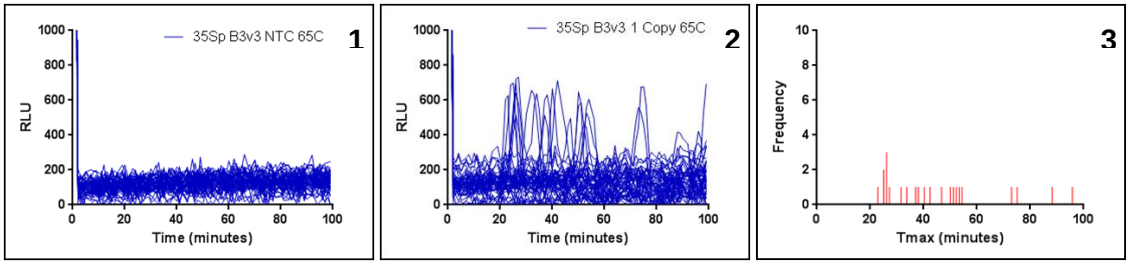


Figure 5.58: (L to R) 35Sp LAMP-BART assay, total assay volume 5µl, 65°C assay temperature (1) the light output against time for 48 NTC replicates (2) the light output from one copy per partition pART7 template against time (3) the frequency distribution of T_{max} results shows the time to peak values from the 48 partitions

The light output was low as a result of the sub-optimal temperature for the luciferase. As a result it is more difficult to differentiate BART peaks from the baseline bioluminescence. All the NTCs are free from contamination. The amplification frequency was 48%, very similar to the values obtained with this assay at 60°C. The fastest T_{max} at 22.87 minutes is slower as is the modal T_{max} at 26.13 minutes and the average T_{max} at 45.31 minutes. The standard deviation for the average T_{max} is 20.83 similar to other single copy assays at 5µl assay volume per partition.

5.6.3.3 Addition of further magnesium

In the next step towards the addition of another polymerase to the reaction mix, the concentration of magnesium ions was increased and the assay temperature maintained at 65°C (Figure 5.59).

Although there are already magnesium ions in the LAMP-BART assay from the Thermopol buffer (1X Thermopol buffer contains 2mM $MgSO_4$), the addition of Taq

polymerase to the mix may require additional magnesium and this assay aimed to test whether additional magnesium is deleterious to the overall assay.

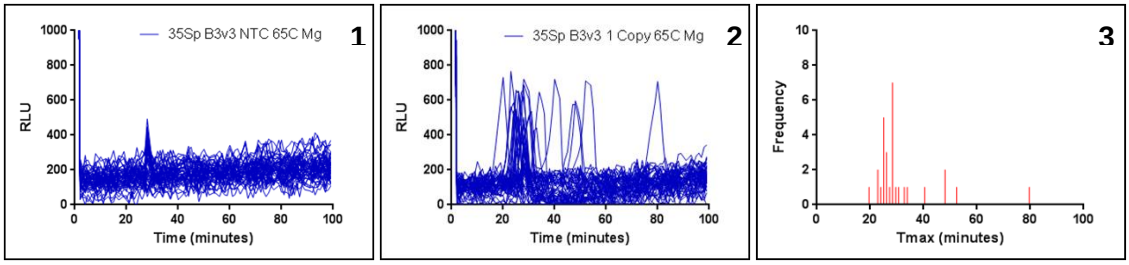


Figure 5.59: (L to R) 35Sp LAMP-BART assay, total assay volume 5µl, 65°C assay temperature, addition of extra 25mM $MgCl_2$ (1) the light output against time for 48 NTC replicates (2) the light output from one copy per partition pART7 template against time (3) the frequency distribution of T_{max} results shows the time to peak values from the 48 partitions

The high temperature provides sub-optimal conditions for the luciferase and as a consequence the light output for this assay was low. The NTC replicates remained clear throughout the assay. The amplification frequency of 60% was near the theoretical maximum for a digital assay assuming 100% efficient primers. The frequency distribution shows an isolated fastest T_{max} value of 19.61 minutes followed by a cluster of frequencies around the modal T_{max} of 28.39 minutes. There are twenty nine positive results with a highest frequency of seven. The average T_{max} of 31.54 minutes is faster than the previous assay results at 65°C. The standard deviation at 12.14 is less than the previous 65°C assay and could be the result of increased amplification efficiency from the Bst polymerase. It was concluded that the addition of extra magnesium to the reaction mix did not reduce amplification frequency in this experiment.

5.6.4 Amplification frequency of genomic template

Frozen aliquots of extracted and purified maize genomic DNA from event MON810 seeds were used fresh for each experiment. The aim of the experiments was to improve the amplification frequency for low copy number genomic template LAMP-BART assays. The results of the assays are in the Appendix (Fig App5.1 to App5.7). In summary, using genomic DNA at 10 copies per partition, no improvement was seen by increasing temperature, magnesium concentration or from the addition of Taq polymerase.

5.7 Discussion

The LAMP-BART assays for the quantification with average time-to-peak and by ultra-quantification had a volume of 20µl. At the single copies of the DNA template, reducing the volume confines the template increasing the relative concentration and increasing the sensitivity (Kalinina et al. 1997). Reducing the assay volume to 5µl showed no improvement to the amplification frequency of the LAMP-BART digital assay; however the volume reduction was small. Kalinina et al. (1997) described nanolitre volumes in micro-capillaries and nanolitre (Kumaresan et al. 2008) and picolitre (Beer et al. 2008) droplets have been described for digital PCR. The reduction in assay volume allowed for loading to 384 well plates for digital BART, but could be required to reduce further for microfluidic platforms. The light output from each partition is likely to be the limiting factor in selecting a minimum assay volume (Gandelman et al. 2010).

The mismatching displacement primer used in the 35Sp LAMP-BART assay and the mismatching LAMP and loop primers in the ADH1 LAMP-BART assay showed a slight reduction in amplification frequency at very low copy number. This slight reduction and the differences in time-to-peak values could be stochastic which would indicate a robustness of the assay for minor mismatching events with target specificity maintained. A single nucleotide polymorphism (SNP) has been located in the 35S promoter sequence (Morisset D. et al. 2009) and resulted in a 16-fold lower sensitivity of a commonly used qPCR assay when compared to a set of primers that targeted a neighbouring sequence of the promoter.

The 35Sp primer sets with B3 version 3 or 4, showed that denaturing the primers reduced the incidence of non-specific primer interactions which were evident towards the end of the LAMP-BART assay. Although denaturing template and primers appears to be beneficial to the assay, one of the desirable characteristics of using isothermal amplification is to conduct the assay at a single temperature which keeps the hardware simple and inexpensive. Either the primers need to be redesigned so that they can be used in native form, or the assay run time could be reduced to below 70 minutes, after which time non-specific primer interactions are typically observed. However reducing the assay time could exclude positive partitions that are late to amplify which would affect digital quantification using amplification frequency.

Increasing the primer concentration did not increase the amplification frequency, however faster times were observed for the first time-to-peak. One possibility for these observations is that the concentration of primers is already optimal and that the

increased concentration had a positive influence on the rate of amplification once initiation had occurred. The variance between replicates remained high suggesting that time limiting steps in LAMP amplification were unaffected by primer concentration.

The conformation of the plasmid template was more problematic for LAMP-BART than qPCR. The amplification frequency for the linearised template was similar to the qPCR assay; however the circular plasmid template produced lower amplification frequencies and slower time-to-peak values. It has been suggested before that for qPCR assays of plasmid template the linearised form provides more accurate results (Chen et al. 2007). For LAMP-BART the assay is compromised by the non-linear form of the template and suggests that the strand invasion by the LAMP primers is less likely to occur. The denaturing of the linearised plasmid gave rise to faster time-to-peak LAMP-BART results and this could be the result of removing the requirement for strand invasion. The amplification frequency did not improve suggesting that all partitions with template were amplified with 100% amplification efficiency with or without template denaturation. Denaturing the nucleic acid template for LAMP assays has been shown to increase assay sensitivity 5-fold (Suzuki et al. 2010) in the detection of cytomegalovirus. Denaturing the maize transgenic genomic template increased the amplification frequency of the 35Sp LAMP-BART assay by approximately 2-fold. The result suggests that the size of the template could be a limiting factor in assay sensitivity. This will require further investigation to establish whether template size affects LAMP amplification by assaying genomic DNA broken into smaller fragments by sonication or by testing smaller genomes.

The 384 well digital BART assays with the linearised plasmid DNA and the attempts to improve amplification frequency with the 35Sp primers, achieved a range of between 40 and 62%. The LAMP amplification with these primers is possibly 100% efficient, however it appears that there is inter-assay and stochastic variation which are not solved by the various attempted enhancements. Delaying the start of the assay for a period at room temperature produced high values of 57% and 62% from two of the three assays, but this pre-incubation step will add further time to the overall assay. The 35Sp digital BART falls just short of being quantitative with linear plasmid when the results are compared to predicted amplification frequencies (Huggett et al. 2013).

Lower amplification frequencies were observed from the LAMP amplification of maize genomic DNA. Similar values were observed with the ADH1 and 35Sp LAMP primer

assays. The initial quantification of the genomic template was assessed by gel electrophoresis and not by spectrophotometry because the gel density method is likely to give more reliable results (Kiddle et al. 2012). Inaccuracies in the initial quantification by spectrophotometry could originate from residual phenol (if used in the extraction), RNA or DNA from organelles that would contribute to the absorbance giving an artificially high value for target DNA concentration. The size of the maize genomic DNA precludes quantification by the Agilent Bioanalyzer.

In comparison to the linearised plasmid template, the amplification frequency of the genomic template was lower for all DNA concentrations below approximately 300 copies per partition. This was improved to 100 copies per partition by a single round of purification of the extracted DNA with phenol:chloroform. Additional purification with phenol:chloroform did not improve the amplification frequency: Increased residual phenol in the extract could have inhibited LAMP amplification. The single round of phenol:chloroform purification could have removed inhibitory compounds that had previously affected both qPCR and LAMP-BART, these would need to have been tightly bound to the DNA given the number of dilutions. The LOD (based on 95% positive partitions) was increased by approximately 3-fold for both qPCR and LAMP-BART by purifying the extract with phenol:chloroform. If the cause of the improvement is the removal of inhibitors then it might be expected that LAMP-BART might be improved to a lesser degree due to the greater tolerance of LAMP to certain inhibitors of PCR (Kaneko et al. 2007); (Francois et al. 2011); (Kiddle et al. 2012). However, the Promega Wizard extraction kit was designed to produce extracts for PCR amplification and not for isothermal amplifications.

The quantification of genomic DNA by digital LAMP-BART remains challenging. If the initial quantification is correct, then the efficiency of LAMP amplification is reduced by factors affecting the initiation of the amplification. Once LAMP is initiated then the newly synthesised DNA can act as template for cycling and elongation. Possible candidates for the low amplification efficiency; are the size of genomic DNA fragments, the methylation of the maize DNA or the tight binding of inhibitory compounds to the DNA.

Chapter 6
Full Dynamic Range Quantification

6.1 Introduction

In Chapters 3 and 4 it was shown that quantification of a linearised plasmid template was possible using average T_{max} down to 20-50 copies due to the extremely high reproducibility of time-to-peak measurements. Below this range of copy numbers down to single copy detection, a number of ultra-quantification methods were found to have potential to allow differentiation between individual copy numbers. Furthermore, digital BART described in Chapter 5, potentially quantifies at and below single copies per partition by using the present/absent approach. These quantitation methods taken together have the potential to quantify over a wide dynamic range of target nucleic acid concentrations. In this chapter the aim was to assess the various methods of LAMP-BART quantification, qPCR, Agilent Bioanalyzer and NanoDrop spectrophotometry on a very low but unknown concentration of the linearised plasmid pUC35S ADH1. This plasmid was used as an alternative linearised plasmid target to the pART7 used in previous chapters, because of the presence of NOSt and maize ADH1 sequences in addition to the 35Sp present in both. The optimised 35Sp primer set used for ultra-quantification and digital LAMP-BART assays of the pART7 template was used for testing both pUC35S ADH1 and pART7. After analysing sequencing results for the pUC35S ADH1 plasmid it was noted that the 35Sp sequence in this plasmid had a mismatch to the 3' end of the B3 displacement primer. In Chapter 5 section 5.3.2, a mismatch between the target sequence and the displacement primer at this point resulted in a slight reduction in amplification frequency at very low copy number and slower assay times.

6.2 Time to peak detection and quantification

First, the linearised pART7 plasmid of defined copy number was diluted to a range of copy numbers per partition from 10 to 10^6 as a standard to quantify the linearised pUC35S ADH1 sample using a 35Sp LAMP-BART assay (Figure 6.1). The pUC35S ADH1 sample of uncertain concentration was serially diluted from 10^{-1} to 10^{-10} using molecular grade water. The LAMP-BART assay using the optimised 35Sp HPLC-grade

primer set for pART7 was used to assay dilutions of pART7 and pUC35S ADH1 on the same 96 well plate to eliminate inter-assay variation.

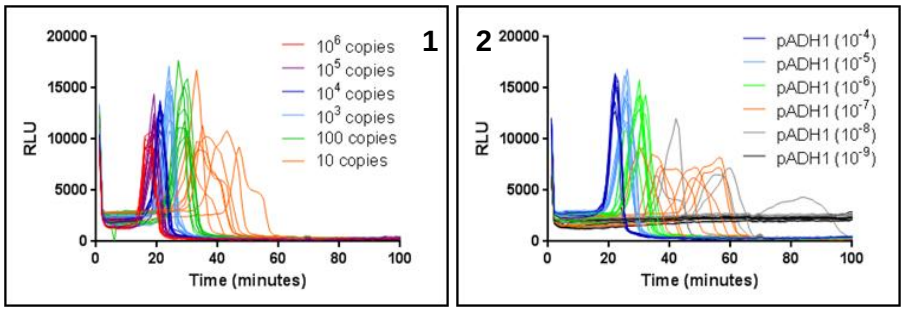


Figure 6.1: 35Sp LAMP-BART assays of linearised plasmid template, light output against assay time for (1) pART7 dilutions series from 10^6 to 10 copies per partition (20µl) (2) pUC35S ADH1 dilution from stock sample from 10^{-4} to 10^{-9}.

Visual inspection of the results for the pART7 assay indicates an increase in variation between replicates at 10 copies per partition with 100% amplification frequency. The pUC35S ADH1 dilution series showed a similar pattern of higher variation between replicates with 100% amplification frequency for 10^{-7} dilution. The more concentrated pUC35S ADH1 dilutions of 10^{-6}, 10^{-5} and 10^{-4} have low variation and are therefore more suitable for quantification using average T_{max} (Figure 6.2).

copies	mean	SD	cv	fastest	s/rate
10000	20.83	0.00	0%	20.83	100%
1000	24.39	0.51	2%	24.12	100%
100	28.50	0.83	3%	27.40	100%

dilution	mean	SD	cv	fastest	s/rate
10^{-4}	21.92	0.00	0%	21.92	100%
10^{-5}	25.76	0.59	2%	25.21	100%
10^{-6}	29.73	1.60	5%	26.31	100%

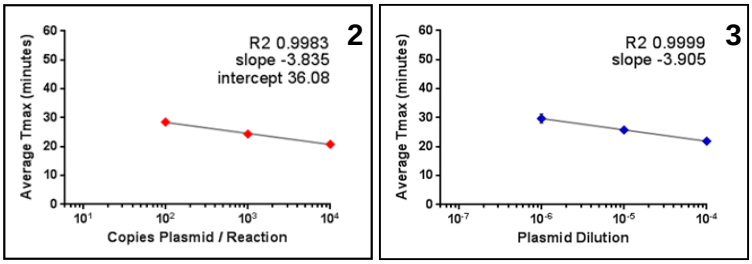


Figure 6.2: 35Sp LAMP-BART assays of linearised plasmid template (1) summary of table of results for pART7 (above) and pUC35S ADH1 (2) average T_{max} for pART7 dilutions series from 10^2 to 10^4 copies per partition (3) average T_{max} for pUC35S ADH1 dilution from stock sample from 10^{-6} to 10^{-4}.

The average T_{max} values for the 35Sp LAMP-BART assay of 10000, 1000 and 100 copies per partition differ by approximately 1 minute from the average T_{max} of the 10^{-4}, 10^{-5} and 10^{-6} dilutions of pUC35S ADH1. The gradient of the slope for the average T_{max} from the two serial dilutions of linearised plasmid target, are comparable. The equation of the line for the pART7 copies per partition against average T_{max} used to calculate the copies/µl for each of the pUC35S ADH1 dilutions (Figure 6.2, Plot 2).

$$\textbf{Copies of target/µl} = \frac{\textbf{average Tmax - intercept with y-axis}}{\textbf{gradient x 0.2 dilution factor}}$$

The dilution 10^{-4} equates to approximately 985 copies/µl, 10^{-5} to 98 copies/µl and the 10^{-6} dilution to 9 copies/µl. From this progression it was calculated that the original pUC35S ADH1 sample (dilution 10^{0}) contained approximately 10^{7} copies/µl. By assuming that the plasmid is 4330 base pairs in length, the template DNA concentration of the sample was calculated to be 0.05ng/µl from the equation below.

$$\text{ng/µl of double stranded DNA} = \frac{\text{Length in base pairs} \times 10^{9} \times 650 \text{ Daltons} \times \text{copies of target/µl}}{\text{Avogadro's constant } (6.022 \times 10^{23})}$$

The data generated in this experiment from the two linearised plasmid templates was compared using three ultra-quantification methods to assess the compatibility of the data (Figure 6.3). Fastest T_{max}, modal T_{max} and standard deviation were selected to indicate this. The pUC35S ADH1 dilutions were converted to copies/partition based on the previous calculations from average T_{max} comparison. The dilution 10^{-4} was converted to 4918 copies/5µl, 10^{-5} to 490 copies/5µl and the 10^{-6} dilution to 45 copies/5µl, for comparison with the copies/5µl pART7.

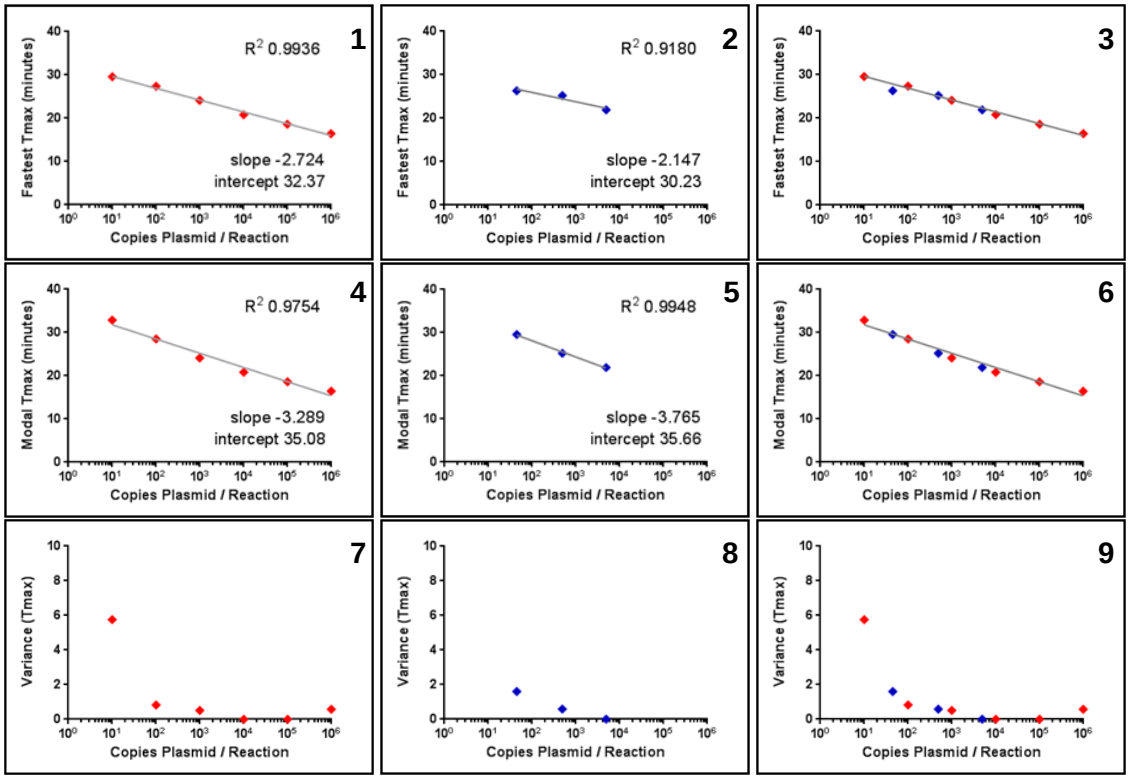


Figure 6.3: 35Sp LAMP-BART assays of linearised plasmid templates (1) fastest T_{max} for pART7 copies per partition (2) fastest T_{max} for pUC35S ADH1 copies per partition (3) overlay of fastest T_{max} results (4) modal T_{max} for pART7 copies per partition (5) modal T_{max} for pUC35S ADH1 copies per partition (6) overlay of modal T_{max} results (7) variance for pART7 copies per partition (8) variance for pUC35S ADH1 copies per partition (9) overlay of variance results

The results of these analyses are shown in Figure 6.3. The fastest T_{max} for the pART7 template correlate to a semi-logarithmic line with an R^2 value of 0.99 (Figure 6.3, Panel 1). The fastest T_{max} values for pUC35S ADH1 (Panel 2), based on the calculation of copy number by the average T_{max} quantification, when overlaid with the pART7 data fit the line with the exception of the 45 copies per partition which is faster than expected. This fastest T_{max} value was 3 minutes faster than the next fastest time-to-peak of 29.6 minutes which would be a closer fit to the line.
The modal T_{max} results for pART7 correlate to the semi-log line with an R^2 value of 0.98 (Panels 4-6). All the modal T_{max} values, from the pUC35S ADH1 dilution converted to copies per partition, closely fit this line.
The overlaid results for variance from the pART7 and pUC35S ADH1 also suggest the average T_{max} quantification calculations of pUC35S ADH1 are appropriate (Panels 7-9). The standard deviation for 45 copies pUC35S ADH1 per partition is 1.6 minutes and correlates to the increasing standard deviation that has previously been observed with reducing copy number.

In summary, the calculation of copy number based on average T_{max} is supported by the variance, modal T_{max} and the fastest T_{max} methods applied to the 35Sp LAMP-BART results of the linearised pUC35S ADH1 plasmid template in this assay, in suggesting that the original sample contained 10^7 template copies per µl, equating to a DNA concentration of 0.05ng/µl. However the presence of a mismatching displacement primer B3 (see 6.1) could have caused the underestimation of this value.

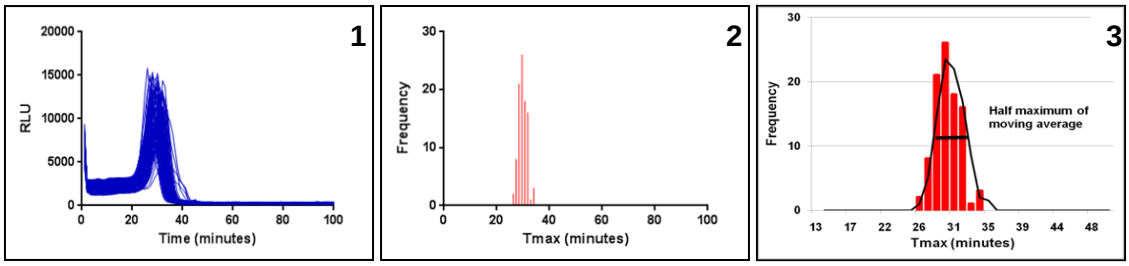
6.3 Ultra-quantification

The aim of the experiments in this section was to attempt to quantify the pUC35S ADH1 linearised plasmid using the ultra-quantification tools from Chapter 4. The LAMP-BART total assay volume in this section was maintained at 20µl and the 35Sp primer set used was previously optimised for the pART7 template.

6.3.1 Ultra-quantification of 10^{-6} pUC35S ADH1 dilution

In the first experiment the 10^{-6} dilution of the pUC35S ADH1 plasmid was used (Figure 6.4). From section 6.2 the indication was that this dilution would have 45 copies per partition which would be in the range for comparison with the ultra-quantification data from Chapter 4. The mismatched displacement primer could slow time-to-peak times which would invalidate comparisons using T_{max} and therefore calculations of

amplification frequency, variance between the replicates and the frequency distribution data were assessed.



dilution	mean	SD	cv	fastest	mode	s/rate
10^{-6}	29.85	1.63	5%	26.28	29.60	100%

4

Figure 6.4: 35Sp LAMP-BART assay of 10^{-6} dilution from original stock of linearised pUC35S ADH1 plasmid (1) the light output against assay time (2) the frequency distribution of T_{max} results showing the spread of T_{max} results after the fastest T_{max}, time and frequency of the modal T_{max} (3) the Full Width Half Maximum of the two point moving average of T_{max} frequency distribution (4) summary table of results

The average T_{max} for this LAMP-BART assay was 29.85 minutes with standard deviation of 1.63. Comparison to the previous assay of this dilution shows a marginal inter-assay variation with 29.73 minutes for the average T_{max} and 1.60 for the standard deviation (Figure 6.5). This is supported by other measurements; fastest T_{max} in this assay was evident at 26.28 minutes and the modal T_{max} is 29.60 minutes, compared to 26.31 minutes and 29.59 minutes for the previous assay.

The highest frequency of T_{max} results is 26 from 95 positive results (27.4% as a percentage of positive results) and the FWHM of the frequency distribution of T_{max} results is 4.27. The amplification frequency of 100% is to be expected from the assumed concentration of template.

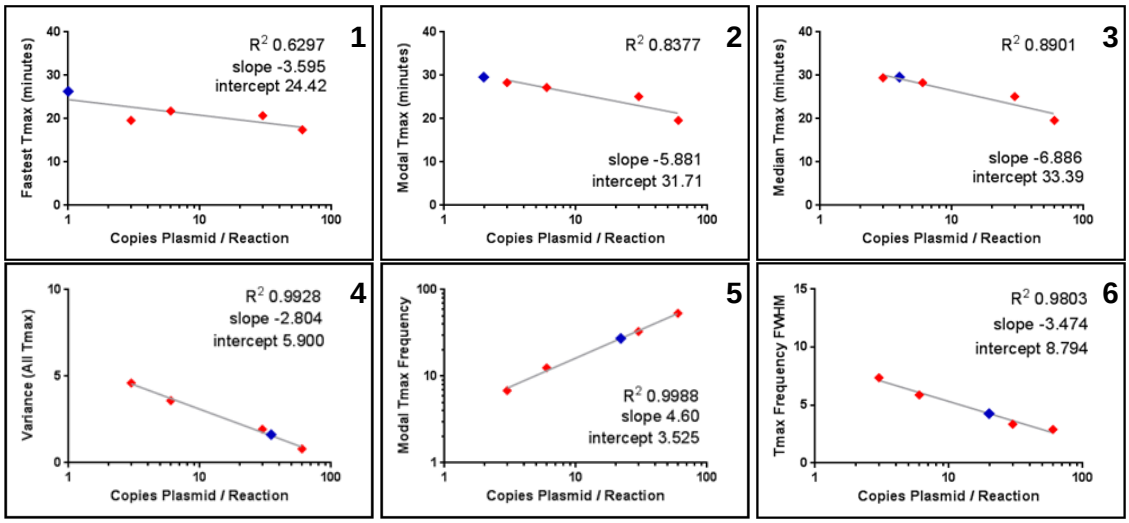


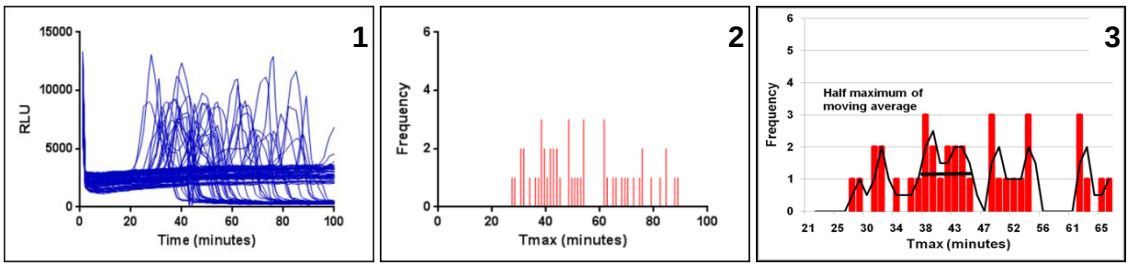
Figure 6.5: 35Sp LAMP-BART assay of 10^{-6} dilution from original stock of linearised pUC35S ADH1 plasmid (in blue) plotted with the 60, 30, 6 and 3 copies pART7 template per partition data from Chapter 4 (in red) (1) fastest T_{max} (2) modal T_{max} (3) median T_{max} (4) variance (5) modal T_{max} frequency (6) full width half maximum of two point moving average T_{max} frequency distribution

By comparing the pUC35S ADH1 10^{-6} dilution results to ultra-quantification measurements of 60, 30, 6 and 3 copies pART7 per partition (Chapter 4), fastest T_{max}, modal T_{max} and median T_{max} measurements indicate a copy number per partition of less than 3 copies. However, the other measurements indicate the template concentration to be between 20 and 30 copies per partition. The single digit copy number results from the measurements based on T_{max} suggest that the time-to-peak data is delayed. This is also evident with the T_{max} results for the pART7 assay, used to compare average T_{max} with the results for pUC35S ADH1 in section 6.2, which was greater than 7 minutes slower for time-to-peak measurements than comparable assays from Chapter 4. Another indicator that the pUC35S ADH1 copy number per partition in the 10^{-6} dilution is greater than 1 to 3 copies is the 100% amplification frequency of positive replicates. The inter-assay variation between the data used in Chapter 4 and this data highlight the requirement for calibrants when using ultra-quantification tools based on T_{max} measurements. Discrepancies between the different ultra-quantification approaches (those based on T_{max} and those based on variance and frequency data) could be used to indicate assay problems from, for example, sequence mismatches caused by single nucleotide polymorphisms.

6.3.2 Ultra-quantification of 10^{-7} pUC35S ADH1 dilution

In a second experiment, the 10^{-7} dilution of the pUC35S ADH1 sample was used and compared to the data from Chapter 4 of 10, 5, 4, 3, 2 and 1 copy of pART7 per partition

(Figure 6.6). The data from section 6.3.1 suggests that this dilution could contain 2 to 3 copies per partition at this level the amplification frequency would be expected to reduce. The total assay volume was kept at 20µl.



dilution	mean	SD	cv	fastest	mode	s/rate
10^{-7}	53.5	17.53	33%	27.35	38.29	53%

4

Figure 6.6: 35Sp LAMP-BART assay of 10^{-7} dilution from original stock of linearised pUC35S ADH1 plasmid (1) the light output against assay time (2) the frequency distribution of T_{max} results showing the spread of T_{max} results after the fastest T_{max}, time and frequency of the modal T_{max} (3) the Full Width Half Maximum of the two point moving average of T_{max} frequency distribution (4) summary table of results

The highest frequency of T_{max} results is 3 from 96 positive results (3.1% as a percentage of positive results) and the FWHM of the frequency distribution of T_{max} results is 7.62. The FWHM value is indicative of approximately 1 copy per partition as is the high assay T_{max} variance of 17.53 minutes. The other ultra-quantification measurements point to less than 1 copy per partition for this assay (Figure 6.7).

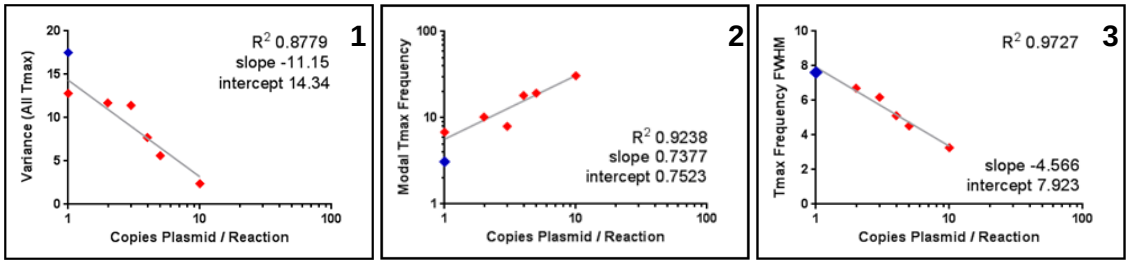


Figure 6.7: 35Sp LAMP-BART assay of 10^{-7} dilution from original stock of linearised pUC35S ADH1 plasmid (in blue) plotted with the 10, 5, 4, 3, 2 and 1 copy pART7 template per partition data from Chapter 4 (in red) (1) variance (2) modal T_{max} frequency (3) full width half maximum of two point moving average T_{max} frequency distribution

The amplification frequency for this assay was 53% which implies 1 to 2 copies per partition based on previous amplification frequency data. Once again the assays appear slow for all values of T_{max} by approximately seven minutes.

The use of the ultra-quantification methods can only indicate that these assays contained low copy numbers based on the amplification frequency, variance, modal T_{max} frequency and T_{max} frequency FWHM. Assuming that the 10^{-7} dilution of pUC35S

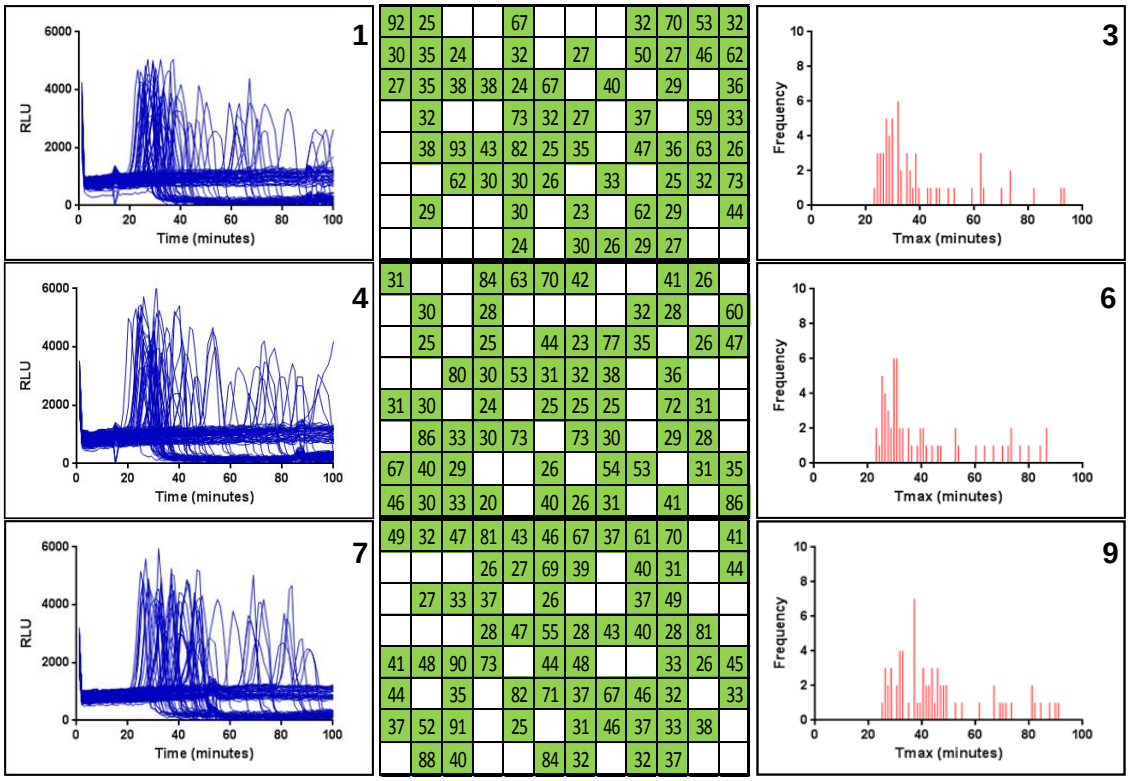
ADH1 contained 1 to 2 copies per partition, the undiluted sample would therefore have a DNA concentration of approximately 0.02ng/µl, which is lower than the concentration suggested by the average T_{max} quantification method.

6.4 Digital BART

The aim of the experiments in this section was to quantify the pUC35S ADH1 linearised plasmid using the digital BART method from Chapter 5. The LAMP-BART total assay volume in this section was reduced to 5µl and the template was added directly to the mastermix. The 35Sp primer set used was previously optimised for the pART7 template, unless otherwise stated.

6.4.1 Digital BART quantification of 10^{-7} pUC35S ADH1 dilution

In these experiments, the 10^{-7} dilution of the pUC35S ADH1 plasmid was used (Figure 6.8). From section 6.3 the indication was that this dilution would have approximately 1 copy per partition which is suitable for quantitation with both ultra-quantification and digital BART quantification. The aim of these experiments was to compare the amplification frequencies between the two LAMP-BART assay protocols for the same pUC35 ADH1 dilution and to investigate the variation between three replicate assays, before diluting the sample further to 10^{-8}. At this dilution, which would equate to a DNA concentration of 0.5 ag/µl (attogram = 10^{-18} grams), only digital BART would be suitable for quantitation.



92	25			67				32	70	53	32
30	35	24		32		27		50	27	46	62
27	35	38	38	24	67		40		29		36
	32			73	32	27		37		59	33
	38	93	43	82	25	35		47	36	63	26
		62	30	30	26		33		25	32	73
	29			30		23		62	29		44
				24		30	26	29	27		

31			84	63	70	42			41	26	
	30		28					32	28		60
	25		25		44	23	77	35		26	47
		80	30	53	31	32	38		36		
31	30		24		25	25	25		72	31	
	86	33	30	73		73	30		29	28	
67	40	29			26		54	53		31	35
46	30	33	20		40	26	31		41		86

49	32	47	81	43	46	67	37	61	70		41
			26	27	69	39		40	31		44
	27	33	37		26			37	49		
			28	47	55	28	43	40	28	81	
41	48	90	73		44	48			33	26	45
44		35		82	71	37	67	46	32		33
37	52	91		25		31	46	37	33	38	
	88	40			84	32		32	37		

Figure 6.8: Three Digital 35Sp LAMP-BART assays of 10^{-7} dilution from original stock of linearised pUC35S ADH1 plasmid, 0.5µl of the dilution per partition with a total assay volume of 5µl (1,4,7) the light output against assay time (2,5,8) the positions of positive results and the associated T_{max} values (3,6,9) the frequency distribution of T_{max} results showing the spread of T_{max} results after the fastest T_{max}, time and frequency of the modal T_{max}.

The amplification frequencies of 63%, 64% and 68% for these three assays are close to the expected frequency for 1 copy per partition with 100% efficiency of amplification. The variation between the amplification frequencies from the three assays is low with a coefficient of variation value of 4%. The amplification frequency from the ultra-quantification assay of the same dilution from section 6.3.2 was 53%. This small variation between assays is likely to be stochastic.

The mean values from the three replicate digital BART assays from the uCount^{SM} internet software are 1.009, 0.9808 and 1.130, giving an average of 1.04 copies per partition with standard deviation of 0.08. This gives 104 copies from the 50µl volume of the pUC35S ADH1 10^{-7} dilution used in the assay. This equates to 2.08 x 10^{7} copies/µl for the original sample or 0.10ng/µl which is twice the DNA concentration suggested by the average T_{max} quantification.

6.4.2 Digital BART quantification of 10^{-8} pUC35S ADH1 dilution

In this experiment, the LAMP-BART digital assay used the 35Sp primer set with B3 version 4 which is a perfect match to the 35Sp sequence of the pUC35S ADH1 template (Figure 6.9). The template was diluted to 10^{-8} and the aim was to calculate the DNA concentration of the original sample using digital BART only.

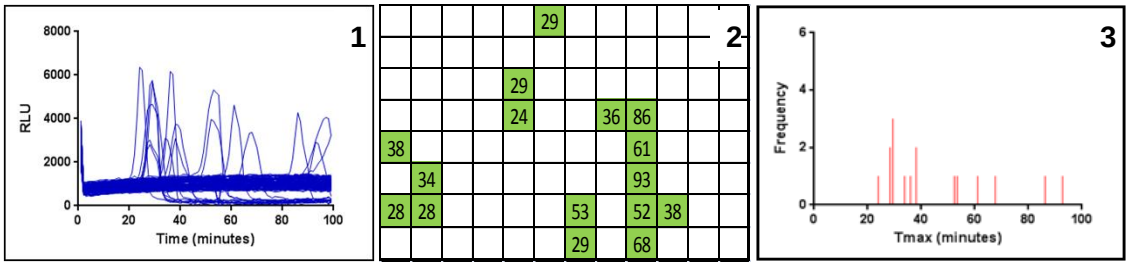


Figure 6.9: Digital 35Sp LAMP-BART assay of 10^{-8} dilution from original stock of linearised pUC35S ADH1 plasmid, 0.5µl of the dilution per partition with a total assay volume of 5µl (1) the light output against assay time (2) the positions of positive results and the associated T_{max} values (3) the frequency distribution of T_{max} results showing the spread of T_{max} results after the fastest T_{max}, time and frequency of the modal T_{max}

The average T_{max}, fastest T_{max}, variance and modal T_{max} are all too high for ultra-quantitation. The low amplification frequency and high variance of T_{max} results have also removed the possibility of calculating the frequency FWHM and maximum frequency measurements for quantification.

Using the uCount^{SM} software at a 99% confidence level, the 16 positive partitions from 96 partitions resulted in a mean of 0.1823 copies per partition with an upper and lower confidence interval of 0.3092 and 0.0729 copies per partition. Therefore for this assay there were between 7 and 31 copies with a mean of 18 copies in the 50µl of the $1x10^{-8}$ dilution of pUC35S ADH1 spread across the partitions. The previous triplicate assays with the mismatched B3 primer gave mean values of 101, 98 and 113 copies in the 50µl of the $1x10^{-7}$ dilution of pUC35S ADH1. Therefore the mismatched B3 primer version 3 used previously had an impact on the quantitation of pUC35S ADH1. Taking the mean value from uCount^{SM} of 0.1823 copies per partition, gives 18 copies from the 50µl volume of the pUC35S ADH1 10^{-8} dilution used in the assay. This equates to 3.6×10^{7} copies/µl for the original sample or 0.18ng/µl which is almost twice the DNA concentration suggested by the digital assay with the 10^{-7} linearised plasmid dilution with the mismatched displacement primer.

In summary, the quantitation of the pUC35S ADH1 linearised plasmid using time-to-peak results relative to a titration of pART7, suggested a concentration of 0.05ng/µl,

although the B3 primer used had a mismatch to the pUC35S ADH1 sequence. Ultra-quantification using previous data for low copy numbers per partition of the linearised pART7 plasmid were impeded by the slow time-to-peak times, but some of the measurements suggested a concentration of the pUC35S ADH1 sample of 0.02ng/µl. The ultra-quantification highlighted the requirements for appropriate comparative data for a particular primer set, data for the reduced total assay volume of 5µl and the need for calibrants to compensate for inter-assay variation. The digital assays with the mismatched displacement primer, suggested that the concentration of the pUC35S ADH1 samples was 0.10ng/µl, but the greater template dilution with matching primers indicated a DNA concentration of 0.18ng/µl. There is almost a tenfold difference between the highest and lowest quantifications of DNA concentration across these methods.

6.6 Quantification by Bioanalyzer and spectrophotometry

The original concentration of the linearised pUC35S ADH1 plasmid was assessed using the Agilent Bioanalyzer (Figure 6.10). Indications from the LAMP-BART and digital BART assays were that the concentration was below the optimum concentrations for accurate measurement of 0.5 to 50ng/µl. No dilutions were therefore prepared of the sample for testing.

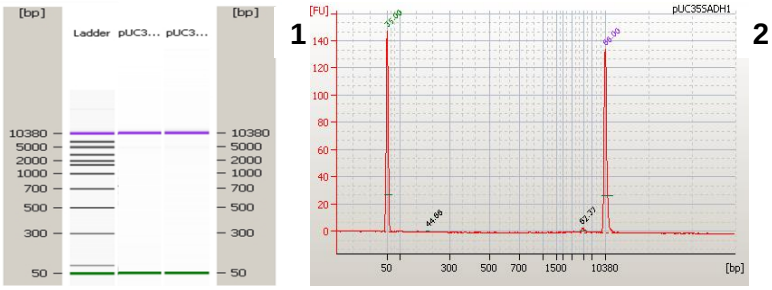


Figure 6.10: Quantification of the linearised pUC35S ADH1 samples using the Agilent Bioanalyzer (1) ladder and upper and lower marker bands with test lanes showing feint bands at approximately 5000bp (2) electropherogram showing the upper and lower ladder markers at a small peak representing the plasmid sample

From panel 1 of Figure 6.10, a faint band is visible at approximately 5000bp in the two lanes containing the undiluted linearised plasmid DNA. As expected the concentration of the sample is too low to be accurately calculated and a value of 0.1ng/µl was returned. This value is in the middle of the range of values from LAMP-BART and digital BART assays of 0.02 to 0.18ng/µl.

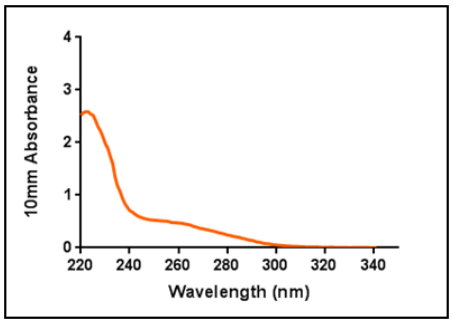
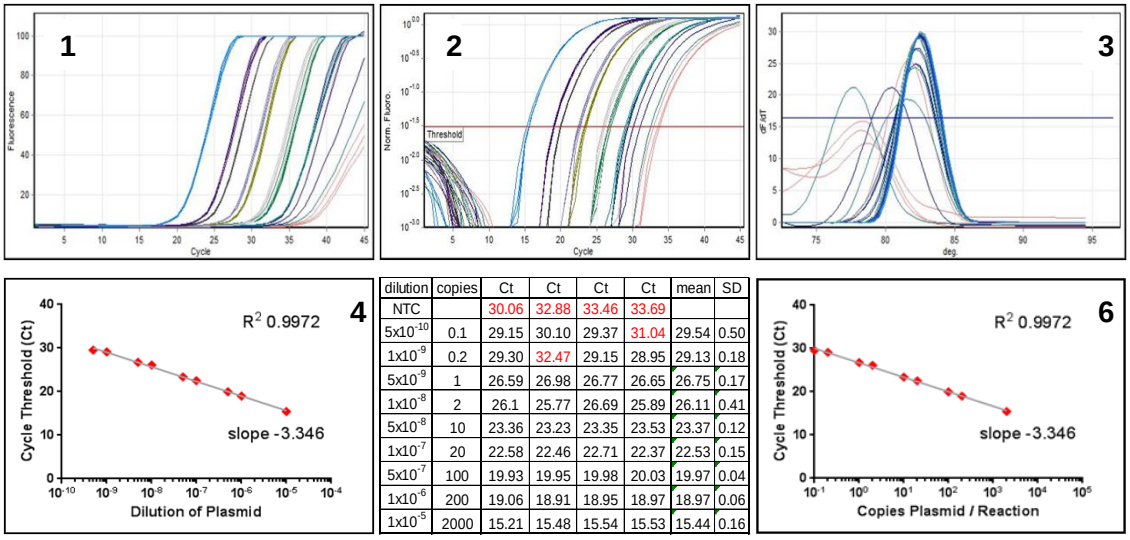


Figure 6.11: Thermo Fisher NanoDrop results for undiluted pUC35S ADH1, graphical representation of the 10mm absorbance with increasing wavelength.

The NanoDrop (Figure 6.11) indicated a DNA concentration for the plasmid of 23.54ng/µl, but the profile of absorbance against wavelength was atypical. From the manufacturer's instructions, the limit of detection for double stranded DNA is 2ng/µl and it is possible that this result reflects this.

6.7 qPCR 35Sp assay of pUC35S ADH1

The linearised pUC35S ADH1 sample was quantified using the qPCR method described in Chapter 2 utilising the 35Sp PCR primers from Fernandez S (2005). 5µl of the diluted template was added to 15µl mastermix containing the primers, MGW and SYBR® Green JumpStart™ Taq ReadyMix™ (Figure 6.12).



dilution	copies	Ct	Ct	Ct	Ct	mean	SD
NTC		30.06	32.88	33.46	33.69		
$5x10^{-10}$	0.1	29.15	30.10	29.37	31.04	29.54	0.50
$1x10^{-9}$	0.2	29.30	32.47	29.15	28.95	29.13	0.18
$5x10^{-9}$	1	26.59	26.98	26.77	26.65	26.75	0.17
$1x10^{-8}$	2	26.1	25.77	26.69	25.89	26.11	0.41
$5x10^{-8}$	10	23.36	23.23	23.35	23.53	23.37	0.12
$1x10^{-7}$	20	22.58	22.46	22.71	22.37	22.53	0.15
$5x10^{-7}$	100	19.93	19.95	19.98	20.03	19.97	0.04
$1x10^{-6}$	200	19.06	18.91	18.95	18.97	18.97	0.06
$1x10^{-5}$	2000	15.21	15.48	15.54	15.53	15.44	0.16

Figure 6.12: qPCR 35Sp assay of linearised pUC35S ADH1 plasmid template titrations from $1x10^{-5}$ to $5x10^{-10}$ NTC, $5x10^{-10}$, $1x10^{-9}$, $5x10^{-9}$, $1x10^{-8}$, $5x10^{-8}$, $1x10^{-7}$, $5x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, (1) fluorescence against amplification cycles showing sigmoidal signals generated by amplification (2) normalised fluorescence with position of the cycle threshold (3) melt curve analysis with threshold position (4) cycle threshold results plotted against the dilution of the template (5) summary table of results with removed values in red and suggested copy numbers per reaction associated with each dilution (6) cycle threshold plotted against suggested copy numbers per reaction

The sigmoidal shape (panel 1 Fig. 6.12) seen with the least dilute 10^{-5} sample (in light blue, furthest left) becomes less steep at approximately 10^{-9}, this is also the level at which the melt curve analysis indicates lower temperatures for the transition from double stranded to single stranded DNA than the 82.5°C for the 35Sp amplicon. The PCR amplification efficiency for this assay is 94% which represents an optimised assay. The cycle threshold values for amplified template plateau at approximately 29, which is associated with the $5x10^{-10}$ and $1x10^{-9}$ dilutions. The assay of single copies of template by this qPCR assay may therefore lie between the $1x10^{-9}$ and $5x10^{-9}$ dilutions of template and arbitrarily the $5x10^{-9}$ dilution was associated with 1 copy per partition and the other dilutions extrapolated from this association. Panel 6 of Fig. 6.12 shows the assumed copy numbers plotted against the cycle threshold values. The graph shows the cycle threshold plateau at approximately 1 copy per reaction. If the 10^{-7} dilution represents 4 copies/µl (20 copies per partition), then this assay suggests that the original concentration of the linearised pUC35S ADH1 contained would be $4x10^{7}$ copies/µl or 0.20ng/µl. This value is very similar to the 0.18ng/µl derived from the digital BART assay with optimised 35Sp primers.

6.8 Digital BART of pUC35S ADH1 using NOSt primers

The construction of the linearised plasmid pUC35S ADH1 brought together three DNA recognition sequences for the LAMP primer sets; 35Sp, NOSt and ADH1.
The NOS terminator primer set was optimised as part of a final year project by student Christian Chess (data not shown) to give the final set used in these experiments. The LAMP primers denoted FIP and BIP were redesigned (details of which are in Chapter 2) and all other LAMP primers were the original version. HPLC grade primers were used.

6.8.1 Digital BART NOSt assay of NTCs

The aim of this first experiment was to ensure that the primers in the digital LAMP-BART assay would not lead to non-specific primer interactions and that the anti-contamination procedures in place continue to be effective (Figure 6.13).

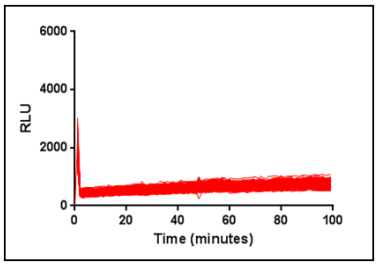


Figure 6.13: Digital BART assay with NOSt primers, assay of NTCs in 96 partitions. Graphical representation of the light output against assay time from the 96 partitions

No peaks were observed from the digital BART NOSt assay and this indicates that non-specific primer interactions and contamination were not evident.

6.8.2 Digital BART NOSt assay of 10^{-8} dilution of pUC35S ADH1

In this experiment, the LAMP-BART digital assay used the NOSt primer set with redesigned FIP and BIP primers for the NOSt sequence of the pUC35S ADH1 template (Figure 6.14). The template was diluted to 10^{-8} with the aim of comparing the results to the 35Sp digital BART results from section 6.4.2.

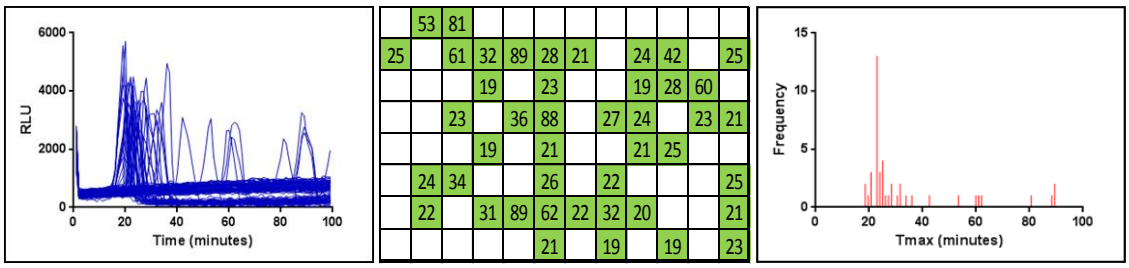


Figure 6.14: Digital NOSt LAMP-BART assay of 10^{-8} dilution from original stock of linearised pUC35S ADH1 plasmid, 0.5µl of the dilution per partition with a total assay volume of 5µl (1) the light output against assay time (2) the positions of positive results and the associated T_{max} values (3) the frequency distribution of T_{max} results showing the spread of T_{max} results after the fastest T_{max}, time and frequency of the modal T_{max}

There is no comparative data for NOSt ultra-quantification and therefore only the amplification frequency was used for quantification.

Using the uCount^{SM} software at a 99% confidence level the 44 positive partitions from 96 partitions resulted in a mean of 0.6131 copies per partition with an upper and lower confidence interval of 0.8784 and 0.3908 copies per partition. Therefore for this assay there were between 39 and 88 copies with a mean of 61 copies in the 50µl of the 10^{-8} dilution of pUC35S ADH1 spread across the partitions. Therefore this dilution of the template has 1.23 copies/µl. This equates to $1.23x10^{8}$ copies/µl or 0.57ng/µl for the undiluted pUC35S ADH1 sample, compared to 0.18ng/µl measured using the 35S

primers. Assuming that there was no variation in dilution, the amplification frequency implies that the NOSt primer set is marginally better optimised than the 35Sp primer set.

6.9 Digital BART of pUC35S ADH1 using ADH1 primers

The linearised pUC35S ADH1 plasmid contains the sequence for maize alcohol dehydrogenase gene ADH1. The ADH1 primer set was redesigned (Chapter 5, section 5.3.3) to replace the mismatching sequences with FIP and B-Loop primers (denoted version 3 for the new primers). The template was again diluted to 10^{-8} with the aim of comparing the results to the 35Sp digital BART results from section 6.4.2 and the NOSt digital results from the previous section (Figure 6.15).

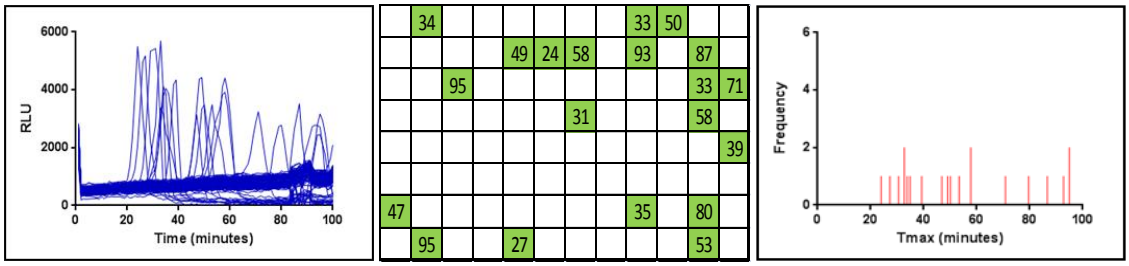


Figure 6.15: Digital ADH1 LAMP-BART assay of 10^{-8} dilution from original stock of linearised pUC35S ADH1 plasmid, 0.5µl of the dilution per partition with a total assay volume of 5µl (1) the light output against assay time (2) the positions of positive results and the associated T_{max} values (3) the frequency distribution of T_{max} results showing the spread of T_{max} results after the fastest T_{max}, time and frequency of the modal T_{max}

As with the NOSt digital LAMP-BART data, there is no comparative data for ADH1 ultra-quantification and as a result only the amplification frequency was used for quantification. From Chapter 5 it was apparent that the ADH1 primer set could result in a small number of false positive results from non-specific primer interactions after approximately 70 minutes of assay run time. In this assay six of the positive results had T_{max} values greater than 70 minutes and a proportion of those could be the result of non-specific primer interactions. Unfortunately the plate was discarded before the LAMP ladder patterns could be investigated by gel electrophoresis. Calculations of copy number per well were carried out both for the total 20 positive partitions and the reduced amplification frequency of 14 positive partitions assuming that all positive peaks above 70 minutes were false positives.

The uCount^{SM} internet software, at a 99% confidence level for the 20 positive partitions from a total of 96 partitions, indicated a mean of 0.2336 copies per partition with an

upper and lower confidence interval of 0.3790 and 0.1082 copies per partition. These values show that for this assay there were between 11 and 38 copies with a mean of 23 copies in the 50µl of the 10^{-8} dilution of pUC35S ADH1 spread across the partitions. Therefore this dilution of the template has 0.46 copies/µl. This equates to $4.6x10^{7}$ copies/µl or 0.21ng/µl for the undiluted pUC35S ADH1 sample.
The reduced assay result of 14 positive partitions indicated a mean of 0.1576 with upper and lower confidence intervals of 0.2738 and 0.05642. These calculations show that there were between 5 and 27 copies with a mean of 16 copies in the 50µl of the 10^{-8} dilution of pUC35S ADH1 spread across the partitions. This equates to $3.2x10^{7}$ copies/µl or 0.15ng/µl for the undiluted pUC35S ADH1 sample, compared to $1.23x10^{8}$ copies/µl or 0.57ng/µl measured using the NOSt primers.

In summary the digital BART assay results from the 35Sp (with the perfectly matching B3 displacement primer), NOSt and ADH1 primer sets of the 10^{-8} dilution of the pUC35S ADH1 linearised plasmid template, are shown in the table below (Figure 6.16):

digital BART primer set	assay positives	uCount^{SM} mean/50µl	uCount^{SM} upper/50µl	uCount^{SM} lower/50µl	sample copies/µl	sample conc. ng/µl
35Sp	18	18	31	7	3.8×10^{7}	0.18
NOSt	44	61	88	39	1.2×10^{8}	0.57
ADH1 $^{(H)}$	20	23	38	11	4.6×10^{7}	0.21
ADH1 $^{(L)}$	14	16	27	5	3.2×10^{7}	0.15

Figure 6.16: Summary table of digital BART quantification of 10^{-8} pUC35S ADH1 dilution, ADH1$^{(H)}$ denotes results based on 20 positive partitions and ADH1$^{(L)}$ denotes results based on 14 positive partitions.

The coefficient of variation for the quantitation of the undiluted sample is 70% due to the result from the NOSt digital BART assay, this value is reduced to 18% with the exclusion of the NOSt calculation. Using the upper confidence interval results for the 35Sp and ADH1 uCountSM copies/50µl and lower result for NOSt gives quantitation values with correlation of variation of 17% between the three assays.

6.10 Discussion

The aim of this chapter was to quantify a single DNA template with multiple target sequences over a wide dynamic range using the average time-to-peak, ultra-quantification and digital LAMP-BART methods. Assumptions were made about the linearised plasmid template used in this chapter and the LAMP-BART assay. It was assumed that the pUC35S ADH1 and pART7 templates would give identical time-to-

peak results at identical copy numbers and that the purity and possible presence of inhibitors were similar for both. The LAMP-BART assay of pART7 and prior data used for ultra-quantification used the 35Sp LAMP displacement primer (version 3) which corrected for the sequence mismatch with this target. It was assumed that the mismatching displacement primer would not affect LAMP-BART time-to-peak results at higher copy number and in the range for ultra-quantification. However, it is probably that this mismatch has an impact at low copy number. For the time-to-peak and ultra-quantification LAMP-BART assays the total reaction volume was 20µl to enable comparison to the previously generated data in Chapter 4. For digital BART the total assay volume was reduced to 5µl. To quantify using a LAMP-BART assay across a wide range of target concentrations this lower assay volume would enable multiple partitions without using precious sample volumes. The higher volume was required for comparative analysis.

The undiluted pUC35S ADH1 plasmid template would be quantifiable by LAMP-BART using average time-to-peak measurements providing that an appropriate calibration of results was in place. However, the sample was too concentrated for quantitation by LAMP-BART ultra-quantification and digital BART and required dilution of between 10^{-6} and 10^{-8}. The undiluted sample appeared to be too dilute for quantification using both the Agilent Bioanalyzer and NanoDrop spectrophotometer techniques.

The 35Sp LAMP-BART assay of the pART7 template of known copy number indicated that the assay displayed increased time-to-peak when compared to the previous data from Chapter 4. The assays of pUC35S ADH1 also appear to have run slowly with a longer assay time before the BART peaks. Although the conditions, primers and reactants are the same for the LAMP-BART assays in this chapter as those in Chapter 4, the variation in assay time may be the result of activity variation between aliquots of Bst polymerase. The ultra-quantification methods based on time-to-peak measurement for the quantification of pUC35S ADH1 were affected by the slow assay and compared poorly to the previous data from Chapter 4. It may be possible to adjust the data by using high and low calibrating assay samples of known copy number to compensate for inter-assay variation.

The triplicate 35Sp digital LAMP-BART assays of the 10^{-7} diluted sample showed low variation between the assays with amplification frequencies of 63%, 64% and 68%. However, a small difference in amplification frequency for the dilution gave a large

difference between copy numbers (approximately 3 x 10^6 copies/µl) when extrapolated to the concentration of the undiluted sample.

LAMP BART 35Sp assay of pUC35S ADH1	sample dilution	undiluted sample copies/µl	undiluted sample conc. ng/µl
average time-to-peak	10^{-4} to 10^{-9}	1.0 x 10^7	0.05
average time-to-peak$^{(V)}$	10^{-7}	2.0 x 10^7	0.09
ultra-quantification 1	10^{-6}	0.6 x 10^7	0.03
ultra-quantification 2	10^{-7}	0.5 x 10^7	0.02
digital 1	10^{-7}	2.0 x 10^7	0.09
digital 2	10^{-7}	2.0 x 10^7	0.09
digital 3	10^{-7}	2.3 x 10^7	0.11
digital 35Sp $^{(B3v4)}$	10^{-8}	3.8 x 10^7	0.18

Figure 6.17: Summary table of 35S LAMP-BART quantification of dilutions of pUC35S ADH1 dilution, average time-to-peak$^{(V)}$ denotes visual interpretation of 10^{-7} dilution to 10 copies per partition, digital 35Sp$^{(B3v4)}$ denotes the replacement of the mismatched displacement primer B3 version 3 to the sequence matching primer B3 version 4.

Summarising the 35Sp quantification of pUC35S ADH1 (Fig. 6.17), the digital assay with the non-mismatching LAMP displacement primer at the lowest dilution (10^{-8}) had the highest value for the copy numbers/µl for the original sample (3.8 x 10^7). However, the value for the lower confidence integral derived from the uCount^{SM} analysis was 1.4 x 10^7 or 0.7ng/µl which is in the range 0.02 to 0.11ng/µl from the other assays. It is probable that the displacement primer mismatch will have had an impact on the pUC35S ADH1 assays especially at low copy number per partition.

The qPCR assay suggested an undiluted sample concentration of 0.20ng/µl but would require calibration of the Ct values for more accurate results. The calculation was based on the observation that the amplification frequency was reduced after 29 cycles and that the 35Sp PCR primers were previously (Chapter 5) able to detect single copies per partition of the linearised pART7 template.

The final digital LAMP-BART assays with the improved primer sets of ADH1 and NOSt suggested that the LAMP 35Sp primer set could be further optimised.

In summary the inclusion of a number of partitions of appropriate calibrating template of known copy number would be essential for those samples that are in the concentration range for quantification using average time-to-peak. The total assay volume of the LAMP-BART assay should be sufficiently low for digital and ultra-quantification analysis of multiple partitions to reduce the consumption of the sample. For future developments

with micro-fluidic chambers this volume may be very low and there needs to be a balance with light output of BART peaks against baseline bioluminescence (if the volume is too low BART peaks will be indistinguishable). Digital LAMP-BART assays require that every target molecule be successfully amplified; therefore the three primer sets require further optimisation to ensure parity of results, although the variation in results between them could be stochastic.

Chapter 7
Discussion and conclusions

7.1 Discussion of aims and objectives

Low DNA template copy number quantification was previously reported to be limited to between 55 to 550 copies per assay for plasmid DNA (Gandelman et al. 2010) and approximately 10^5 copies per assay for genomic DNA (Kiddle et al. 2012), with the LAMP-BART coupled assay. Other detection approaches used with LAMP have reported LOQ values of 2000 copies of lambda DNA using turbidimetry (Mori et al. 2004), approximately 210 copies hepatitis B DNA virus using fluorescence (Cai et al. 2008) and 1000 copies of human cytomegalovirus (Nixon et al. 2014). In this thesis, the LAMP-BART assay with 35Sp primers of the linearised plasmid pART7 template consistently achieved quantification between 10 and 100 copies per assay using average time-to-peak from replicates. After optimisation of the assay, the LOQ (based on average time-to-peak) was nearer to the lower copy number in that range and ultra-quantification and digital BART reduced this value further to the threshold of absolute quantitation.

Improved quantitative capability of LAMP-BART

The LAMP-BART assay of LAMP amplicon gel bands showed a linear correlation of average time-to-peak against assay time for 10 orders of magnitude on a semi-logarithmic scale. This is higher than the usual linear dynamic range reported for qPCR of 5-6 orders of magnitude (Bustin et al. 2009). The linear dynamic range for the ChAT plasmid (Gandelman et al. 2010) was 7 orders of magnitude. The LAMP-BART assay is limited by increasing variance between replicates at very low copy number but the linear dynamic range is potentially far wider than qPCR. The highest concentration of template that could remain correlated to the linear line could be limited by the increasing concentration of non-target DNA; which has been shown to negatively influence the BART peak times at concentrations greater than 110ng per assay. Therefore the maize genomic template the linear dynamic range is likely to be narrower than for the plasmid templates.

Parameters affecting LAMP-BART assay kinetics

The specificity and sensitivity of LAMP-BART were unaffected by 100ng/µl foreign DNA (Gandelman et al. 2010) with plasmid DNA. The LAMP reaction has also been shown to tolerate 200ng non-target DNA but above this level results were unreliable (Lee et al. 2009). In this thesis, the presence of non-target DNA in the assay was shown to have a positive influence on LAMP-BART assay kinetics. No influence was observed with non-target DNA on qPCR. The range of non-target DNA of 30 to 110ng/assay using both maize genomic DNA and sonicated salmon sperm DNA carrier improved sensitivity, reproducibility and time-to-peak values were shorter (Kiddle et al. 2012). For subsequent assays with linearised plasmid and genomic DNA template the carrier DNA was adjusted to 100ng/assay.

The initiation of LAMP amplification requires the strand invasion of the double stranded DNA template by loop-forming LAMP primers which are subsequently displaced by displacement primers (Notomi et al. 2000). Standard LAMP reactions contain two loop-forming primers (denoted FIP and BIP), two displacement primers (F3 and B3) and two loop primers (LF and LB) for rapid, sensitive results. In Chapter 3, the assay successfully amplified the DNA template without the presence of displacement primers albeit with reduced sensitivity and increased variability between replicates. LAMP amplification with loop-forming primers only could aid the optimisation of a primer set by adopting a two-step approach with loop-forming primers first followed by the optimisation of the displacement primers. The experiments showed that one of the displacement primers increased assay sensitivity more than the other one; this primer could be improved for greater amplification efficiency. The presence of a mismatch between the 3' terminus of a displacement primer and the template sequence resulted in a slight decrease in assay sensitivity which was less than the reported loss of sensitivity in a qPCR assay due to a SNP in the target sequence (Morisset et al. 2009). Improved primers were designed based on template sequencing results and the improved results were observed with HPLC grade primers.

Another LAMP-BART parameter that was investigated was the APS concentration. APS concentration had previously been shown to have little effect on time-to-peak times over a range of APS concentrations (Gandelman et al. 2010). However at very low copy number the time of the BART peak could be slowed to a greater extent than at low copy numbers by increased APS concentration. The effect of this was to increase differentiation between copy numbers at very low copy number. Increased

APS concentration (and increased luciferase concentration) increased the light output in the assay which could be beneficial for LAMP-BART assays with very low total assay volumes (such as for digital-BART and micro-fluidic approaches).

LAMP amplification was first described at an assay temperature of 65°C (Notomi et al. 2000). Subsequently the assay temperature has been optimised for each particular assay in a range of 60°C (Nyan et al. 2014) to 68°C (Wassermann et al. 2014). LAMP-BART is limited by the thermostability of the luciferase enzyme and 55°C has been used previously (Gandelman et al. 2010). In this thesis, the LAMP-BART 35Sp assays showed indications of non-specific primer interactions below 60°C and slight loss of amplification frequency and low light output above 60°C. Therefore the assay temperature for the 35Sp primer set remained at 60°C.

A new Bst polymerase (Bst 2.0; NEB, US) has been shown to amplify at a faster rate than the wild type (Tanner et al. 2012); (Poole et al. 2012) and could therefore reduce the LAMP-BART assay time. The shorter assay time would have less demand on the stability of the luciferase and could allow the selection of an alternative to Promega Ultra-glo™ with improved kinetics.

Improved quantification of genomic DNA

The improvements to the parameters affecting LAMP-BART assay kinetics for linearised plasmid templates were utilised in genomic DNA template quantification and detection. Firstly non-linear plasmid template was compared to linear and the conformation was shown to compromise sensitivity and reproducibility between replicates. The size and complexity of genomic template remained problematic for the LAMP-BART assay, but the assay sensitivity was improved for Promega Wizard kit extracted maize genomic DNA with phenol:chloroform purification and ethanol precipitation. The denaturation of the genomic DNA improved sensitivity to a greater extent than the denaturing of the linear plasmid DNA. This suggests that the initiation of amplification that is limiting the sensitivity and an approach to denaturing the template without heating needs to be found. A number of LAMP assays rely on an initial heat denaturing step for increased sensitivity (Lee et al. 2009). Those that do not use pre-treatment tend to describe smaller DNA molecules (Cai et al. 2008) or RNA LAMP assays. Hot-start polymerases used in qPCR require an initial denaturing step which denatures the template for the start of thermal cycling and this can lead to greater sensitivity when compared to LAMP assays (Paris et al. 2008). Attempts to increase

amplification frequency at low copy numbers with genomic DNA with alterations to the LAMP-BART assay parameters were not as effective as those that centred on the template itself. Further investigations into the effect of the size of the template on LAMP-BART would increase understanding in this area. Although estimation of the initial concentration of genomic DNA is challenging, the improvements to the template and the assay did improve the detection and quantification when compared to 35Sp results in Kiddle et al. (2012).

Detection of single copy numbers

The detection of a single copy of a DNA template relies not only on the efficiency of the amplification and detection, but also on the accuracy of the initial quantification of the template. Single copy detection of the linearised plasmid pART7 with LAMP-BART using 35Sp primers was achieved based on initial quantification by two quantification techniques (NanoDrop spectrophotometer and Agilent Bioanalyzer) independently assayed at Cardiff and at Lumora (Ely). The initial quantification of maize genomic DNA cannot be achieved, due to template size, by Agilent Bioanalyzer and spectrophotometry could be affected by contaminants, RNA and DNA from organelles. Agarose gel with a comparative ladder of known concentrations was used and estimates of concentration from analysis of fluorescence intensity with ImageJ software. The detection of single copies of genomic DNA at low amplification frequency was based on initial estimations by this method and should be treated with caution. The theoretical limit of detection for qPCR is 3 copies per assay based on 95% amplification frequency and this is almost matched in this thesis by the LAMP-BART assay of pART7 with 35Sp primers (LOD 5 copies per assay; 93% amplification frequency for 4 copies per assay). LAMP sensitivity has been reported for various assays to be as low as 10 copies per assay (Wang et al. 2013); (Lucchi et al. 2010); (Kim et al. 2011). Heat treatment of the template before LAMP amplification can increase sensitivity, as observed with genomic DNA in this thesis, but the diagnostic test is no longer isothermal. LAMP detection has also been recorded at 10pg (Rigano et al. 2014), 1pg (Njiru et al. 2008) and 50fg (Moradi et al. 2014) and in this thesis at attogram amounts.

Differentiation of individual copy numbers

The detection of single copies with LAMP-BART enabled a comparison of data from 1 copy, 2 copies, 3 copies, 4 copies and 5 copies per partition to develop quantitation methods to differentiate between individual copy numbers at ultra-low levels. This

differentiation cannot currently be achieved using qPCR. A number of the quantitation techniques were based around the time-to-peak data; fastest T_{max}, average T_{max}, median T_{max}. Others were based on the characteristics of the replicate peaks; variance of replicate positive results, full width half maximum of two point moving average of the frequency distribution data, percentage maximum T_{max} frequency and the amplification frequency. Less successful methods were based on the peak morphology; average peak height and full width half maximum of the peaks. The less successful methods were affected by variation between vials within a batch of Bst polymerase and were therefore less robust. Inter-assay variation could affect those techniques based on time-to-peak and assay calibration could correct this. The variance was observed to increase with decreasing copy number and appears to be a characteristic of LAMP amplification. There was a small range of copy numbers that this phenomenon could be useful for which for the 35Sp LAMP-BART assay of pART7 was between 1 and 10 copies per partition. In isolation many of the techniques showed potential, but in combination as part of an ultra-quantification algorithm they could be a powerful method.

Digital LAMP-BART

The detection of single copies of the linearised plasmid DNA template also facilitated the development of a GM digital BART assay. Amplification frequency for the 35Sp LAMP-BART assay of pART7 ranged from approximately 30 to 60% and attempts were made unsuccessfully to achieve the upper end of this range consistently. The percentage of positive partitions from a single copy assay assuming Poisson distribution of the template across the partitions would be approximately 63% within confidence levels. Digital LAMP has been reported in the scientific literature (Gansen et al. 2012); (Zhu et al. 2012) but it is uncertain whether heat treatment of the template was used before amplification to increase sensitivity (Sun et al. 2013). An alternative linearised plasmid with the 35S promoter, NOS terminator and maize ADH1 gene sequences was studied to show that other primer sets could potentially be used for GM digital BART. The amplification frequency for the digital LAMP-BART of genomic DNA was low and showed some improvement from purification and pre-amplification heat treatment. A consistent value for amplification efficiency could facilitate the quantification of very low copy numbers by the number of positive partitions with genomic DNA. Digital PCR developments such as droplet PCR continue to strive towards absolute quantification, but remain reliant on expensive and laboratory-based technology.

Full dynamic range quantification with LAMP-BART

Quantitation using the benchmark technology of qPCR for molecular diagnostics is limited by the linear dynamic range that can be achieved (Bustin et al. 2009) in a single assay. LAMP-BART has the potential to quantify over a far wide dynamic range using the various techniques of average T_{max}, ultra-quantification and digital BART. An assessment was made with the linearised plasmid pUC35S ADH1 to use the various methods and to understand the various factors that need to be addressed for a single assay full dynamic range approach. For comparative data using the time-to-peak quantification methods, assay calibration is required to compensate for inter-assay variation. Non-mismatching primers with comparable amplification efficiencies would also be required. The quantification results from digital BART using 35Sp, NOSt and ADH1 primer sets were close to the estimation from the qPCR assay. The initial concentration of the linearised plasmid sample was too low for quantification by Agilent Bioanalyzer or NanoDrop spectrophotometer. Using ultra-quantification methods highlighted the slow time-to-peak times when compared to the other approaches less dependent on the timing of the BART peak. The range of results from the methods indicated a problem with the LAMP-BART 35Sp assay of pUC35S ADH1.

7.2 Conclusions and future directions

The ultimate aim has been to develop quantification approaches to a single LAMP-BART assay that can quantify the amount of template in a sample over a full dynamic range. This would be an advantage over qPCR as would reports of the tolerance of LAMP to a range of inhibitors to a greater concentration than qPCR (Francois et al. 2011); (Kaneko et al. 2007); (Edwards et al. 2014); (Kiddle et al. 2012), although the improved tolerance could depend on the nucleic acid quantification method (Nixon et al. 2014). Reducing the cost and complexity of qPCR for point-of-care environments has brought about the development of qPCR microfluidic devices (Jangam et al. 2013); (Verdoy et al. 2012); (Song et al. 2012). Microfluidic devices have also been used for LAMP based diagnostics (Myers et al. 2013).

The development of a simple, sensitive, specific, cheap and rapid diagnostic LAMP-BART nucleic acid detection and quantification system for low resource settings is an exciting possibility using microfluidic chip technologies.

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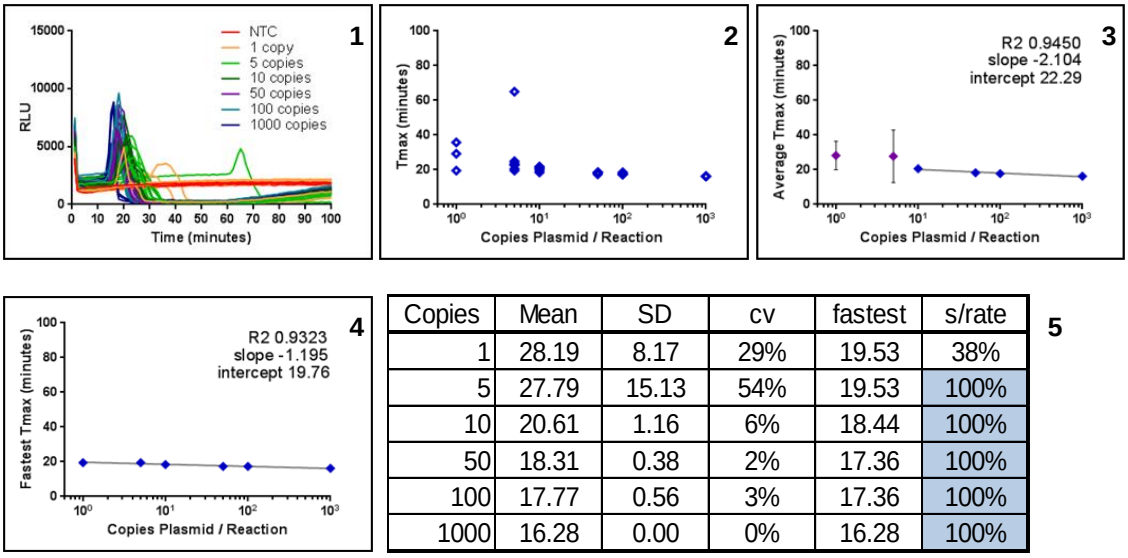
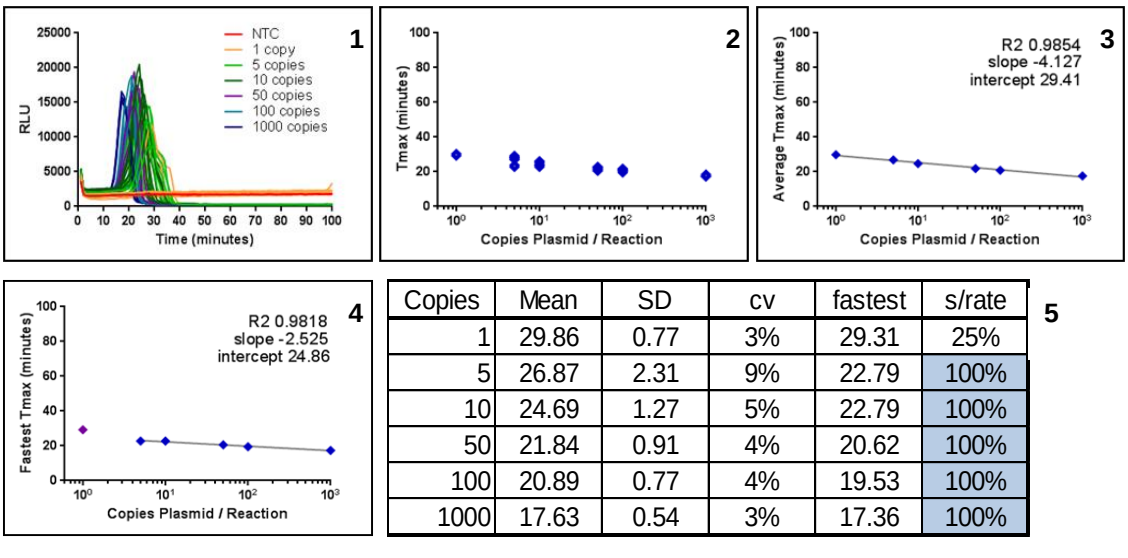
Appendix



5

Copies	Mean	SD	cv	fastest	s/rate
1	28.19	8.17	29%	19.53	38%
5	27.79	15.13	54%	19.53	100%
10	20.61	1.16	6%	18.44	100%
50	18.31	0.38	2%	17.36	100%
100	17.77	0.56	3%	17.36	100%
1000	16.28	0.00	0%	16.28	100%

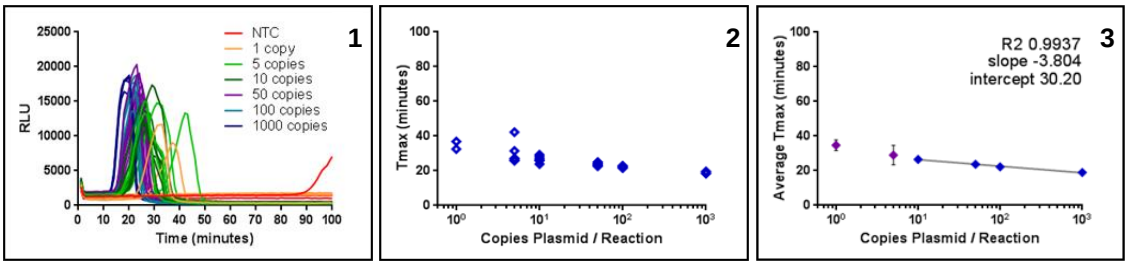
Figure App3.1: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with standard concentration of APS (250µM) (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results



5

Copies	Mean	SD	cv	fastest	s/rate
1	29.86	0.77	3%	29.31	25%
5	26.87	2.31	9%	22.79	100%
10	24.69	1.27	5%	22.79	100%
50	21.84	0.91	4%	20.62	100%
100	20.89	0.77	4%	19.53	100%
1000	17.63	0.54	3%	17.36	100%

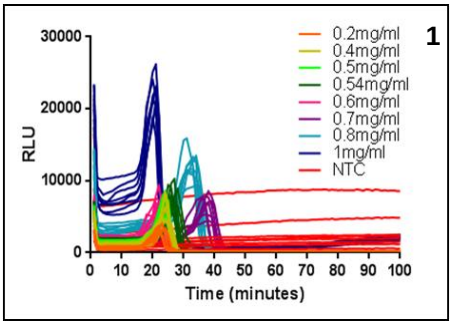
Figure App3.2: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with twice the standard concentration of APS (500µM) (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results



5

Copies	Mean	SD	cv	fastest	s/rate
1	34.72	3.06	9%	32.55	25%
5	29.02	5.67	20%	26.04	100%
10	26.58	1.64	6%	23.86	100%
50	23.73	0.91	4%	22.78	100%
100	22.24	0.58	3%	21.69	100%
1000	18.97	0.62	3%	18.43	100%

Figure App3.3: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 with three times the standard concentration of APS (750µM) (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results



2

Copies	Luciferase	Mean	SD	cv	fastest	s/rate
100	0.2mg/ml	22.45	1.00	4%	21.76	100%
100	0.4mg/ml	23.81	0.38	2%	22.85	100%
100	0.5mg/ml	23.81	0.38	2%	22.85	100%
100	0.54mg/ml	26.80	1.15	4%	25.03	100%
100	0.6mg/ml	21.08	1.29	6%	18.50	100%
100	0.7mg/ml	37.25	1.26	3%	36.98	100%
100	0.8mg/ml	32.63	1.01	3%	31.55	100%
100	1.0mg/ml	20.27	0.56	3%	19.59	100%

Figure App3.4: (1) light output against time for the concentrations of Ultra-Glo® luciferase (5.4mg/ml batch E140X 25724903) in the LAMP-BART 35Sp assay of 100 copies pART7 per partition (2) summary table of results

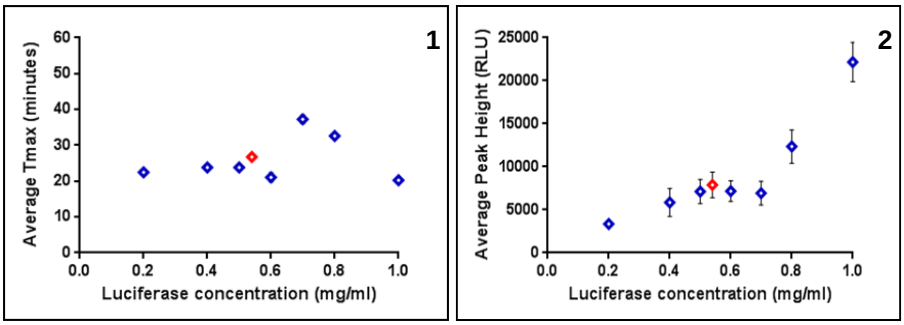
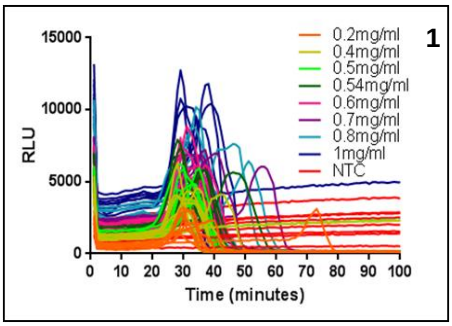


Figure App3.5: (1) average Tmax against concentrations of Ultra-Glo® luciferase (5.4mg/ml batch E140X 25724903) in the LAMP-BART 35Sp assay of 100 copies pART7 per partition (2) average peak height against luciferase concentration



2

Copies	Luciferase	Mean	SD	cv	fastest	s/rate
10	0.2mg/ml	37.10	14.79	40%	28.27	100%
10	0.4mg/ml	31.84	5.09	16%	28.27	88%
10	0.5mg/ml	32.00	2.66	8%	28.27	88%
10	0.54mg/ml	34.12	5.76	17%	28.27	100%
10	0.6mg/ml	32.62	2.73	8%	29.35	100%
10	0.7mg/ml	37.24	8.49	23%	29.35	100%
10	0.8mg/ml	36.29	7.66	21%	30.44	100%
10	1.0mg/ml	33.24	3.97	12%	29.35	88%

Figure App3.6: (1) light output against time for the concentrations of Ultra-Glo® luciferase (5.4mg/ml batch E140X 25724903) in the LAMP-BART 35Sp assay of 10 copies pART7 per partition (2) summary table of results

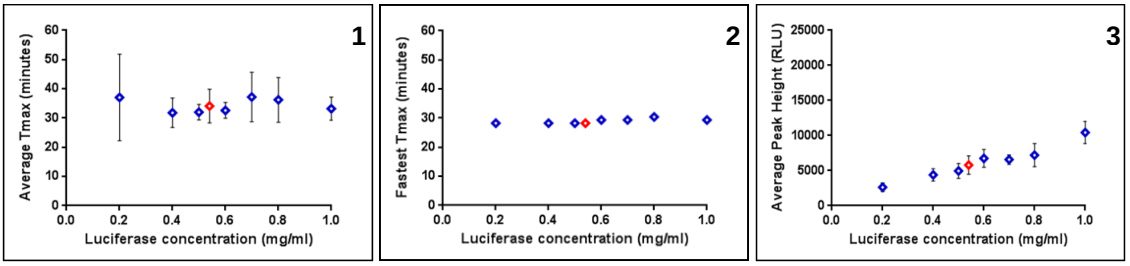
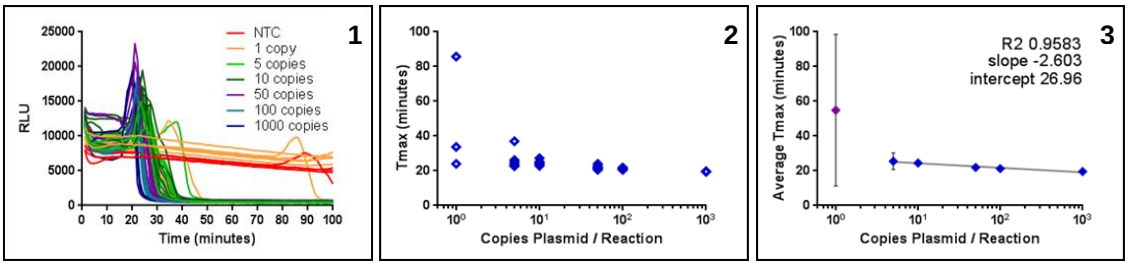
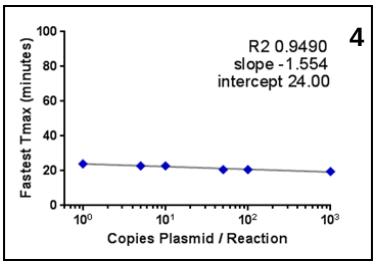


Figure App3.7: (1) average Tmax against concentrations of Ultra-Glo® luciferase (5.4mg/ml batch E140X 25724903) in the LAMP-BART 35Sp assay of 10 copies pART7 per partition (2) fastest Tmax against luciferase concentration (3) average peak height against luciferase concentration

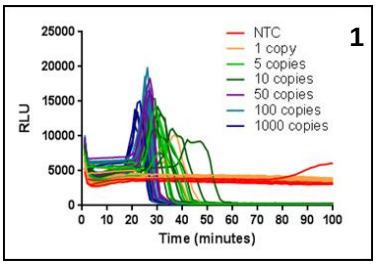


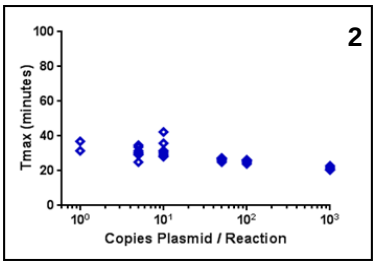


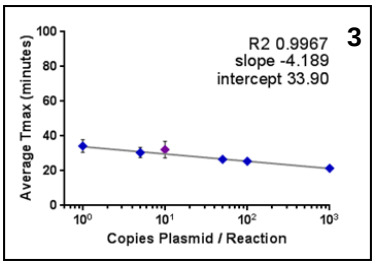
5

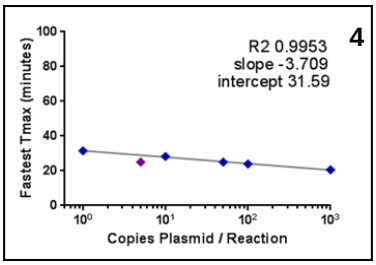
Copies	Mean	SD	cv	fastest	s/rate
1	54.92	43.71	80%	24.01	38%
5	25.50	4.83	19%	22.91	100%
10	24.55	1.30	5%	22.91	100%
50	21.96	1.08	5%	20.73	100%
100	21.28	0.58	3%	20.73	100%
1000	19.64	0.00	0%	19.64	100%

Figure App3.8: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 at **55°C** assay temperature (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results





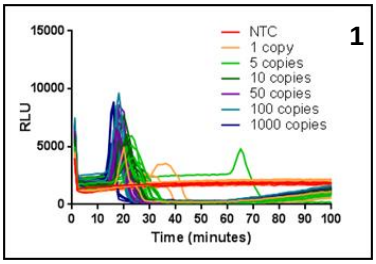


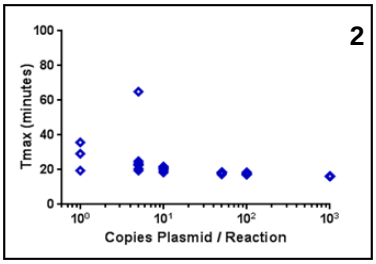


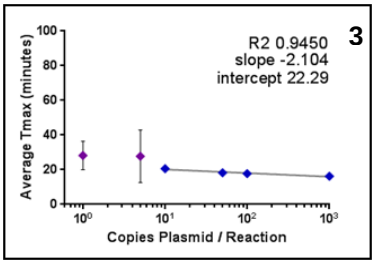
5

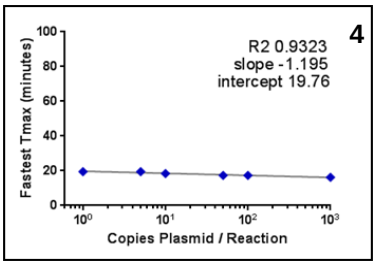
Copies	Mean	SD	cv	fastest	s/rate
1	34.25	3.63	11%	31.54	25%
5	30.58	2.99	10%	25.01	100%
10	32.21	4.78	15%	28.27	100%
50	26.65	0.82	3%	25.01	100%
100	25.56	0.82	3%	23.93	100%
1000	21.48	1.04	5%	20.66	100%

Figure App3.9: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 at **57°C** assay temperature (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results





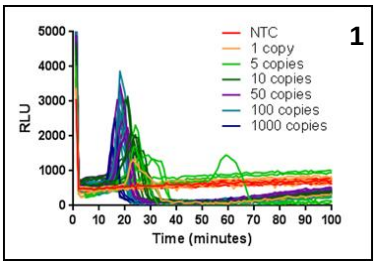


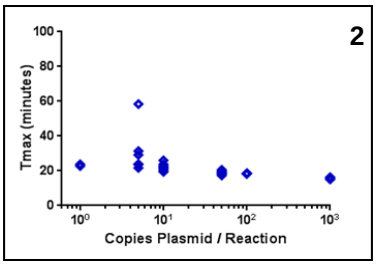


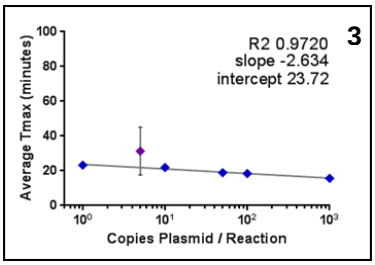
5

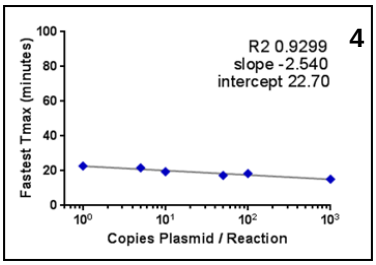
Copies	Mean	SD	cv	fastest	s/rate
1	28.19	8.17	29%	19.53	38%
5	27.79	15.13	54%	19.53	100%
10	20.61	1.16	6%	18.44	100%
50	18.31	0.38	2%	17.36	100%
100	17.77	0.56	3%	17.36	100%
1000	16.28	0.00	0%	16.28	100%

Figure App3.10: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 at **60°C** assay temperature (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results





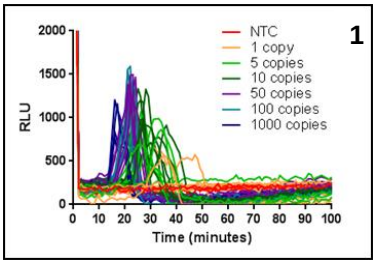


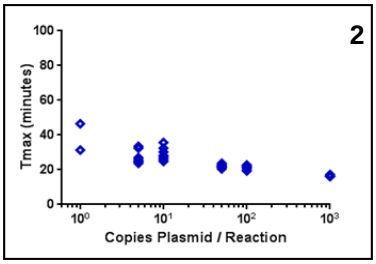


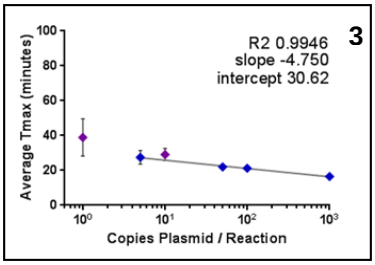
5

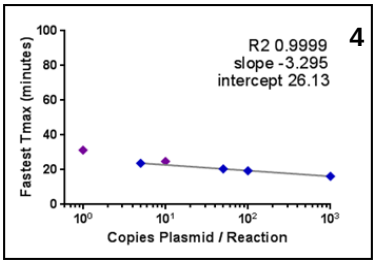
Copies	Mean	SD	cv	fastest	s/rate
1	23.29	0.76	3%	22.75	25%
5	31.41	13.76	44%	21.67	75%
10	21.94	2.22	10%	19.50	100%
50	18.96	1.00	5%	17.34	100%
100	18.42	0.00	0%	18.42	100%
1000	15.72	0.62	4%	15.18	100%

Figure App3.11: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 at **63°C** assay temperature (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results









5

Copies	Mean	SD	cv	fastest	s/rate
1	38.96	10.71	27%	31.39	25%
5	27.53	3.89	14%	23.82	88%
10	29.09	3.59	12%	24.90	100%
50	22.06	0.99	5%	20.57	100%
100	21.25	1.29	6%	19.49	100%
1000	16.52	0.54	3%	16.25	100%

Figure App3.12: GM-LAMP-BART 35Sp assay of linearised plasmid pART7 at **65°C** assay temperature (1) light output against time for the serial dilution of 10^3 to 10^0 copies per partition (2) time-to-peak against template copies per partition (3) average Tmax against template copies per partition (4) fastest Tmax against template copies per partition (5) summary table of results

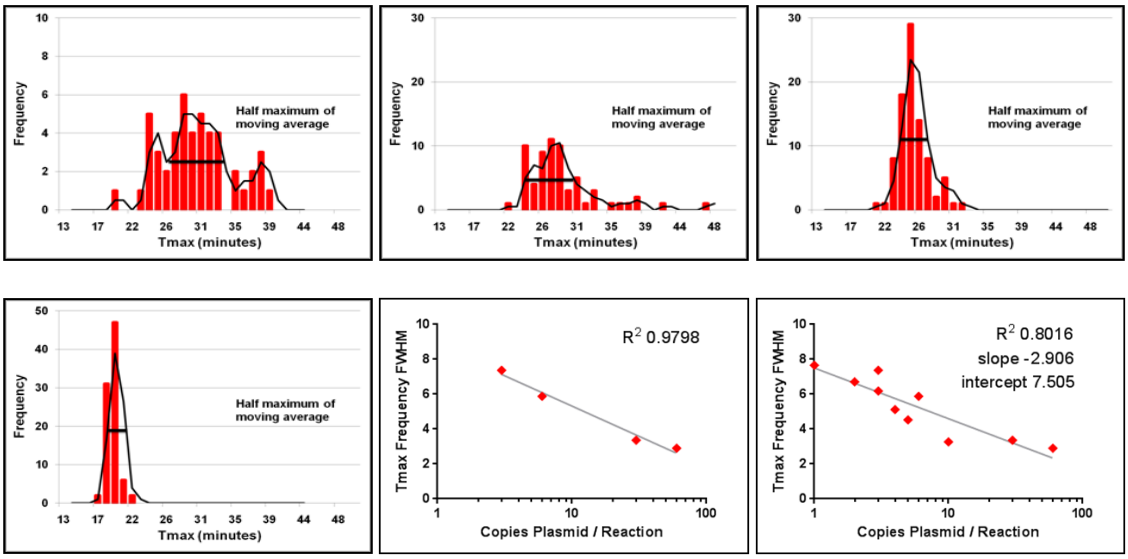


Figure App4.1: (L to R) (1) frequency distribution with two point moving average indicated by a black line and the FWHM by a black horizontal bar for 3 copies per partition (2) for 6 copies per partition (3) for 30 copies per partition (4) for 60 copies per partition

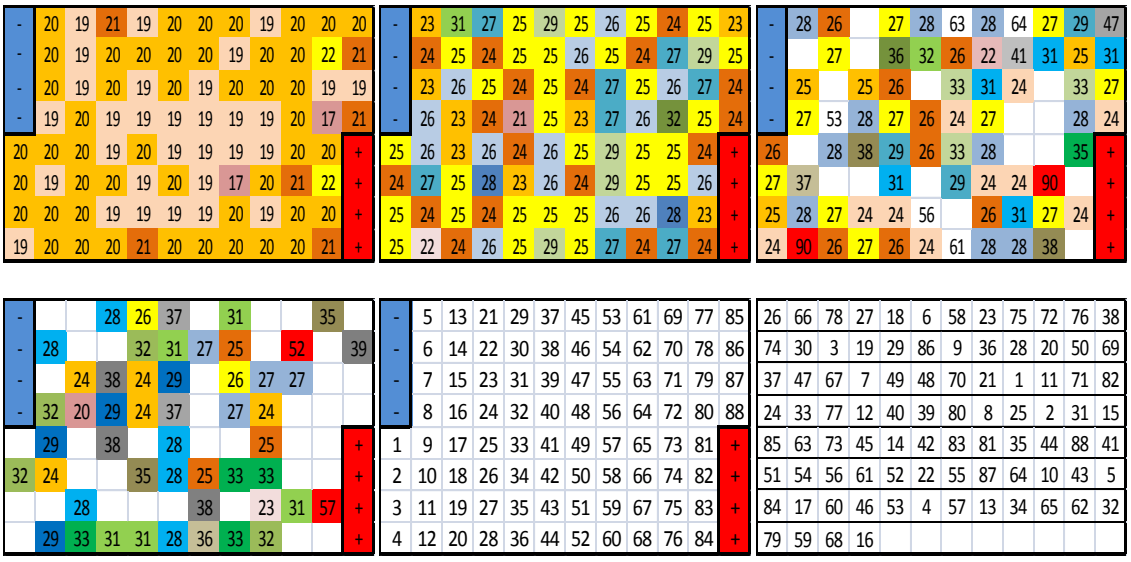
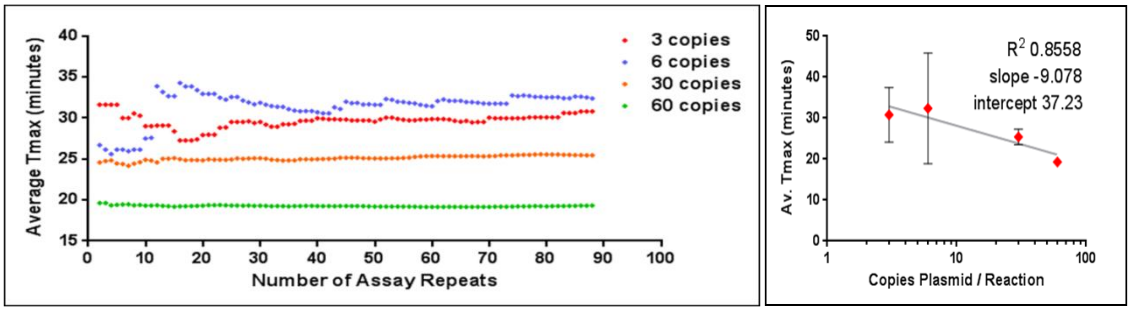


Figure App4.2: (L to R) T_{max} data for (1) 60 copies per repeat (2) 30 copies per repeat (3) 6 copies per repeat (4) 3 copies per repeat (5) order of loading and position number of repeat (6) position number on the plate for associated T_{max} values from randomly generated non-repeating integer set between 1 and 88 (read left to right, top to bottom) www.random.org



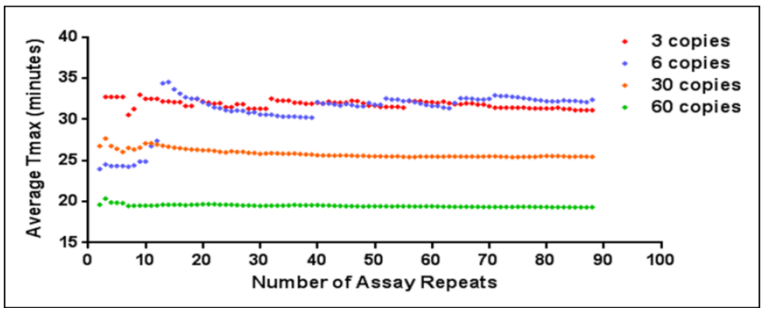


Figure App4.3: Average T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate.

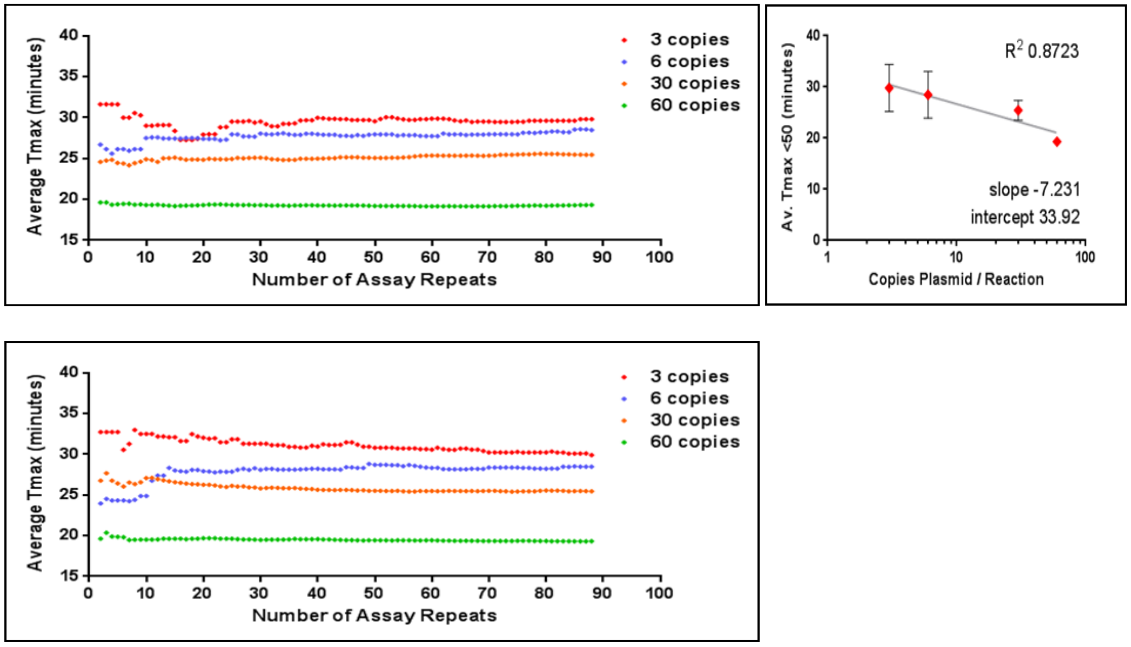
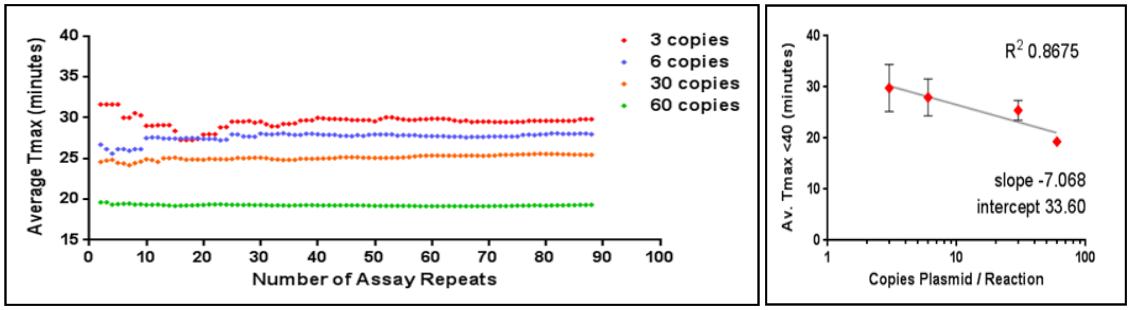


Figure App4.4: Average T_{max} for values less than 50 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate.



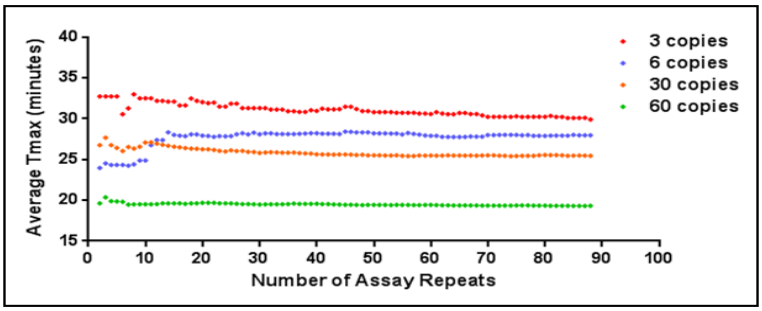


Figure App4.5: Average T_{max} for values less than 40 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate.

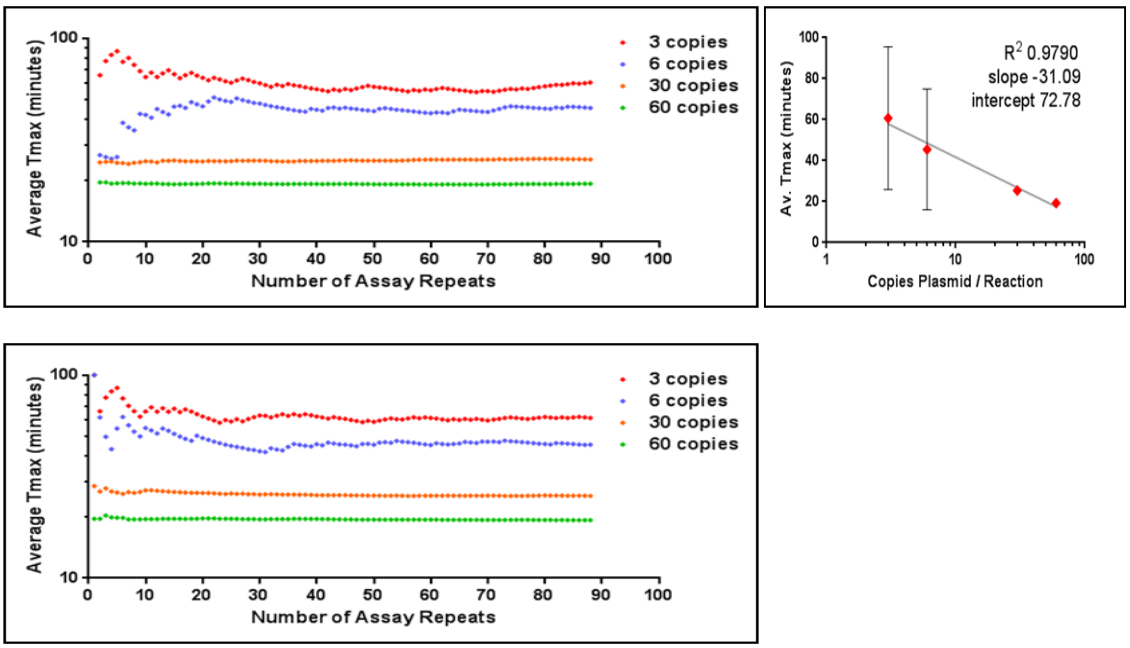
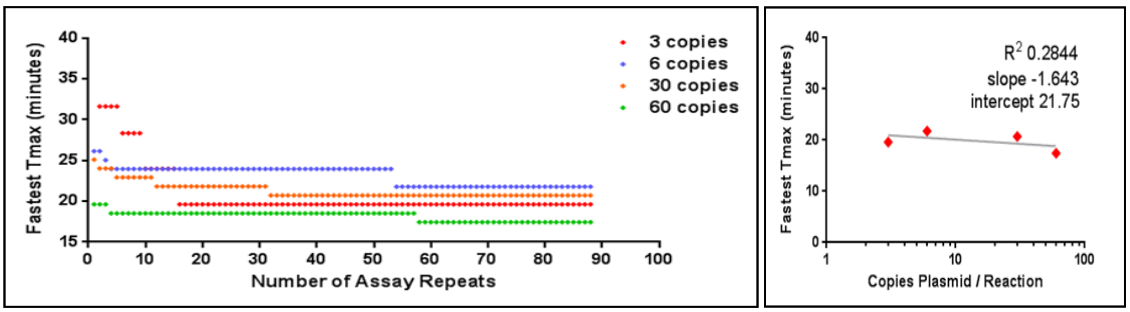


Figure App4.6: Average T_{max} for all data with negative results given a T_{max} value of 100 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate.



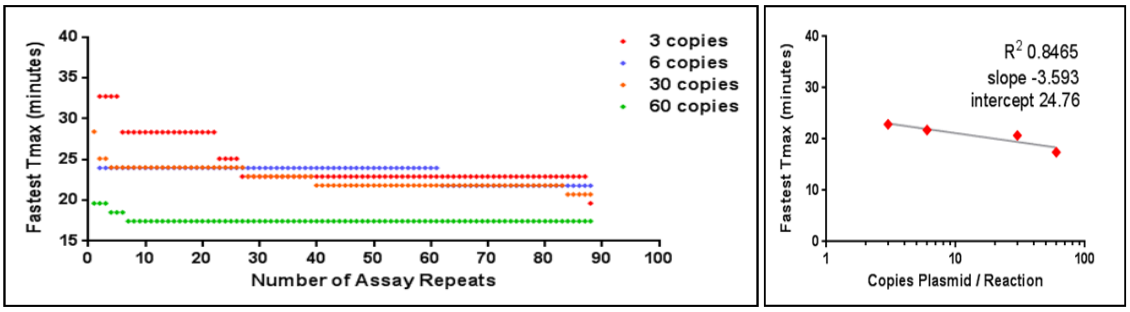


Figure App4.7: Fastest T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate (4) revised chart with the second fastest T_{max} for 3 copies per partition substituted

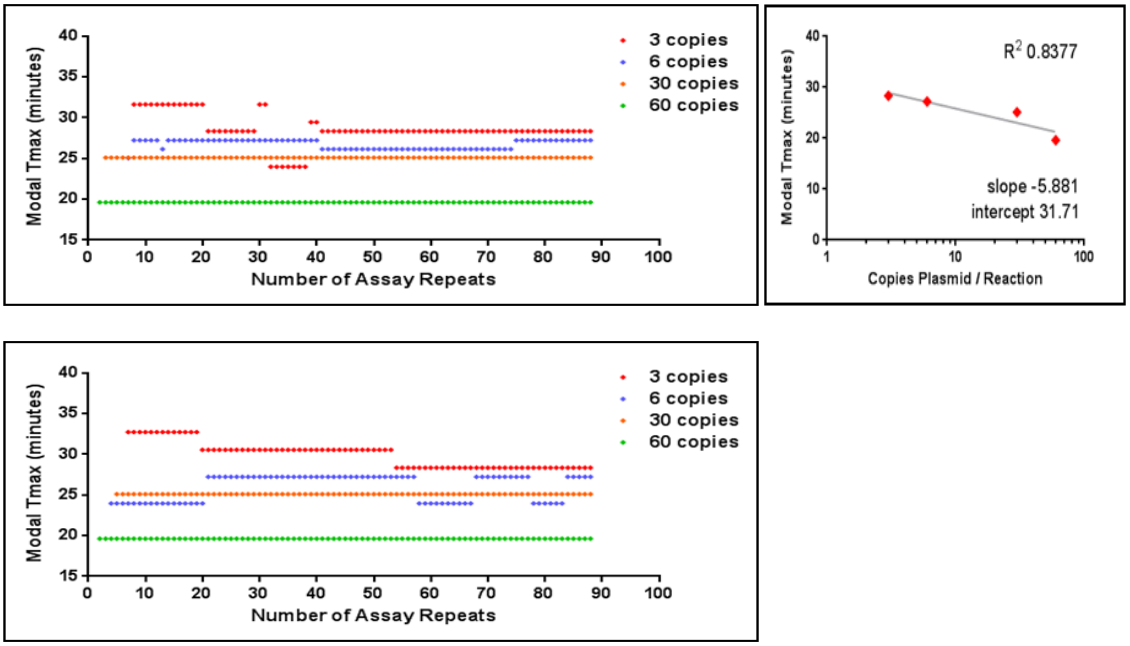
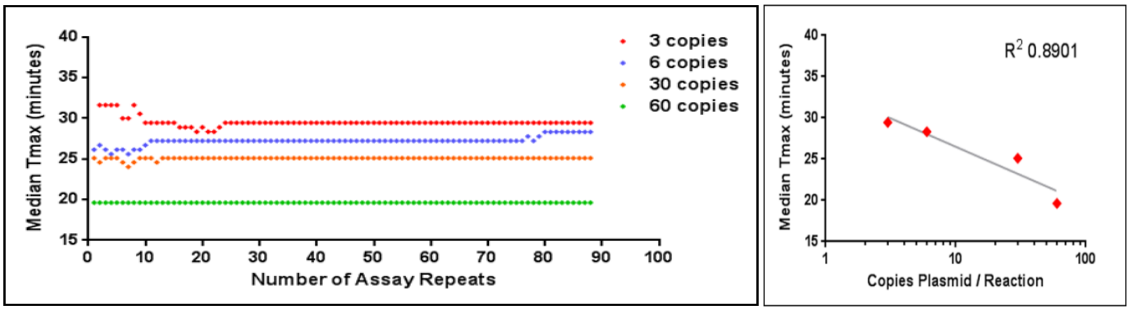


Figure App4.8: Modal T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate



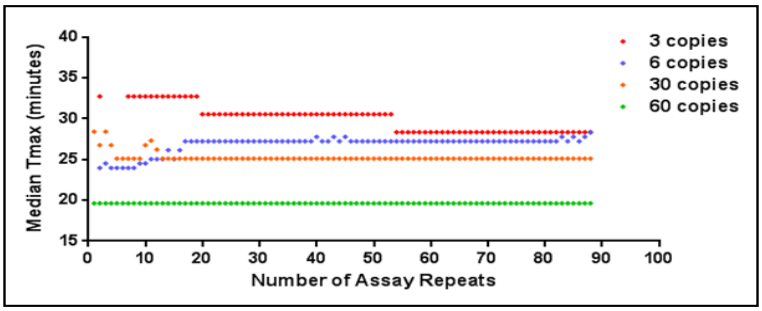


Figure App4.9: Median T_{max} (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate

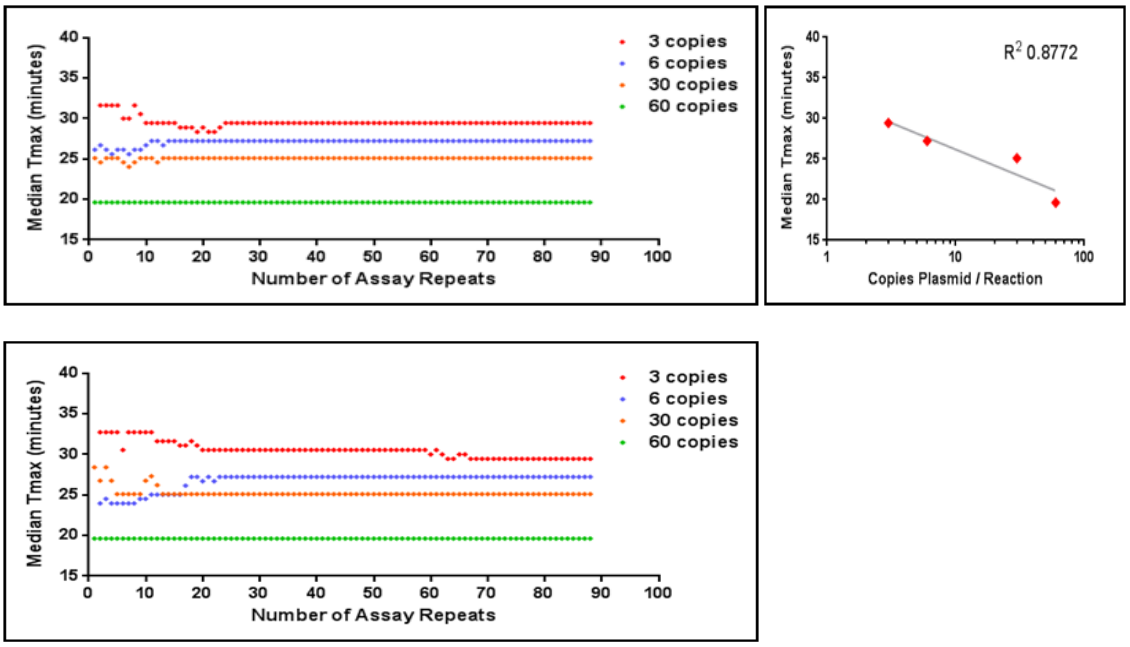
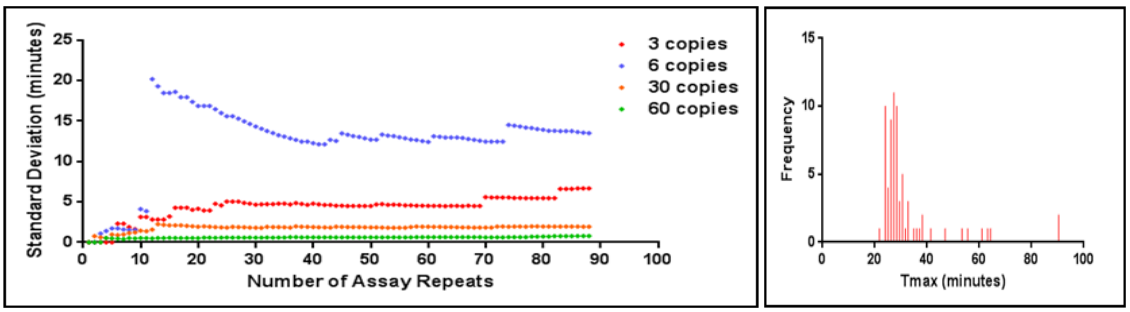


Figure App4.10: Median T_{max} for values less than 40 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate



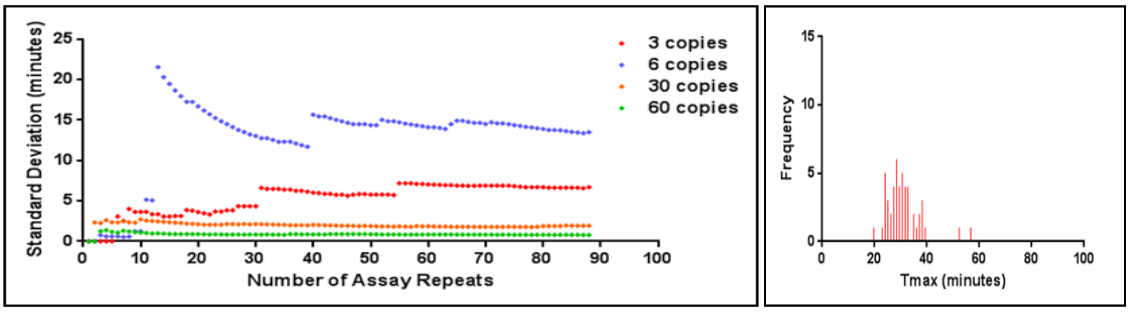


Figure App4.11: Standard deviation of average T_{max} times (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) frequency distribution of 6 copies per partition showing the incidence of T_{max} values up to 100 minutes (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate (4) frequency distribution of 3 copies per partition showing the incidence of T_{max} values up to 100 minutes.

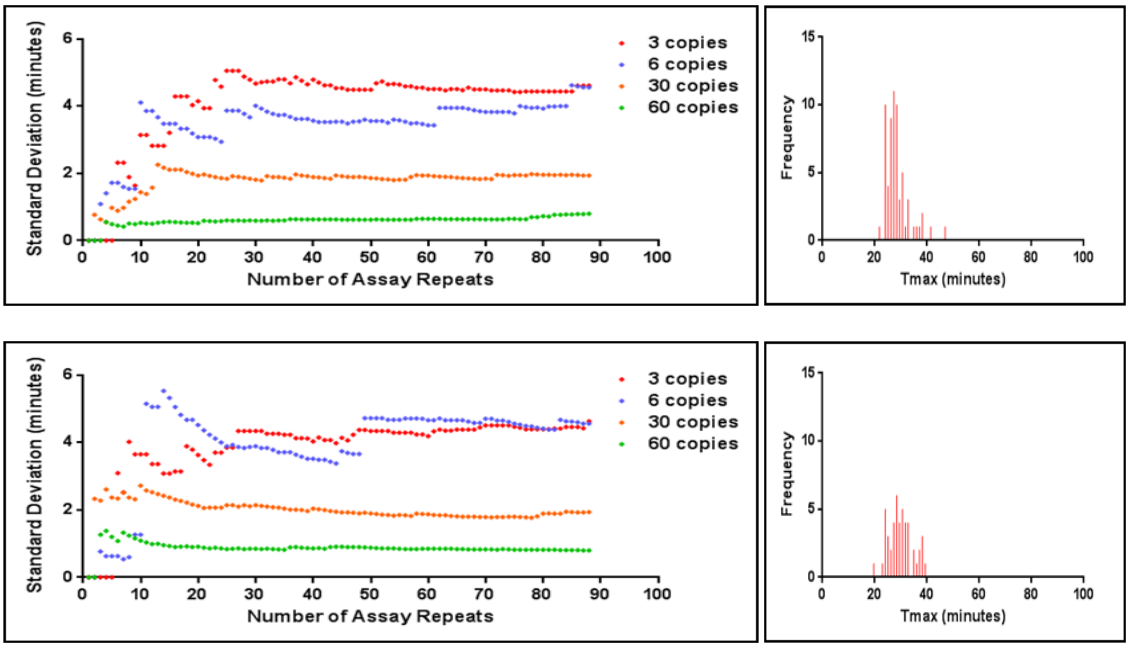
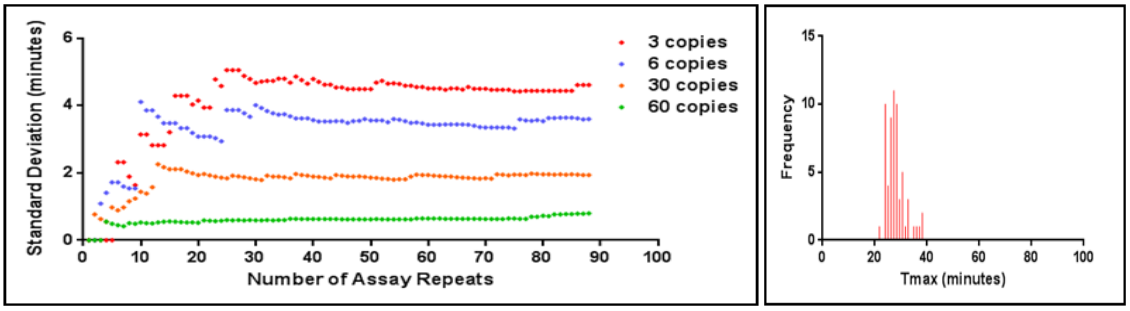


Figure App4.12: Standard deviation of average T_{max} times below 50 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) frequency distribution of 6 copies per partition showing the incidence of T_{max} values up to 50 minutes (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate (4) frequency distribution of 3 copies per partition showing the incidence of T_{max} values up to 50 minutes.



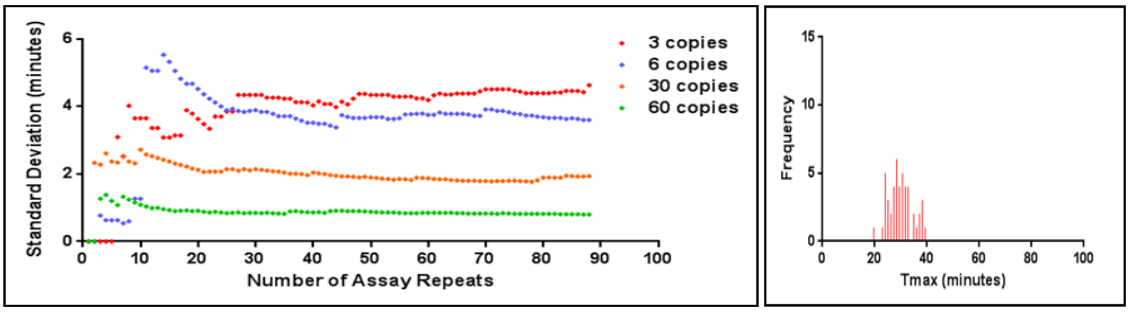


Figure App4.13: Standard deviation of average T_{max} times below 40 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) frequency distribution of 6 copies per partition showing the incidence of T_{max} values up to 40 minutes (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate (4) frequency distribution of 3 copies per partition showing the incidence of T_{max} values up to 40 minutes.

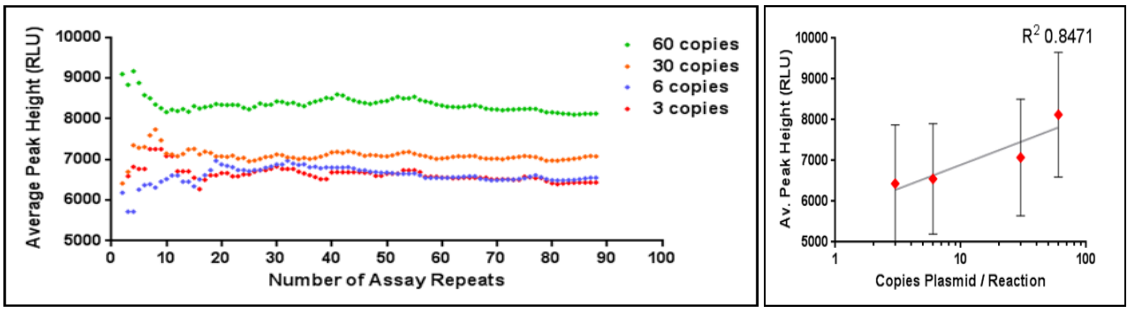


Figure App4.14: Average T_{max} peak height (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition

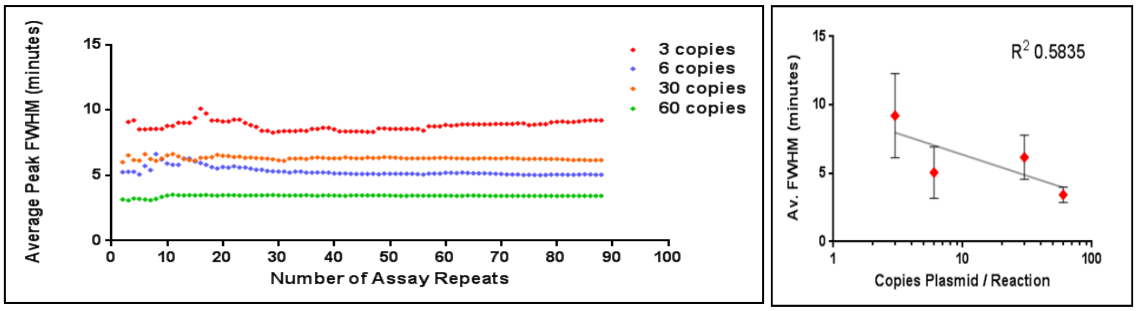
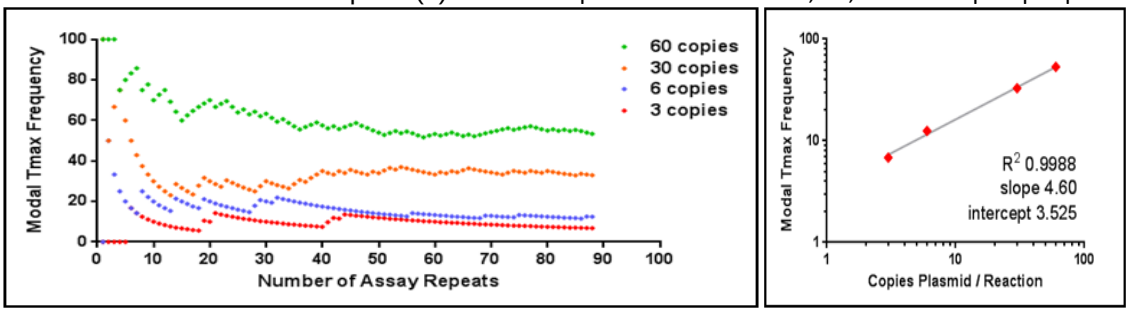


Figure App4.15: Average peak FWHM (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition



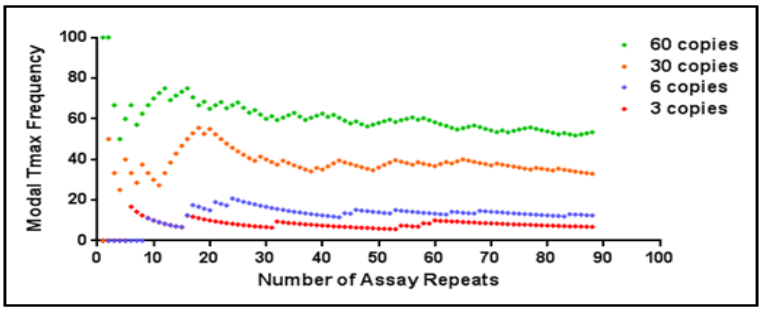


Figure App4.16: Percentage modal T_{max} frequency (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition (3) with increasing number of assay repeats for T_{max} values from randomised positions on the plate

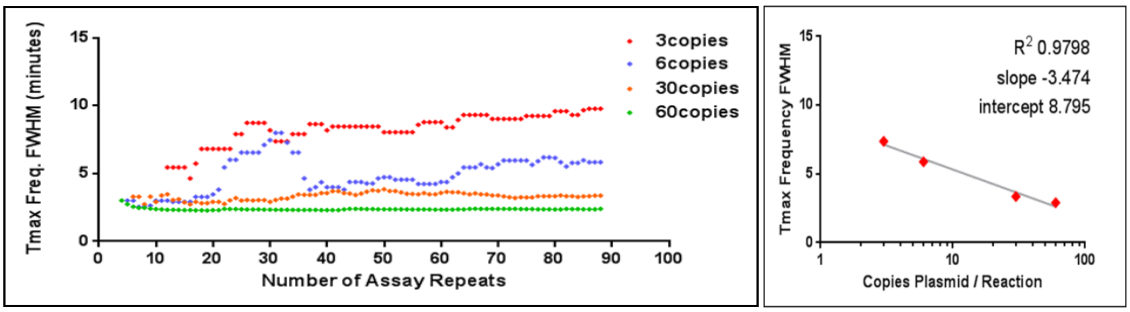


Figure App4.17: Full width half maximum of the moving average of Tmax frequencies (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 88 replicates at each of 60, 30, 6 and 3 copies per partition

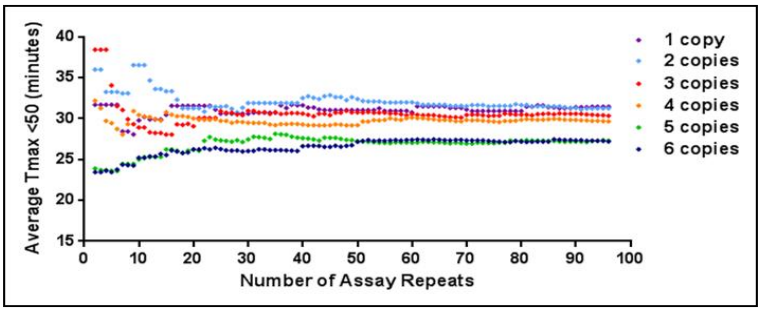


Figure App4.18: Average T_{max} for values less than 50 minutes (1) with increasing number of assay repeats for the data in the order it was loaded to the plate (2) for all 96 replicates at each of 6, 5, 4, 3, 2 and 1 copies per partition BARTmaster reduced total volume assay
Standard assay conditions for ADH1 assay

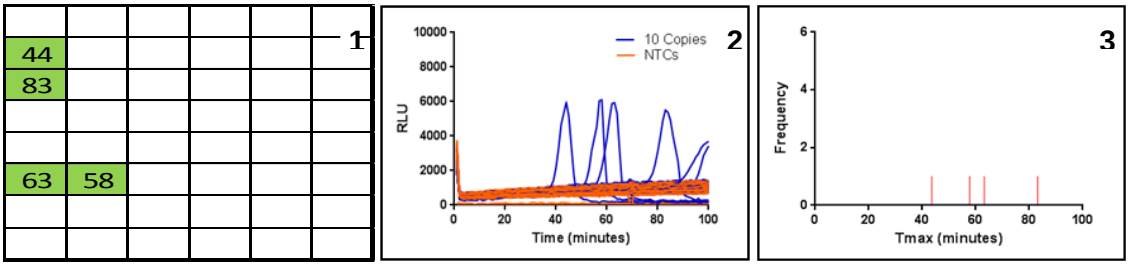


Figure App5.1: (L to R) ADH1 LAMP-BART assay of 10 copies genomic template per partition 96 well plate, total assay volume 5µl, 1 x concentration denatured primers, 60°C assay temperature (1) table to show the spread of positive results in green with associated Tmax value (2) the light output from positive results against time, 10 copies per partition in blue and NTCs in orange (3) the frequency distribution of Tmax results

Standard assay conditions for 35Sp assay

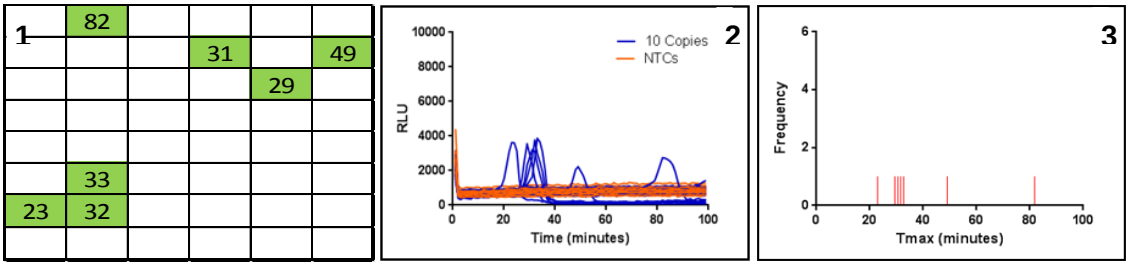


Figure App5.2: (L to R) 35Sp LAMP-BART assay of 10 copies genomic template per partition 96 well plate, total assay volume 5µl, 1 x concentration denatured primers, 60°C assay temperature (1) table to show the spread of positive results in green with associated Tmax value (2) the light output from positive results against time, 10 copies per partition in blue and NTCs in orange (3) the frequency distribution of Tmax results

Standard assay conditions for 35Sp assay with additional magnesium

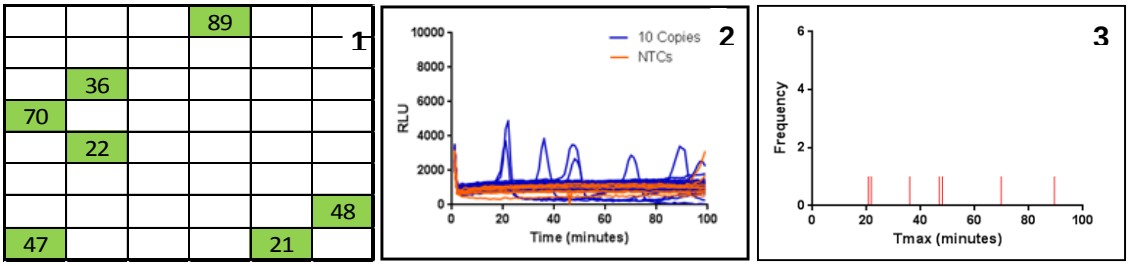


Figure App5.3: (L to R) 35Sp LAMP-BART assay of 10 copies genomic template per partition 96 well plate, total assay volume 5µl, 1 x concentration denatured primers, 60°C assay temperature, additional magnesium (1) table to show the spread of positive results in green with associated Tmax value (2) the light output from positive results against time, 10 copies per partition in blue and NTCs in orange (3) the frequency distribution of Tmax results

35Sp assay temperature 62°C

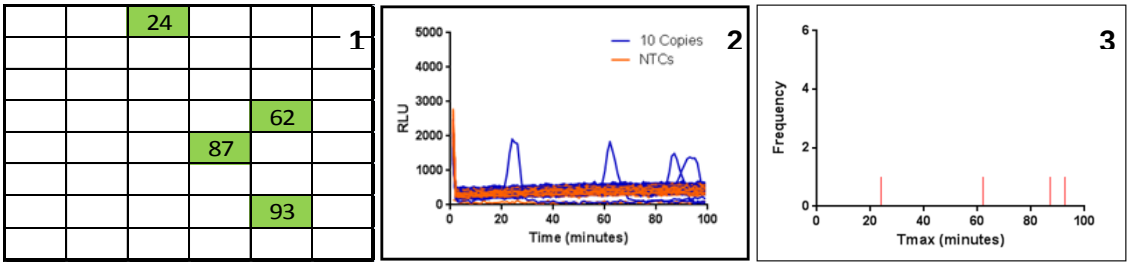


Figure App5.4: (L to R) 35Sp LAMP-BART assay of 10 copies genomic template per partition 96 well plate, total assay volume 5µl, 1 x concentration denatured primers, 62°C assay temperature (1) table to show the spread of positive results in green with associated Tmax value (2) the light output from positive results against time, 10 copies per partition in blue and NTCs in orange (3) the frequency distribution of Tmax results

35Sp assay temperature 65°C

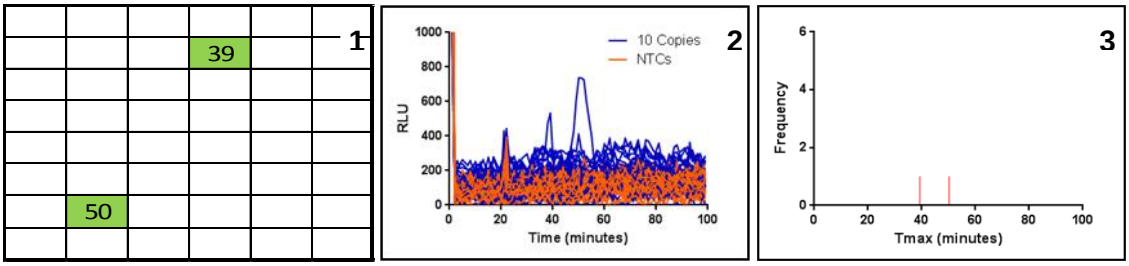


Figure App5.5: (L to R) 35Sp LAMP-BART assay of 10 copies genomic template per partition 96 well plate, total assay volume 5µl, 1 x concentration denatured primers, 65°C assay temperature (1) table to show the spread of positive results in green with associated Tmax value (2) the light output from positive results against time, 10 copies per partition in blue and NTCs in orange (3) the frequency distribution of Tmax results

35Sp assay temperature 65°C with 1.5 x primer concentration

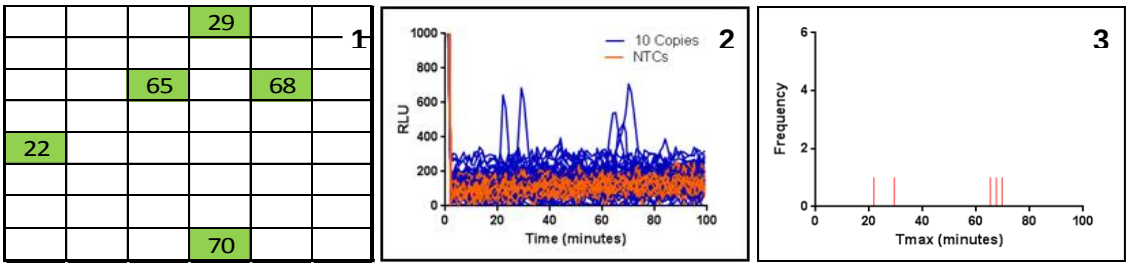


Figure App5.6: (L to R) 35Sp LAMP-BART assay of 10 copies genomic template per partition 96 well plate, total assay volume 5µl, 1.5 x concentration denatured primers, 65°C assay temperature (1) table to show the spread of positive results in green with associated Tmax value (2) the light output from positive results against time, 10 copies per partition in blue and NTCs in orange (3) the frequency distribution of Tmax results

35Sp assay temperature 65°C with additional magnesium and Taq polymerase

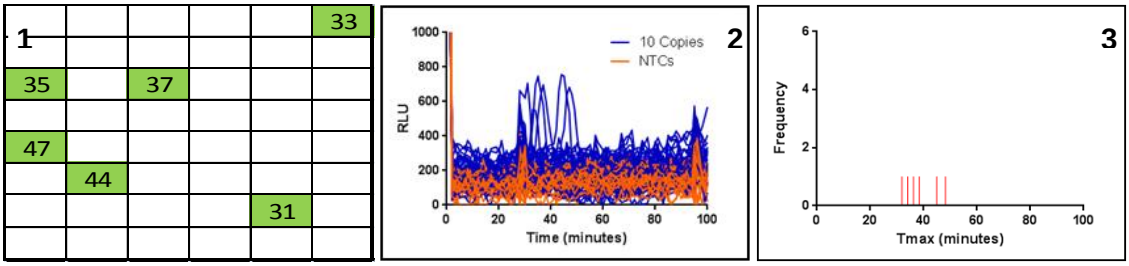


Figure App5.7: (L to R) 35Sp LAMP-BART assay of 10 copies genomic template per partition 96 well plate, total assay volume 5µl, 1 x concentration denatured primers, 65°C assay temperature, additional magnesium, non-hot start Taq polymerase (1) table to show the spread of positive results in green with associated Tmax value (2) the light output from positive results against time, 10 copies per partition in blue and NTCs in orange (3) the frequency distribution of Tmax results