

The development of a point-of-care diagnostic for the porcine respiratory disease complex

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Abstract

The porcine respiratory disease complex (PRDC) consists of bacterial and viral pathogens, which commonly cause pneumonia in pigs and result in high economic losses worldwide. The pathogens involved include *Actinobacillus pleuropneumoniae* (APP), *Mycoplasma hyopneumoniae* (MH), *Pasteurella multocida* (PM), *Streptococcus suis* (SS) and *Glaesserella parasuis* (GPS), Swine influenza, Porcine circovirus-2 and Betaarterivirus suis. This Thesis describes the development of a diagnostic platform that can be used pen-side to identify the main PRDC pathogens.

Analysis of four isothermal amplification methods determined that loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) produced rapid and specific amplification. LAMP achieved detection limits of 80, 20, 8, 8 and 20 copies/ μ L in the detection of APP, MH, PM, SS and GPS, respectively. RPA achieved 10, 20, 80 and 10 copies/ μ L for APP, MH, PM, and SS, respectively. Furthermore, LAMP and RPA achieved clinical sensitivities of 80.39% and 84.31% (n=61), 71.05% and 78.95% (n=123), 80.0% and 53.33% (n=92), 45.24% and 40.48% (n=121), and 79.66% (n=111) detecting APP, MH, PM, SS and GPS, respectively. LAMP conditions were successfully optimised and lyophilised, enabling ambient storage. Lyophilised LAMP displayed a slightly delayed amplification, but increased sensitivity compared to freshly prepared reactions.

Additionally, an equipment-limited tissue extraction protocol was developed, and although less genetic material was extracted compared to the gold standard method, it was significantly more rapid (5 vs 70 minutes). 10 clinical lung samples were compared in sample-to-result using fluorescence-, lateral flow-, and colorimetric-LAMP detection methods with equivalent results.

The colourimetric-LAMP was developed into a PRDC panel, and its usability was well received in a demonstration performed to a group of veterinarians. Implementing a rapid diagnostic tool that can rapidly (<35 minutes) identify and aid in the diagnosis of these important swine pathogens will prove a powerful tool to veterinarians, ensuring appropriate and prompt treatment.

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Abbreviations

Abbreviation	Meaning
(NH ₄) ₂ SO ₄	Ammonium sulphate
β	Beta coefficient
ΔpH	Differential pH
12s	12S rRNA
AA	Amino acid
Ab	Antibody
Ag	Silver
Ag-Cl	Silver-chloride
APHA	Animal and Plant Health Agency
APP	<i>Actinobacillus pleuropneumoniae</i>
Apx	Repeat-in-toxins
ASSURED	Affordable, sensitive, SPE, user-friendly, rapid, and robust equipment free and deliverable to end users
AUC	Area under the curve
BALF	Bronchoalveolar lavage fluid
BB	Bromothymol blue
BHI	Brain heart infusion
BHQ-1	Blackhole quencher 1
BIP	Backward inner primer
bp	Base pairs
BSA	Bovine serum albumin
Bsm	<i>Bacillus smithii</i> DNA polymerase
Bst	<i>Bacillus stearothermophilus</i> DNA polymerase
Bst LF	<i>Bacillus stearothermophilus</i> large fragment DNA polymerase
BVS	Betaarterivirus suid
BVS1	Betaarterivirus suid 1
BVS2	Betaarterivirus suid 2
Cal	Calmagite
CB1	Custom buffer 1
CB2	Custom buffer 2
cDNA	complementary DNA
CFU	Colony forming units
CLS-TC	Cell lysis solutions for tissues and cells
CMOS	Complementary metal–oxide–semiconductor
COVID-19	Coronavirus disease 2019
cps	Capsule synthesis
C _t	Cycle threshold
Ctrl	Control
cytB	<i>Cytochrome B</i> gene
DbsE	Disulphide bond formation protein E

DHB	Defibrinated horse blood
DIG	Digoxigenin
DIVA	Differentiate between infected or vaccinated animals
dLAMP	digital LAMP
dsDNA	Double-stranded DNA
EBT	Eriochrome Black T
ECHA	European Chemicals Agency
EDTA	Tris-acetate ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
ETDA	Ethylenediaminetetraacetic acid
Exo	Exonuclease III
FAM	Carboxyfluorescein
FDA	U.S. Food and Drug Administration
FDM	Freeze drying microscopy
FET	Field-effect transistor
FEU	FDA emergency use
FIP	Forward inner primer
FITC	Fluorescein
FN	False negative
FP	False positive
Fpg	Formamidopyrimidine DNA glycosylase
FRET	Fluorescence resonance energy transfer
g	Centrifugal force
Gal	Galactose
gDNA	genomic DNA
GHS	Globally Harmonized System
GoX	Glucose-oxidase
GPS	<i>Glaesserella parasuis</i>
GTC	Guanidine thiocyanate
GuHCl	Guanidine hydrochloride
HA	Hemagglutinin
HCl	Hydrochloric acid
HDA	Helicase dependant amplification
hgDNA	human genomic DNA
HNB	Hydroxynaphthol blue
HRP	Horseradish peroxide
IC	Internal control
IDT	Integrated DNA technologies
IP	Intellectual property
IPA	Isopropanol

ISFET	Ion-sensitive field-effect transistor
IVD	In vitro diagnostics
KAc	Potassium acetate
KCl	Potassium chloride
KOH	Potassium hydroxide
LAMP	Loop mediated isothermal amplification
LB	loop backward
LBB	Lysis and binding buffer
LBS	Lysis and binding solution
LctD	L-lactate dehydrogenase
LF	Loop forward
LFT	Lateral flow test
LNA	Locked nucleic acid
LoD	Limit of detection
LPS	Lipopolysaccharide
M	Matrix protein
MgAc	Magnesium acetate
MgSO ₄	Magnesium sulphate
MH	<i>Mycoplasma hyopneumoniae</i>
MOSFET	Metal oxide semiconductor field effect transistor
mtD	mitochondrial DNA D-loop region
NA	Neuraminidase
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NASBA	Nucleic acid sequence-based amplification
NEAR	Nicking and extension amplification reaction
NEB	New England Biolabs
NEP	Nuclear export protein
Nfo	Endonuclease IV
NP	Nucleoprotein
NPV	Negative predictive value
NR	Neutral red
NS1	Non-structural segment
NSP2	Non-structural protein 2
NT	Non-typable
NTC	Negative template control
ORF	Open reading frame
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline

PCB	Printed circuit board
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV1	Porcine circovirus 1
PCV2	Porcine circovirus 2
PCV3	Porcine circovirus 3
PCV4	Porcine circovirus 4
PCVD	Porcine circovirus disease
PEG	Polyethylene glycol
PEG8	8 kDa polyethylene glycol
PEG35	35 kDa polyethylene glycol
PM	<i>Pasteurella multocida</i>
PMWS	Post-weaning multisystemic wasting
PoC	Point-of-Care
PPV	Positive predictive value
PR	Phenol red
PRDC	Porcine respiratory disease complex
PRDS	Porcine dermatitis and nephropathy syndrome
PRRS	Porcine respiratory and reproductive syndrome
BVS	Porcine respiratory and reproductive syndrome virus
PSR	Polymerase spiral reaction
qPCR	quantitative polymerase chain reaction
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RFU	Raw fluorescence units
RHV	Human rhinovirus
RI	RNase inhibitor
RIPA	Radioimmunoprecipitation assay
RNase	Ribonuclease
RPA	Recombinase polymerase amplification
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SA	Sialic acid
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SAMRs	Self-avoiding molecular recognition systems
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
SEN	Sensitivity
Si ₃ N ₄	Silicon nitride
SiO ₂	Silicon dioxide
SIV	Swine influenza

SNP	Single nucleotide polymorphism
SPE	Specificity
SS	<i>Streptococcus suis</i>
SSBP	Single-stranded binding protein
ssDNA	single-stranded DNA
Taq prb	TaqMan probe
TB	Thymol blue
Tc	Total collapse temperature
TE	Tris-EDTA
tHDA	thermostable helicase dependant amplification
THF	Tetrahydrofuran
Tm	Melting temperature
TMB	Tetramethylbenzidine
TN	True negative
TP	True positive
Tris-HCl	Tris-hydrochloride
TTP	Time to positive
UDG	Uracil-DNA glycosylase
VtaA	Virulence-associated trimeric autotransporters
VTM	Viral transport medium
WHO	World Health Organization
WS	WarmStart
WT	Wild-type
XB	Xylidyl blue

Chapter 1: Introduction

The global pork industry

Pork accounts for more than a third of the meat produced globally, with over 100 million tonnes being produced every year throughout the world (Willoughby, 2022). In 2022, China accounted for the majority of the pork produced (51 million tonnes), followed by the European Union (23 million tonnes), and the United States (12 million tonnes) - with the UK producing 1 million tonnes of meat per year (Wilkins, 2021) (**Figure 1.1A**). Accordingly, China is the largest consumer of pork products consuming 156 kg per capita every year, whilst the UK consumes around 21 kg per capita (**Figure 1.1B**).

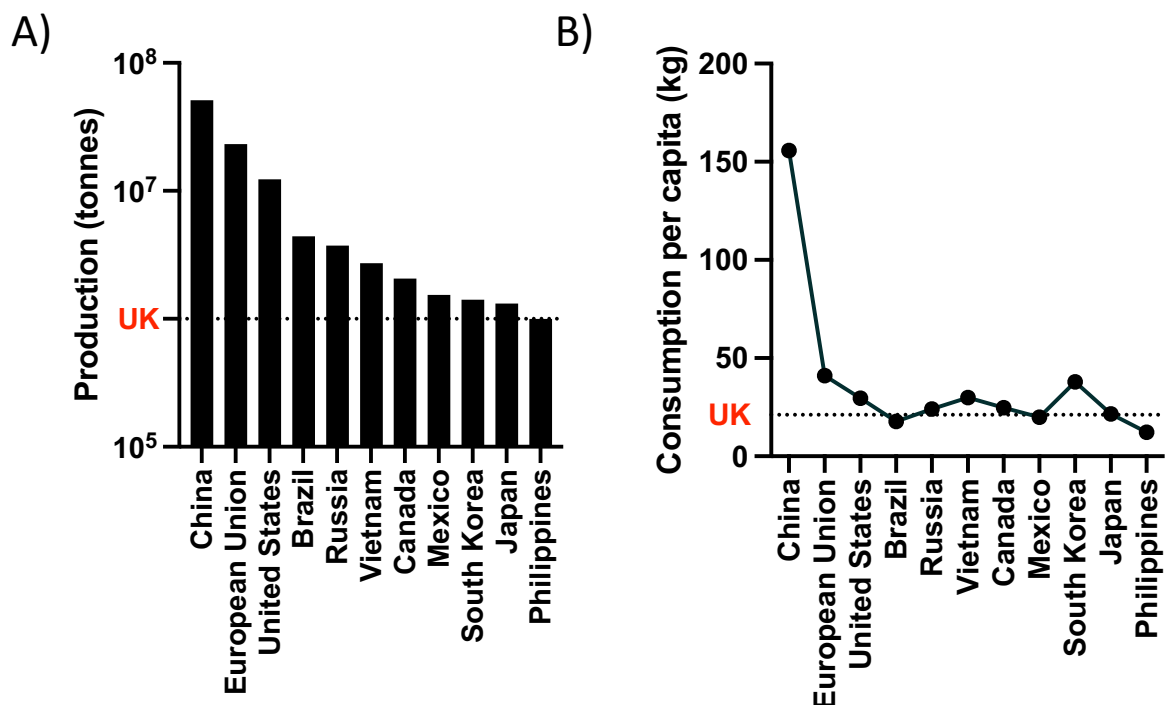


Figure 1.1. Global production and consumption of pork products. The production in metric tonnes (**A**) and the consumption per capita (**B**) of pork products by major countries in 2022. The position of the UK is shown by the dotted line in each graph. Source: (Willoughby, 2022, United States Department of Agriculture Foreign Agricultural Service, 2022).

The global pork market was valued at USD \$236 billion in 2019, and is set to increase to \$258 billion by 2027 (Allied Market Research). The UK pig industry is a major agricultural market - worth GBP £1.44 billion (Shahbandeh, 2021). Historically, the UK pork market has mirrored that of the European Union in being highly consumer-driven i.e., valuing sustainability, product traceability and food safety which has resulted in high production costs, relative to other countries (Hartog, 2005). Several factors have generated additional pressure on UK farms in recent years, with increased feed costs, strict incentives aimed at decreasing the use of antibiotics and new environmental regulations, inflating farm overheads, and reducing profit margins. The situation has been compounded by intense competition from foreign suppliers and stagnating meat prices (Strak, 2017).

A major liability to producers profit margins is the health status of the herd. Poor health incurs high treatment costs as well as increased feed costs, as a result of animals taking longer to reach their finishing weight.

Therefore, ensuring a high health status through disease prevention and farm biosecurity measures is of the utmost importance to maintaining profit margins.

Porcine respiratory diseases in Great Britain

Respiratory diseases in pigs cause significant economic losses to the swine industry. Historically, respiratory conditions such as rhinitis, bronchitis, pneumonia, and pleurisy have been a considerable problem; viral pathogens such as classical swine fever and Aujeszky's disease have been heavily implicated with these conditions. Whilst classical swine fever was eradicated from Great Britain in 1966, sporadic outbreaks have been reported throughout the years, the last being in East Anglia in 2000, affecting 16 farms and resulting in the culling of 75,000 pigs. The last case of Aujeszky's disease (caused by the Herpes Simplex Virus) in Great Britain was in 1989, with Aujeszky's disease officially eradicated in 1991. Whilst eradicating these diseases has been a monumental achievement for the swine industry, an upsurge of viral infection in the pig population has been seen over recent years.

Pathogens involved in the porcine respiratory disease complex (PRDC)

Respiratory pathogens can cause an infection either singularly or in combination. The primary pathogen is the first unique pathogen that can infect the host, facilitating the co-infection by a secondary or opportunistic pathogen. An opportunistic pathogen is an organism that would not be considered to cause disease in the absence of a primary infection. Respiratory infections, either individually or combined with other pathogenic agents, form a complex coinfection commonly referred to as the porcine respiratory disease complex (PRDC). PRDC infections are considered the most common and costly condition affecting the pig industry. Typically, a primary viral infection predisposes the animal to a secondary bacterial infection. The three viral pathogens commonly associated with PRDC infections are swine influenza virus (SIV), Betaarterivirus suis (BVS), and porcine circovirus virus type 2 (PCV2); whilst BVS and PCV2 are not strictly respiratory pathogens, they are known to affect the respiratory tract frequently and can facilitate secondary bacterial respiratory infections. Highly virulent bacterial pathogens such as *Actinobacillus pleuropneumoniae* (APP) and *Mycoplasma hyopneumoniae* (MH) are considered to be primary PRDC pathogens that induce gross lesions within the lungs. Secondary bacterial pathogens which commonly contribute to PRDC disease include *Pasteurella multocida* (PM), *Glaesserella parasuis* (GPS) and *Streptococcus suis* (SS). **Figure 1.2** shows the distribution of respiratory infections diagnosed by the Animal and Plant Health Agency (APHA) over the past 10 years.

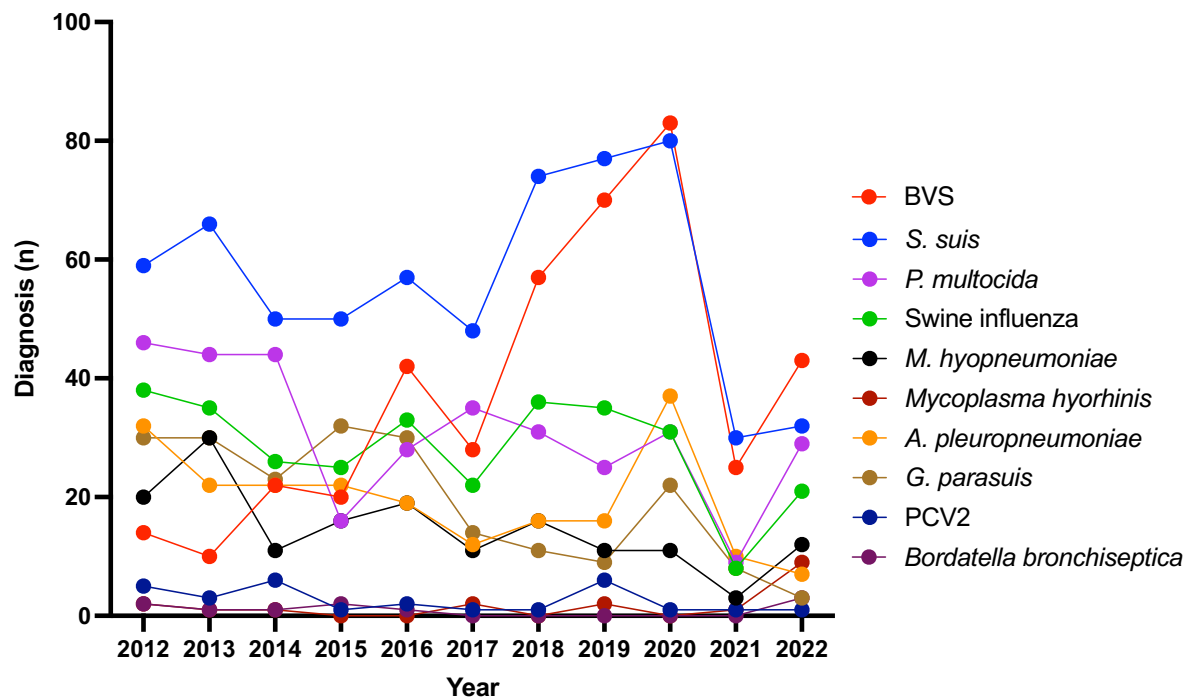


Figure 1.2. Total diagnosis of respiratory pathogens per year. The total number of outbreaks of respiratory pathogens within UK farms, as reported by the Animal and Plant Health Agency (APHA) between 2012 and 2022.

The high prevalence of PRDC pathogens in Europe results in major economic losses to the swine industry. The high economic burden associated with PRDC pathogens is due to the high cost of treatment, reduced growth performance and high mortality rates. Mortality and morbidity rates can be as high as 4-6% and 30-70%, respectively, in endemic farms (Kim et al., 2003). For example, porcine reproduction and respiratory syndrome (PRRS) caused by BVS can result in annual losses as high as EUR €698,379 per farm (Nathues et al., 2017). With greater losses if PRRS is in combination, as it frequently is, with other respiratory diseases.

The complex nature of PRDC infections results in varying clinical outcomes. To understand the interplay between each pathogen, I will first outline how each pathogen behaves when infecting a host.

Bacterial pathogens

1.1. *Actinobacillus pleuropneumoniae* (APP)

1.1.1. Pathogen overview

APP is a small, Gram-negative, rod-shaped, facultatively anaerobic bacterium. APP was first isolated in 1957 (Pattison et al., 1957), and was identified as the causative agent of porcine necrotising pleuropneumonia in 1963 when Richard Shope confirmed Koch's postulates using APP isolated from the lungs of infected animals (Shope, 1963). Initially, APP was assigned to the *Haemophilus* family due to the cultural, morphological, and biological similarities to *Haemophilus influenzae*. In 1983, APP was re-characterised as belonging to the *Pasteurellaceae*

family, within the *Actinobacillus* genus, when DNA hybridisation experiments showed little similarity to *H. influenzae* but high similarity to *Actinobacillus lignieresii* (Pohl et al., 1983).

APP strains are grouped into two biovars based on their requirement for nicotinamide adenine dinucleotide (NAD). Strains requiring NAD for growth, due to a truncated *nadV* gene (Xu et al., 2008), are assigned as biovar 1, whilst strains that are independent of NAD are assigned as biovar 2. Strains can also be assigned to one of 19 serovars, based on differences in their capsule synthesis (*cps*) genes (Bossé et al., 2018). Serovars 5a and 5b were historically distinct (Nielsen, 1986); however, recent analysis shows that they are conserved. As such, in this Thesis serovar 5 is considered one serovar. The distribution of serovars are geologically distinct, with serovar 8 predominating in England and Wales (Li et al., 2016). Of note is that within the UK, antimicrobial resistance genes are found 23-times more frequently compared to Norwegian serovar 8 APP strains (Cohen et al., 2021) which suggests that the UK has historically had a much higher selective pressure due to increased antibiotic usage.

1.1.2. Virulence factors

Specific serovars are associated with higher levels of virulence than others due, in part, to their production of two or more repeat-in-toxins (Apx). There are four Apx toxins that confer varying cytotoxic and haemolytic properties. ApxI is highly haemolytic and strongly cytotoxic and is produced by serovars 1, 5, 9, 10, 11, 14 and 16. ApxII is weakly haemolytic and moderately cytotoxic and is expressed by all serovars except serovar 10 and 14. ApxIII is non-haemolytic and strongly cytotoxic and is expressed by serovars 2, 3, 4, 6, 8 and 15. The last Apx toxin, ApxIV, is produced by all APP isolates and is, therefore, commonly used as a species-specific marker in APP diagnostics. APP serovars expressing ApxI and ApxII are considered the most pathogenic, with clinical signs developing as quickly as 4-12 hours post-infection. Other virulence factors which contribute to the pathogenicity of a strain include modulators to iron metabolism. For example, host produced catecholamines bind to APP and facilitate iron uptake and bacterial growth (Li et al., 2015). Additionally, many genetic differences between breeding stocks are attributed to differences in the gene expression profile of the iron-transport protein, transferrin, which alters the iron availability to APP (Reiner et al., 2014). Biofilm formation is another prevalent virulence factor, with the majority of APP field isolates able to form biofilms (Kaplan and Mulks, 2005, Bossé et al., 2010). Biofilm formation has not only been implicated in the increased survival of APP outside the host but also in increased resistance to host defences and tolerance to antibiotics and is therefore associated with bacterial persistence (Loera-Muro et al., 2021, Izano et al., 2007). The ability of an APP strain to form a biofilm is dependent on poly-N-acetylglucosamine production, encoded by the *pga* operon, which is activated when the bacterium is subject to extracytoplasmic stress (Bossé et al., 2010). Biofilm formation also favours co-infection, as the presence of *S. suis* has been shown to increase APP biofilm formation (Wang et al., 2020).

1.1.3. Clinical signs and transmission

Porcine pleuropneumonia can occur with different clinical manifestations. In the least severe form, subacute disease, symptoms are mild, and animals recover from any clinical signs. However, they remain chronically infected with structural lung abnormalities, as determined at slaughter, including pleuropneumonia with areas of necrotic and haemorrhagic lesions within the lung (**Figure 1.3**). Similar lesions can be observed with infections caused by PM or *Actinobacillus suis*. During acute APP disease, clinical symptoms include anorexia, fever (40.5-41.0°C), and respiratory distress, with morbidity ranging from 10-100% and mortality 1-10%. Severe outbreaks involving the introduction of animals subclinically infected with APP into naïve herds, can result in peracute disease with clinical signs such as high fever (>41.0°C), cardiovascular fever, laboured breathing, apathy, and sudden death. Typically, mortality is so rapid that no clinical signs are observed until deceased animals are found with haemorrhagic foamy nasal discharge (Sassu et al., 2018). Losses of up to 70% of the herd can occur within a 24-48 hour period.

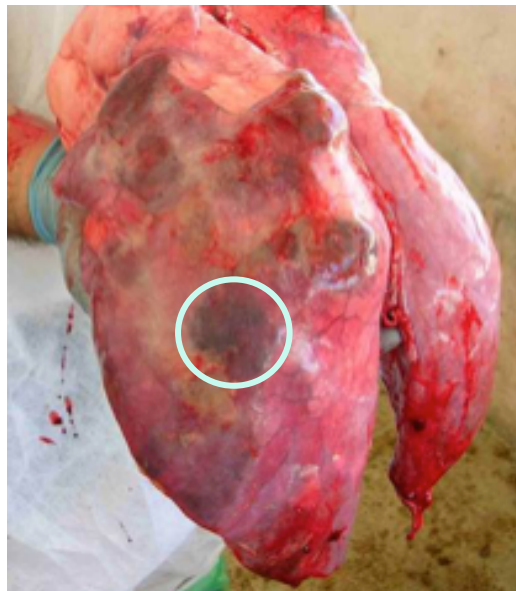


Figure 1.3. Clinical manifestation of *A. pleuropneumoniae* infections. Porcine lungs displaying necrotic lung lesions, highlighted within the circle. Necrotic lesions within the lungs are characterised as multifocal black areas of the lung, a hallmark of APP infection. Image used with the permission of Professor Andrew Rycroft (Royal Veterinary College).

Pigs and boars are the only known natural reservoir for APP, although a case of local tissue necrosis has been reported after a needlestick injury of a live attenuated vaccine candidate in humans (Rycroft et al., 2011). Despite the exact pathogenic mechanisms of APP being unknown, it is believed that both high and low virulence strains reside within the tonsillar crypts causing asymptomatic colonisation, but stressors (e.g., environmental or relating to herd management changes) can result in an outbreak of disease (Gottschalk and Broes, 2019). This is supported by data that infections are typically clonal and endemic areas are frequently reinfected with the same serovar rather than a newly introduced strain (Cohen et al., 2021). Transmission between individuals in a herd occurs through multiple routes, such as short-distance aerosols by coughing (indirect) (Chiers et al., 2002). However, indirect transmission is highly inefficient (Tobias et al., 2013). The transmission coefficient (β) between

infected piglets with no direct contact (i.e., aerosol alone) was found to be $\beta=0.006$ per day, 10 times lower than the transmission rate between animals which had direct contact, $\beta=0.06$ per day (Tobias et al., 2014). Direct contact occurs through the nose-to-nose exchange of nasal secretions and environmental contamination through the saliva (Gottschalk and Broes, 2019). Although APP has been found to survive for up to a month at low temperatures (4°C), there have been reports that APP can survive for longer durations at high temperatures in drinking water from Mexican farms, attributed to the bacterium's ability to form biofilms (Loera-Muro et al., 2021).

1.2. *Mycoplasma hyopneumoniae* (MH)

1.2.1. Pathogen overview

MH is the causative agent of porcine enzootic pneumonia, the most frequently found respiratory disease in pigs both in the UK and worldwide (White, 2009). MH is a small (0.2-0.3 μm) pleomorphic, facultative anaerobe. The bacterium lacks a cell wall, causing it to lack a defined shape, its small size and fragmentation of the cell wall makes Gram-staining unfeasible. Like other members of the *Mycoplasmataceae* family, MH is difficult to culture, requiring complex media and lengthy incubation times, with contamination or overgrowth by other bacteria in samples a common occurrence.

MH was the first isolated in 1965 (Mare and Switzer, 1965); a testament to the difficulties in growth is that the original type strain was lost due to it being non-pathogenic in subsequent cultures. Therefore, the type strain for MH became strain 11, also deposited in 1965, by a different laboratory group (Goodwin et al., 1965). The reference strain, MH232 is used frequently in studies today (largely due to its virulence and transformative properties) was first isolated in 1990 and originated from strain 11 which was serially passaged in homogenised lungs (Zielinski and Ross, 1990).

1.2.2. Virulence factors

MH contains several known virulence-associated genes, most of which are classed as adhesins, and contribute to MH adherence to the ciliated epithelium in the upper respiratory passage beneath the mucosal layer. Nearly 30% of the genes within the MH genome encode predicted adhesion proteins, although many are yet to be functionally characterised (Felde et al., 2018), such as that encoded by the *mhp165* gene. Adhesion of MH to the upper respiratory tract results in the loss of cilia and, ultimately, apoptosis of epithelial and goblet cells (DeBey and Ross, 1994). The adhesin P97, encoded by the *p183* gene, is a critical adhesin involved in MH infection. P97 mutants display reduced adherence to cilia and respiratory epithelial cells (Clampitt et al., 2021). A 60% reduction in adherence was observed when P97 was competitively bound with monoclonal antibodies recognising the protein (Zhang et al., 1995). A P97 mutant does not result in the complete loss of the ability of MH to adhere to cilia and epithelial cells due to several paralogs of P97 found throughout the MH232 genome (Minion et al., 2004). MH infection triggers a host immune response, with local inflammation spreading to the

surrounding alveoli resulting in alveolitis, pneumonia and airway obstruction. The reduced ciliary clearance of the airways predisposes the host to secondary infections resulting in severe pneumonia.

MH also synthesises a strong immunogenic factor, P36, which is a cell surface antigen not produced by other *Mycoplasma* strains infecting swine or other species (Stipkovits et al., 1991). Therefore, due to P36 being produced early in the infection, it has historically been used in MH species identification (Zou et al., 2011). Additionally, MH is able to readily form biofilms through the release of extracellular DNA (Raymond et al., 2018a), which confers an increase in resistance to antimicrobials and host clearance mechanisms.

1.2.3. Clinical signs and transmission

The clinical signs of MH infections are typically non-specific, but the primary classical indication of MH is a dry, non-productive cough that can persist for months. At slaughter, macroscopic consolidation in the cranioventral and diaphragmatic lobes of the lungs are observed (**Figure 1.4**). Lung scoring systems that aid in the diagnosis of MH, based on the location and severity of the lung lesions at slaughter have shown a strong correlation with the infection profile (Garcia-Morante et al., 2016). Interestingly, these classical lesioned areas of MH infection do not necessarily correlate to where in the lungs bacteria are found in the highest abundance (Robbins et al., 2019).

MH is transmitted horizontally through direct contact, such as nose-to-nose exchange of nasal secretions, including vertical transmission, where infected sows rapidly infect their suckling piglets (Batista et al., 2004). A large percentage of infected pigs become chronic carriers, capable of infecting naïve animals, with high rates of reinfections (2.6-10% per year), observed (Maes et al., 2008).

Indirect transmission has been reported with repeated outbreaks occurring in completely repopulated farms (Goodwin, 1985), with MH capable of surviving at least 8 days outside of the host (Browne et al., 2017). Additionally, it has been suggested that viable MH can spread via aerosol as far as 9.2 km (Otake et al., 2010). MH infections are thought to be clonal within an endemic farm. Recently supported by sequencing of the hypervariable *p146* gene demonstrated that the same MH sequence type can be found over several years within the same geographical location (You et al., 2020).

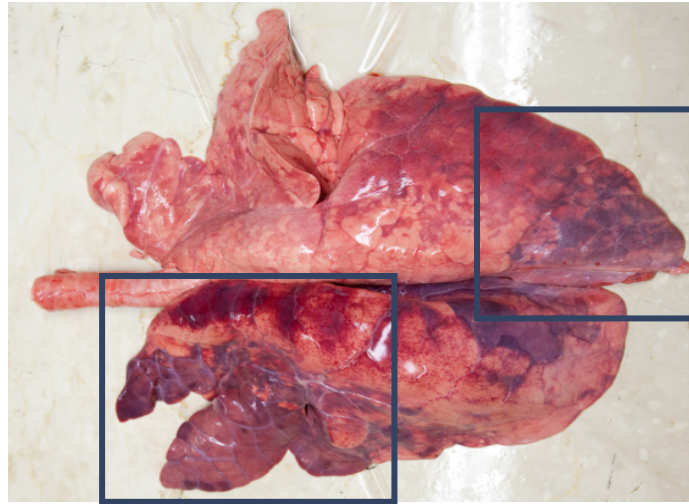


Figure 1.4. Clinical signs of *M. hyopneumoniae* infection. Porcine lungs display pulmonary consolidation, indicated by pulmonary haemorrhage within the lungs to the cranioventral regions (**left**) and the diaphragmatic lobes (**right**), highlighted within the squares. Image used with the permission of CEVA animal health.

1.3. *Pasteurella multocida* (PM)

1.3.1. Pathogen overview

PM is a small, Gram-negative, non-motile, non-spore-forming, facultatively anaerobic coccobacillus which causes atrophic rhinitis in pigs. In the late 1800s, microorganisms were found to cause haemorrhagic septicaemia in a range of animals, and in 1883 Burrill named the bacterium *Micrococcus gallicus* (Burrill, 1883). However, several other genera were assigned to the bacterium in the proceeding years. In 1887, Trevisan proposed the name *Pasteurella* in homage to the work Pasteur performed on the bacterium (Trevisan, 1887), which remains the classification today. Throughout the early 19th century, several bacteria were incorrectly classified as members of the *Pasteurellaceae* family – due to the non-descript requirement for bacteria to display bipolar (accumulation of dye at both poles of the bacterium) staining characteristics with Wright-Giemsa dye. By the turn of the century, *P. multocida* was considered the most appropriate name, and by 1932 only this species was a member of the genus *Pasteurella*. Through the next 40 years, other bacteria (e.g., *Pasteurella haemolytica*, *Pasteurella pneumotropica*, *Pasteurella gallinarum*) were also assigned to the genus. However, classification disagreements raged into the 1970s after years of difficulties in discriminating between *Actinobacillus* and *Pasteurella* genera (Mraz, 1969, Sneath, 1982). Even today, no single phenotypic feature has been found that can reliably distinguish *Pasteurella* species from *Actinobacillus*. Extensive taxonomic reclassification occurred in the 1980s after DNA-DNA hybridization experiments, and a definitive list of *Pasteurella* members was published (Sneath, 1982, Mutters et al., 1985). PM has since been classified into three subspecies (*multocida*, *septica* and *gallicidia*), with most porcine isolates belonging to the *multocida* subspecies, although other subspecies have been isolated from UK pigs (Davies, 2004). Thus, *P. multocida* is currently taxonomically referred to as *P. multocida* subsp. *multocida*. For ease, from herein PM will refer to the *P. multocida* subsp. *multocida* strains.

PM strains express a carbohydrate capsule, which allows the strains to be grouped in five capsule types based on major capsular antigens (Carter, 1952). Capsule types A, B, D and F are reported to infect pigs. Capsule type A is the most common and is associated with porcine pneumonia, followed by capsule type D, which is a common cause of atrophic rhinitis. Capsule types B and F are rare in pigs. PM strains can be additionally grouped into 16 different serovars (formally serotypes) based on the lipopolysaccharide (LPS) antigen (Heddleston et al., 1972).

1.3.2. Virulence factors

The PM LPS is known to be associated with virulence (Fernández De Henestrosa et al., 1997), playing a key role in inducing a host inflammatory response (Harper and Boyce, 2017). Several other virulence factors have been associated with the pathogenicity of PM strains, including fimbriae, adhesins and toxins. Thus virulence genotyping has proved a useful typing method (Liu et al., 2021). A major virulence factor of PM is a bacteriophage-encoded toxin, *P. multocida* toxin. Strains can be toxin or non-toxin-producing; both are considered virulent. However, non-toxin-producing strains are largely associated with opportunistic secondary infections. The toxin is associated with cell adhesion molecules to mediate attachment and receptor-mediated endocytosis, which results in progressive atrophic rhinitis (atrophy of the nasal turbinate's - a curved bone that causes air to flow steadily across the nasal mucosa). These toxigenic strains of PM, belong mostly to serotype D with some belonging to serotype A (Kielstein, 1986, Foged, 1992). However, the toxigenicity status of the isolate has not yet been linked to increased involvement in the PRDC and, in this Thesis, the focus is on the universal detection of PM isolates.

1.3.3. Clinical signs and transmission

There are two clinical disease stages of PM infection, acute and sub-acute disease. In acute disease, signs include pneumonia, high temperature, nasal discharge, rapid breathing, and blue discolouration to the extremities. In the sub-acute disease that usually affects animals 10-18 weeks of age, the pneumonia is less severe but is frequently associated with complications of heart sac inflammation and pleurisy. Other clinical signs include coughing, nasal discharge, and emaciation, which are associated with increased mortality.

Lung lesions usually exude into the bronchi and bronchioles but more frequently, lung tissue necrosis and sepsis are observed. Additionally, focal haemorrhagic pleuropneumonia can be seen in the cardiac lobe, coinciding with the dissemination of the infection to the cardiac sac causing fibrinous pleuritis and pericarditis of the heart (**Figure 1.5**).



Figure 1.5. Clinical signs of *P. multocida* infection. Porcine lungs displaying extensive haemorrhagic pleuropneumonia in the cardiac lobe with fibrin on the pleura (**left**). Porcine pericardium and heart tissue with diffuse fibrinous pericarditis (**right**). Image used with the permission of the publications author (Oliveira Filho et al., 2015).

PM is considered both a commensal and an opportunistic pathogen, often colonising cells within the oral, nasopharyngeal, and upper respiratory tract and under stress conditions, spreading to the lungs. The general term for PM-induced disease is pasteurellosis. The instance of PM dissemination from the lungs into the heart tissue, causing pericardial sac inflammation, is reported to be as high as 73% (Pors et al., 2011). PM can disseminate from the lungs into the bloodstream, resulting in sepsis, as well as systemic spreading into other internal organs.

Pigs can carry the bacterium for many months; carrier animals spread disease between herds, as the bacterium can colonise the respiratory tract and the tonsils. The spread is by droplet infection between pigs, by direct pig-to-pig (nose-to-nose) contact, or indirectly through contact with objects such as equipment or clothing (Register and Brockmeier, 2019). Again, stressors have been associated with increased disease severity and an increased likelihood of transmission (Desrosiers, 2011).

1.4. *Streptococcus suis* (SS)

1.4.1. Pathogen overview

SS is a small β -haemolytic Gram-positive bacterium. Like other *Streptococcus* species, SS is a commensal of the upper respiratory tract that can cause invasive systemic disease. In 1954 *Streptococcus* was first reported to cause bacteraemia and meningitis in piglets (Field et al., 1954). However, it was not until 1979 that SS was isolated from pigs displaying respiratory illness (Koehne et al., 1979). Although isolates of SS were grouped into the Lancefield groups, as with all *Streptococcus* species, it wasn't until 1987 that DNA hybridisation experiments defined SS to be a genetically homogeneous species, distinct from other streptococci (Kilpper-Balz and Schleifer, 1987). Recently, a divergent clade of SS, only showing 65% similarity with the "wild-type" (WT) clade, has been reported to cause clinical disease, and isolates have been found in the UK (Baig et al., 2015).

Whilst SS frequently infects pigs, it is a pathogen of zoonotic importance, with over 1,500 human infections being reported between 2002-2013 (Goyette-Desjardins *et al.*, 2014). The human carriage rate within the nasopharynx is 5.3% after prolonged contact with pigs, compared to 0.0% of those with no contact with pigs (Strangmann *et al.*, 2002). Most human SS infections occur in South-Asian countries with intensive porcine farming and cultural cuisine promoting the consumption of uncooked porcine products. In Vietnam, where a traditional dish, *Tiết canh*, is frequently made with fresh porcine blood - SS is, alarmingly, the most common cause of adult meningitis (Thi Hoang Mai *et al.*, 2008).

SS is classified into different serotypes dependent on the CPS structure. There are currently 29 capsular serotypes, originally 35 - with serotypes 20, 22, 26, 32, 33 and 34 reclassified as other pathogens (Hill *et al.*, 2005; Okura *et al.*, 2016; Hatrongjit *et al.*, 2020). Capsule differences arise from genetic differences within the *cps* gene cluster, with serovar 1, 14, 2 and ½ almost identical, differing by a single nucleotide polymorphism (SNP) in the *cpsK* gene (Okura *et al.*, 2013; Van Calsteren *et al.*, 2016; Roy *et al.*, 2017). Certain serovars are more associated with invasive diseases than others, such as serovar 2, which is commonly isolated in systemic cases and is responsible for the majority of human cases (Goyette-Desjardins *et al.*, 2014).

Seroprevalence of SS varies in different geological regions, with serovars 1, 2, 7, 9 and 14 predominating in Europe. However, the seroprevalence is complex, as certain serotypes are more associated with pulmonary disease (serotypes 4, 2, 3 and 7). Therefore, the seroprevalence in the UK is complicated, with studies reporting differing serotypes based on their site of isolation. A study in 2015 using 185 UK isolates reported that serotypes 1 and 2 were the most prevalent cause of systemic disease, with serotypes 4 and 6 more associated with respiratory illness, and the vast majority of non-clinical isolates were non-typeable (NT) (Weinert *et al.*, 2015). Strains associated with systemic disease are less likely to be present as commensal bacteria within the upper respiratory tract. However, a greater overlap was seen with serotypes found in carrier animals, classed as “non-clinical”, than those causing respiratory illness (**Figure 1.6**). This difference may be due to differences in farm location or seasonal differences in sample collection. Interestingly, significantly higher rates of recombination were seen in non-clinical isolates than in clinical isolates (Weinert *et al.*, 2015).

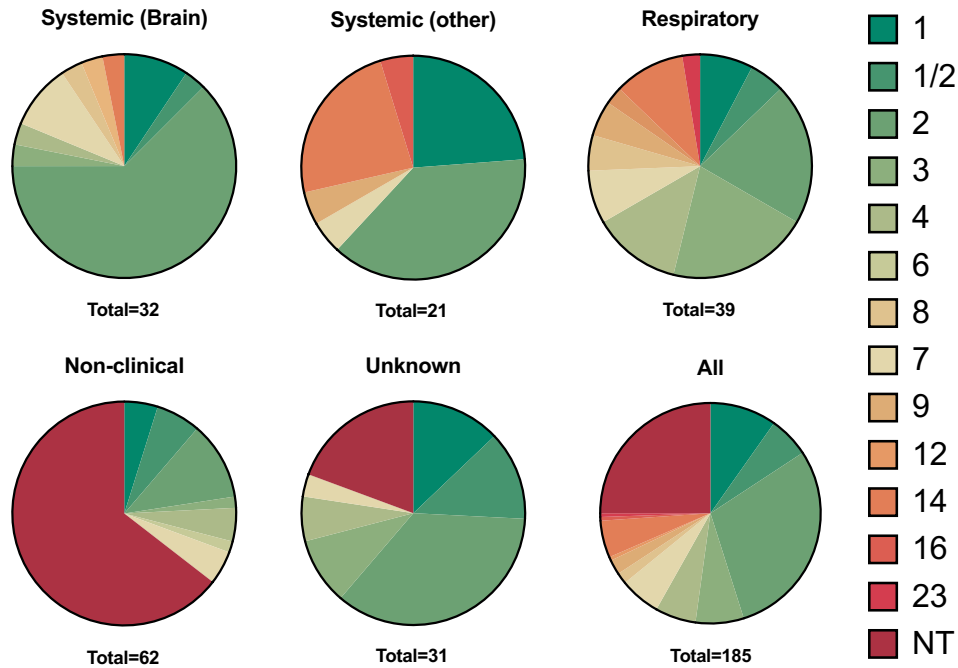


Figure 1.6. Distribution of *S. suis* serotypes. Serotypes of SS isolated from different tissue types from UK farms, with non-typeable isolates designated as “NT”. Data reanalysed from that published (Weinert et al., 2015).

1.4.2. Virulence factors

Several virulence mechanisms have been associated with the ability of isolates to cause active disease, including 51-virulence-associated genes, extensively reviewed (Fittipaldi et al., 2012). Virulence associated genes have been found that increase the adhesion of SS to the extracellular matrix, including fibronectin-binding proteins, such as FbpS (Musyoki et al., 2016) - found in all but two SS serotypes (De Greeff et al., 2002). However, a recent genome-wide comparison between virulent and non-virulent isolates concluded that the presence of virulence factor genes is a poor indicator of the isolates' ability to cause disease (Nicholson et al., 2020).

The most extensively studied virulence factor is the SS capsule. The capsule has been found to contribute to SS resistance to phagocytosis (Segura et al., 2004, Chabot-Roy et al., 2006, Lecours et al., 2011) and survival in whole blood (Auger et al., 2018). Unencapsulated SS mutants have been found to display increased adhesion to epithelial cells (Lalonde et al., 2000, Benga et al., 2004). Thus, during the early stages of infection SS may downregulate capsule expression (Fittipaldi et al., 2012). Additionally, a recent study found that switching the capsule of an isolate can alter the ability of the bacterium to adhere to porcine tracheal epithelial cells, as well as affect host-mediated responses (Okura et al., 2021). Furthermore, like the majority of PRDC pathogens, SS is capable of forming biofilms involving complex expression of genes (Wang et al., 2018). The formation of biofilms help protect SS from antimicrobials and contributes to bacterial persistence (Lewis, 2012).

1.4.3. Clinical signs and transmission

SS is a commensal and opportunistic pathogen with the instance of oral colonisation in UK herds varying between farms, 4-92% (Zou et al., 2018). Once dissemination from the commensal sites to the lower respiratory tract occurs, the bacterium can cause gross and/or microscopic lesions within the lungs (**Figure 1.7**). Certain factors, such as fibronectin binding proteins, e.g., muramidase-related protein (Wang et al., 2015) or SS protein endopeptidase O (Liu et al., 2018a), can also contribute to further infection if the bacterium enters the bloodstream, where it can infiltrate multiple organs such as the spleen, liver, kidneys, and heart, resulting in inflammation. The pathogen can also cross the blood-brain barrier and cause meningitis (Dutkiewicz et al., 2017). Clinical presentations include meningitis, arthritis, endocarditis, septicaemia and sudden death (Gottschalk and Segura, 2019). Airborne transmission of the bacterium has been clearly demonstrated over short distances (pens 40 cm apart) (Berthelot-Hérault et al., 2001), but transmissibility over large distances (c. >10 km) has not been reported. Most infections are typically caused by nose-to-nose (horizontal transmission) or from sow to offspring (vertical transmission). The clinical signs of SS infection are difficult to differentiate from infections caused by other pathogenic bacterial species, with early signs including high fever with no other obvious clinical signs of disease. Within the lungs, lesions and polyserositis, similar to that seen in GPS can be observed in weaning piglets (Obradovic et al., 2021). Systemic disease frequently starts with the signs of septicaemia, intermittent fever, depression, and lameness. In peracute cases, mortality can be sudden, without the onset of clinical signs.



Figure 1.7. Gross lesions as a result of *S. suis* infection. Lung of healthy pig (**left**) and lung of pig infected with SS without meningoencephalitis (**centre**) and non-meningoencephalitis (**right**). Image used in accordance with copyright terms (Liu et al., 2018b).

1.5. *Glaesserella parasuis* (GPS)

1.5.1. Pathogen overview

GPS is a small, pleomorphic, Gram-negative bacterium – a commensal and opportunistic pathogen that belongs to the Pasteurellaceae family. Originally isolates of GPS were assigned as *Haemophilus suis* after isolation from the respiratory tract of pigs infected with swine influenza (Lewis and Shope, 1931). However, isolates of *H. suis* which did not require the X (haem) factor for growth, with X factor independent strains being given the prefix

‘para-’ and were redesignated as *Haemophilus parasuis* (Biberstein and White, 1969). More recently, analysis based on 16S rRNA and *rpoB* genes suggested that GPS is not closely related to *Haemophilus sensu stricto*, and in 2020, after extensive phylogenetic analysis of 70 GPS genomes, *H. parasuis* was reclassified as *Glaesserella parasuis* in homage to K. Glässer who first described the polyserositis disease in pigs in 1910, known as Glässer’s disease (Dickerman et al., 2020).

In 1955, Bakos described four serovars (A-D) based on precipitation testing, which had expanded to seven serovars by 1986 (Morozumi and Nicolet, 1986), proceeded by a further seven serovars with the final serovar being identified in 1992 (Kielstein and Rapp-Gabrielson, 1992). The recent phylogenetic analysis also classed isolates into two distinct clades, as a result of combined alignments of 938 genes (Dickerman et al., 2020). Strains of GPS differ significantly in their virulence. Serovars 1, 5, 10, 12, 13 and 14 are classified as highly virulent, whilst serovars 2, 4, 5, and 15 are less virulent, and serovars 3, 6, 7, 9 and 11 are considered avirulent (Kielstein and Rapp-Gabrielson, 1992, Nielsen, 1993). However, it has been recently reported that a “non-virulent” serovar 7 isolate is capable of causing clinical disease (Dazzi et al., 2020).

Polyserositis in pigs was first described by Glässer in 1910, termed Glässer’s disease, with pigs being the only known natural reservoir for GPS. GPS colonises the upper respiratory tract of weaning pigs, and it is not unusual for several strains (serovars) to be present on a single farm (Cerdà-Cuellar et al., 2010). Historically, Glässer’s disease has been considered a sporadic, stress-induced disease, often activated at 3-4 months of age during the stress of weaning or relocation. As swine production intensified, a shift was observed in older populations falling ill without obvious stressors, attributed to poor health management, over-stocking, or co-infections with other respiratory infections (Palzer et al., 2008, Li et al., 2017)

1.5.2. Virulence factors

Several key factors have been reported that are associated with the ability of GPS to pass from the upper to the lower respiratory tract, including the adhesion to and invasion of epithelial cells. Clinical isolates show a distinct increase in biofilm formation compared to ‘colonising’ strains isolated in the nasal cavity (Bello-Ortí et al., 2014). Virulent isolates have also been shown to possess an increased ability to evade the innate immune system via IgA degradation and resistance to phagocytosis (Costa-Hurtado et al., 2013). The dissemination of isolates into the bloodstream is associated with the bacterium’s ability to disrupt the adheren (Hua et al., 2018) and tight junctions (Wang et al., 2021), increasing the permeability of the vascular tissues resulting in systemic diseases. It has been reported that isolates from the upper respiratory tract or the lung are more likely to be part of a co-infection, whereas isolates from the joints or meninges are likely to be highly virulent, being able to survive serum-killing (Cerdà-Cuellar and Aragon, 2008). A group of virulence-associated genes, the virulence-associated trimeric autotransporters (*vtaA*) have been attributed to the virulence of GPS strains, with groups 1 and 2 associated with clinical strains (Pina et al., 2009). Subsequently, *vtaA* grouping has been shown to be a strong indicator for GPS strain virulence (Howell et al., 2014, Olvera et al., 2012) .

1.5.3. Clinical signs and transmission

Acute outbreaks of Glässer's disease can result in high mortality (5-10% in younger pigs). Classical signs of the disease include high fever, cough, abdominal breathing, swelling of the joints, as well as nervous system signs - such as tremors (Aragon et al., 2019). At slaughter, characteristic pericardial thickening (polyserositis), referred to as "fluffy heart", and necrotic fibrinous lesions within the lungs can be observed (**Figure 1.8**). Additional clinical signs include fluid collection within the abdominal wall containing white blood cells (peritoneal fibrosis exudate) and fluid within the joints. Fibrinous polyserositis lesions can also be found in chronically infected animals encroaching on the intestines and spleen. The pathology of a GPS outbreak is rapid and widespread with a loss of up to 60% of the herd within 72 hours.

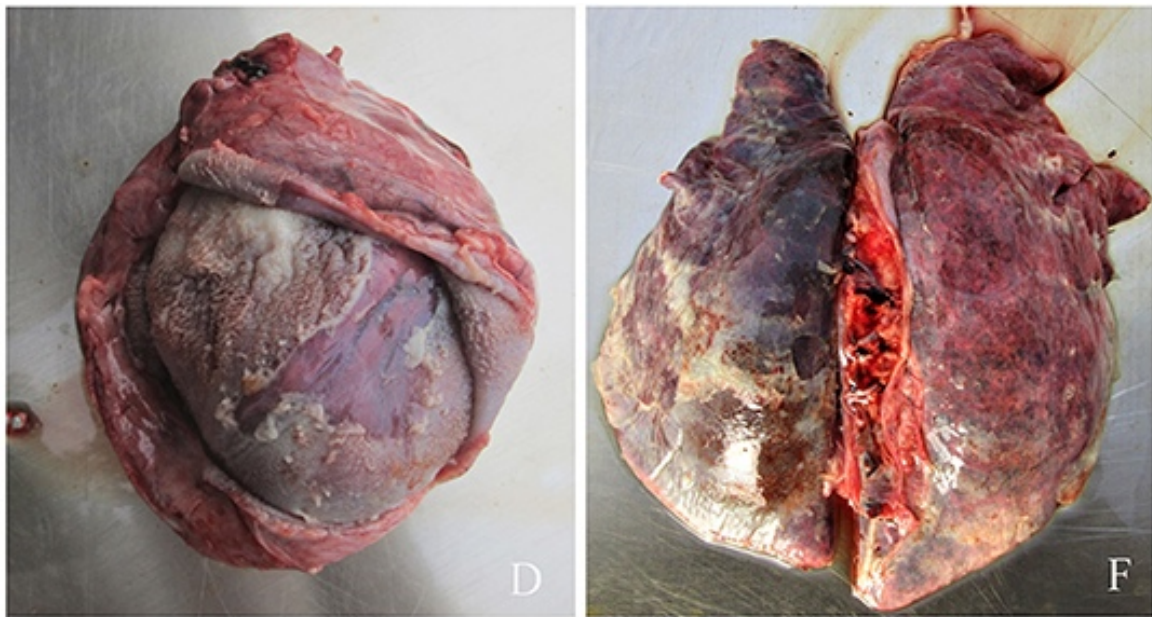


Figure 1.8. Classical signs of Glässers disease caused by *G. parasuis*. Thickening of the cardiac sac "fluffy heart" (**left**). Bleeding and necrosis of the lungs (**right**). Image used in accordance with copyright terms (Qi et al., 2021).

Viral pathogens

1.6. Swine influenza (SIV)

1.6.1. Pathogen overview

SIV is a single-stranded negative-sense RNA virus, belonging to the *Orthomyxoviridae* family of viruses. The *Orthomyxoviridae* family is comprised of four genera, influenza A virus, influenza B virus, influenza C virus and influenza D virus. SIV belongs to the influenza A genus. SIV can cause high morbidity (up to 100%) whilst mortality is generally low.

The SIV genome contains eight RNA segments that encode 12 proteins: polymerase basic protein 2 (PB2) is a 759 amino acid (AA) protein that aids mRNA cap recognition of the polymerase subunit. Polymerase basic protein 1 (PB1) is a 757 AA protein that is responsible for RNA elongation and endonuclease activity of the polymerase subunit. Polymerase acidic protein (PA) is a 716 AA protein that is part of the polymerase subunit and has protease activity. Hemagglutinin (HA) is a 550 AA surface glycoprotein that is responsible for receptor binding and fusion with cells. Nucleoprotein (NP), an RNA binding protein and regulator of nuclear import. Neuraminidase (NA), a 454 AA surface glycoprotein with sialidase activity and mediates viral release. Matrix segment (M) that encodes for a 252 AA M1 matrix protein, and a 97 AA M2 ion channel. A non-structural segment (NS1) which encodes a 230 AA interferon antagonist, and a 121 AA nuclear export protein (NEP) (Palese and Schulman, 1976, Hutchinson et al., 2010). The eight segments are named in descending order of AA length (**Figure 1.9**).

The segmented genome of influenza viruses allows antigenic shift, which is where a virus strain acquires a HA and/or a NA segment from a different subtype. These major events are of great concern, as the subtype may allow the virus to infect a new host species, in which that host has little or no protection - they are immunologically naïve and, therefore, can result in a pandemic strain. HA is present at a higher abundance than the NA protein, a ratio of 4:1 (Bouvier and Palese, 2008). The HA protein recognises N-acetylneuraminic (sialic) acid oligosaccharides on the surface of host cells, with the HA having specificity (SPE) for α -2,3- or α -2,6-linkage that occurs between sialic acid (SA) and galactose (Gal). Pigs are of high importance in zoonotic influenza disease, as the porcine trachea contains both SA-Gal linkage types (Ito et al., 1998), whilst the human respiratory tract contains these linkages in different abundances (Shinya et al., 2006). Therefore, within the swine population, different influenza subtypes can be present that usually wouldn't be present in humans, increasing the potential for novel subtypes. Thus, the porcine population has been known as a 'mixing vessel' for influenza A (Ma et al., 2009).

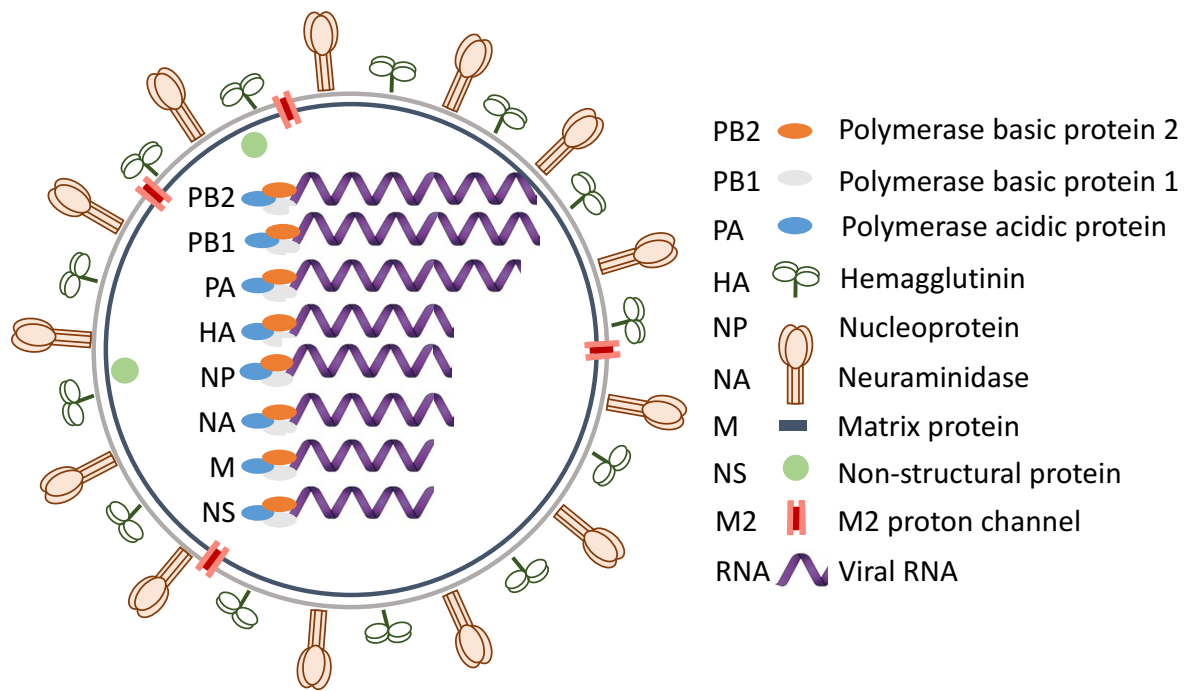


Figure 1.9. Structure of influenza A virion. The viral particles of influenza A containing the eight RNA genome segments encoding the viral polymerase PA, PB1 and PB2 as well as the viral proteins HA, NP, N, M, NS and M2 (see main text for details). Figure created in PowerPoint.

SIV can also undergo antigenic drift, in which errors made by the RNA polymerase during replication accumulate and result in a structural difference to the HA protein. Different SIV subtypes have slightly different nucleotide substitution rates, with H1 ranging from 4.37×10^{-3} per site per year (Lam et al., 2008), whilst the H3 subtype is estimated to have a rate of 6×10^{-3} per site per year (Lewis et al., 2014). The rate of antigenic drift in SIV is thought to be less than what occurs with influenza viruses that infect humans, as the decreased lifespan of swine results in less influenza immunity and, therefore, less selection pressure for the virus (Ryt-Hansen et al., 2020).

Influenza viruses are assigned a subtype via their two main surface glycoproteins, i.e., HA and NA, e.g., H1N2. With three main influenza subtypes infecting pigs: H1N1, H1N2 and H3N2. In 1986, the first case of SIV was detected in the UK, found to belong to the H3N2 subtype, and has been shown to have been present within the pig population from as early as 1971 (Chapman et al., 1977). Since 1997, no H3 influenza strains have been detected in pigs within the UK (APHA, 2020b). Presently, within the UK, only the H1N2 and pandemic H1N1 2009 pandemic strain (H1N1 pm09) SIV strains are circulating (APHA, 2020b), with the H1N1 pm09 strain being the most prevalent (44.83%), followed by H1N2 (41.38%) and H1N1 (13.79%) (Garrido-Mantilla et al., 2019). For epidemiological purposes, the subtyping of SIV outbreaks is extremely important.

1.6.2. Virulence factors

The majority of influenza proteins have been implicated in modulating the virulence of the viral infection, extensively reviewed (Janke, 2014). In natural SIV infection, strains which exhibit a higher mortality have been found to have frequently undergone a triple genomic reassortment (Ma et al., 2010). Additionally, highly virulent

strains have been associated with numerous AA changes to nearly all SIV proteins (Jang et al., 2020). The difficulties in pin-pointing specific viral mutations which confer an increased virulence exemplifies the high degree of genetic drift that occurs within the influenza family of viruses. It has been concluded that high virulence is likely due to increased viral replication in the lower respiratory tract, with high virion abundance and increased elicitation of host immune responses, namely cytokines (Janke, 2014).

1.6.3. Clinical signs and transmission

Clinical signs of SIV infection include high fever, lethargy, sneezing, nasal discharge, persistent dry coughing, difficulty breathing and decreased appetite (Reeth, 2007). In the cases of the uncomplicated disease, i.e., no co-infection, an animal is expected to recover from SIV within 3-5 days, with the viral shedding only occurring for 6-10 days (Sun et al., 2015a, Bragstad et al., 2013). However, reinfections can occur, typically in nursery areas when the pigs are 5-7 weeks of age, SIV is seen in combination with BVS and is called the “blue-flu” (White, 2006). Carrier animals can be asymptotically harbouring the virus for several months, and additionally, maternal antibodies reduce the clinical presentation of the disease but not the spread of the virus (Allerson et al., 2013, Cador et al., 2016). Therefore, nursery units are thought to suffer from endemic forms of influenza.

Swine influenza is spread through direct contact, nasal secretions, dried mucus, or aerosol. It has been suggested that aerosol transmission can occur over 2-4 km away, although open to question as the virus was unable to be isolated from air samples (Corzo et al., 2013, Zhang et al., 2013).

1.7. Porcine circovirus (PCV)

1.7.1. Pathogen overview

PCV is a small non-enveloped single-stranded DNA (ssDNA) virus belonging to the *Circovirus* family of viruses. PCV viruses are the smallest virus known to replicate inside the eukaryotic cells (Mankertz, 2008). In 1974, a virus was found to be present in a culture of porcine kidney cell line, and was named PCV (Tischer et al., 1974). However, there was no evidence that inoculation with PCV resulted in any clinical signs of diseases in pigs. Therefore, PCV was not considered pathogenic (Tischer et al., 1986, Allan et al., 1995). In 1991, a new syndrome was reported in North America that caused weight loss, respiratory signs and jaundice in piglets, described as post-weaning multisystemic wasting syndrome (PMWS) (Clarke, 1997). In 1998, PCV was found to be the etiological agent of PMWS (Ellis et al., 1998, Ellis et al., 1999). Subsequently, the PCV virus responsible for PMWS was found to be genetically distinct from the original virus characterised by Tischer in 1974 and was thus named PCV type 2 (PCV2) (Meehan et al., 1998). With the original PCV virus described by Tischer (1974) now referred to as PCV type 1 (PCV1) and is still not considered to cause disease. PCV2 has been found to be circulating in Europe as early as 1962 (Jacobsen et al., 2009), and in 2016 a new PCV variant was found, named PCV type 3 (PCV3) isolated from a clinical case of porcine dermatitis and nephropathy syndrome (PRDS), also associated with PCV2 (Palinski et al., 2017). Recently, PCV type 4 (PCV4) was found in China, again causing a similar clinical presentation to PCV2 and PCV3, but was also isolated from pigs with no clinical sign of disease (Zhang et al.,

2019a). Although the distribution of PCV4 is unknown, no reports of the virus have arisen from anywhere outside of China. PCV2 still remains the most prevalent cause of PMWS, with a worldwide distribution.

The genomes of PCV are less than 1.8 Kb, consisting of two open reading frames (ORFs) located in opposite orientations on PCVs closed single standard genome. The ORFs only consist of a single capsid protein (ORF2) and two replicase proteins (ORF1) (**Figure 1.10**). The ORF1 locus is highly conserved in all viruses in the *Circovirus* family, whilst the capsid protein encoded by ORF2 is less conserved. Therefore, ORF1 is used as an evolutionary marker to infer the origin of the virus. An analysis of circoviruses determined that the closest ancestor to PCV3 is clade 1, whilst PCV1 and PCV2 were closely related to clade 2 of bat circoviruses (Li et al., 2018, Zhang et al., 2019a). Therefore, the consensus is that PCV1-4 originated in bats and transferred to pigs in two independent genetic diversification events.

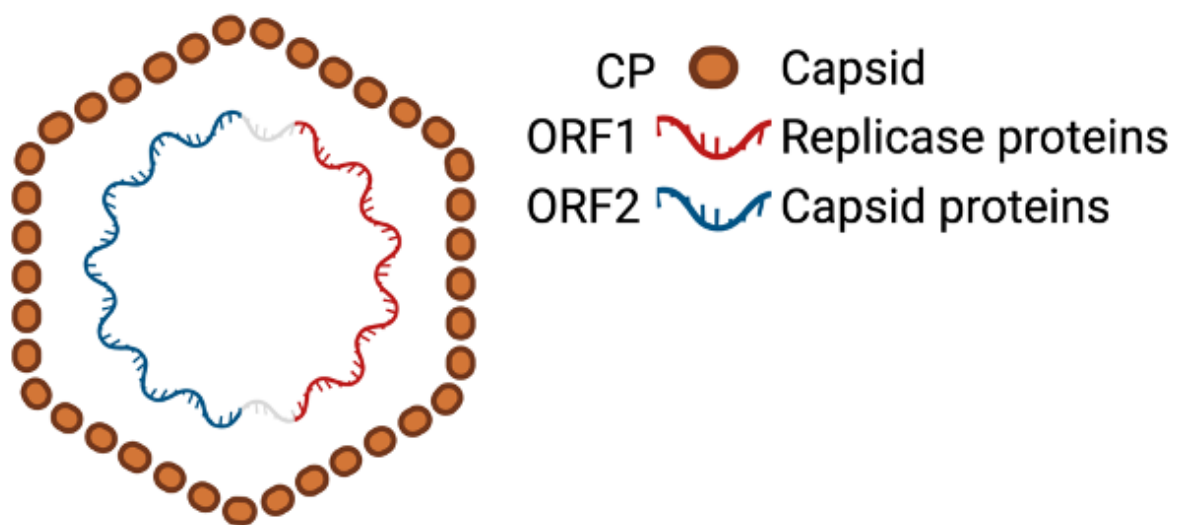


Figure 1.10. Arrangement of PCV virion. The circular ssDNA genome containing two ORFs is surrounded by an outer envelope consisting of capsid proteins. Figure created with BioRender.

Each PCV type has been characterised into several genotypes based on whole-genome and capsule gene variations, with these genetic shifts thought to alter the pathogenicity and vaccine immunity of the virus (Xiao et al., 2015). There has been limited genetic sequencing of PCV1 and due to the lack of PCV1 association with disease, no genotypes have been described. There are currently eight genotypes of PCV2 (a-h) (Franzo and Segalés, 2018), although there is contention as to whether PCV2d and PCV2g are distinct genotypes or intermediates (Xiao et al., 2015). There are three genotypes of PCV3 (a-c) (Fu et al., 2018), and two genotypes of PCV4 (a and b) (Xu et al., 2022). Additional subclades can be present within a genotype (e.g., PCV3a2).

PCV1 and PCV2 show high sequence identity (<80%), with 86% and 65% AA identity shared between their ORF1 and ORF2, respectively. Whilst PCV3 sequence identity with PCV2 is much lower, at <50% overall similarity, with 48% and 26-36% AA identity across their ORF1 and ORF2, respectively (Palinski et al., 2017). PCV4 shares 51.5% sequence identity with PCV2 and 47.2% and 45% AA identity across their ORF1 and ORF2, respectively (Zhang et al., 2019a).

1.7.2. Virulence factors

The substitution rates vary considerably between PCV types and genotypes (Li et al., 2018). For example, PCV2 displays a substitution rate of 10^{-3} substitution/site/year (Franzo et al., 2016). Whilst PCV3 is considered to have limited genetic variability with a substitution rate of 10^{-5} substitution/site/year (Franzo et al., 2019), supported by the sequence similarity found between archived (1993) and recent (2018) samples (Klaumann et al., 2018). There are several genetic alterations that have been associated with the virus's ability to evade host immune defences (Darwich and Mateu, 2012, Franzo et al., 2016). Viral divergence occurs through random point mutations, genome rearrangements, recombination, and translocation (Witzany, 2006). With more than one genotype being present within a host, and genomic recombination common within PCV2 strains (Correa-Fiz et al., 2018). PCV3 can also possess an epitope variation at AA site 24 of ORF2, suggested to confer escape from the host immune system, with the epitope found in strains of PCV3b indicating a positive selection pressure over PCV3a (Li et al., 2018).

1.7.3. Clinical signs and transmission

Infection with PCV2, PCV3 or PCV4 can result in a range of clinical syndromes. referred to as porcine circovirus disease (PCVD). The focus of this Thesis will be PCV2, as it is the most clinically relevant to disease in the UK. PCV2 can cause both a subclinical infection, as well as systemic disease. Subclinical infection is associated with economic loss due to slower fattening rates. The clinical signs of PCV2 systemic disease include; wasting, dyspnoea, paleness, and diarrhoea (Allan and Ellis, 2000). At slaughter, gross lesions can be found within the lungs and kidneys, with enlargement of the lymph nodes and kidneys accompanied by white spots. Direct contact is considered the most effective form of transmission (Andraud et al., 2008), with viral shedding being higher in the systemic disease form through secretions and excretions of infected pigs (Rose et al., 2012). In direct (aerosol) transmission is known to also frequently occur (Mateusen et al., 2004, Pensaert et al., 2004), with additional evidence of indirect PCV2 transmission, with PCV2 dissemination between pens occurring through worker transit between contaminated units (Dvorak et al., 2013, Cao et al., 2018, López-Lorenzo et al., 2019).

PCV2d is the most prevalent within the UK, although PCV3 has been found at high viral loads in stillborn or neonatal piglets, with a low prevalence (9%) of heart tissue being PCR positive with PCV3 (APHA, 2021). Therefore, PCV2 will be the diagnostic focus of this Thesis, as it is widespread throughout the UK. It is noteworthy that PCV3 co-infection can significantly worsen the clinical presentation of PRRS (Zhai et al., 2017).

As PCV2 is the most prevalent, studied and clinically relevant PCV type that is associated with respiratory disease presentation within the UK, PCV2 will be the main Circovirus focus of this Thesis.

1.8. Betaarterivirus suid (BVS)

1.8.1. Pathogen overview

PRRS is caused by an enveloped single-stranded positive-sense RNA virus, BVS, formally the PRRS virus (BVS). BVS belongs to the *Arteriviridae* family of viruses and was first described in America in 1987 (Dial and Parsons, 1989) and in 1991 in Europe as a virus causing anorexia and fever in breeding sows and their piglets. The disease was known as 'mystery pig disease' or swine infertility and respiratory syndrome in North America (Loula, 1990), whilst in the UK it was known as blue ear disease (White, 1991), as the Dutch name of 'abortus blauw', due to the common sign of sows with blue ears. In 1991/2 the BVS was first isolated in the Netherlands (Wensvoort et al., 1991), and in 1992 Koch's postulates were satisfied (McCullough et al., 1992).

There are two types of BVS that cause PRRS in pigs, BVS-1 and BVS-2. Initially, BVS-1 was found to be circulating in Europe and BVS-2 in North America, strains were compared and found to be genetically different (Nelsen et al., 1999). BVS-1 and BVS-2 isolates only share 55-70% homology within their nucleotide sequence and 50-80% AA similarity (Nelsen et al., 1999, Suárez et al., 1996). The genome of BVS is typically between 14.9-15.5 kb with 10 ORFs. The majority of the BVS genome (60-70%) encodes non-structural proteins located towards the 5' end of the genome. Structural proteins are encoded at the 3' end of the genome, such as N, M, GP2-5 and E, which are involved in the formation of the viral envelope and RNA packing (Snijder and Meulenbergh, 1998).

1.8.2. Virulence factors

There is a large genetic variation between BVS strains, helping to sustain endemic levels of the disease in most parts of the world. Driven by BVS displaying the highest instance of mutation seen in any RNA virus, the calculated rate of nucleotide substitution is $4.7\text{-}9.8 \times 10^{-3}$ per site per year (Hanada et al., 2005). Recombination is another important ability of BVS isolates, resulting in vast genetic diversity. BVS-2 recombination frequently occurs within the GP2 and GP4 regions, which are factors involved in cell entry, and thought to play a role in cell tropism and replication capacity (Guo et al., 2021). Strains of BVS that are highly pathogenic carry a significant AA deletion in the non-structural protein 2 (NSP2) (Zhao et al., 2015a, Canelli et al., 2017), which mediates a reduction in the host inflammatory response (Du et al., 2021), including decreased pro-inflammatory cytokine expression (Sun et al., 2012, Gómez-laguna et al., 2013), as well as replicating in the thymus ultimately leading to dysfunction and a subsequent abhorrent immune response (Butler et al., 2014). Furthermore, the emergence of a highly pathogenic BVS-1 has been observed in Europe, (Stadejek et al., 2013, Balka et al., 2018). These highly pathogenic strains have been shown to replicate at an increased rate compared to typical BVS strains (Ferrari et al., 2018).

1.8.3. Clinical signs and transmission

Clinical signs of PRRS include respiratory distress in young pigs, and reproductive failure in pregnant sows resulting in stillborn and aborted fetuses (Goyal, 1993). Pulmonary dysfunction can be accompanied by increased respiratory rate, lethargy, fever, occasionally coughing and sneezing, and enlargement of the lymph

nodes (Rossow et al., 1994, Wagner et al., 2011). At slaughter, mild to severe lung lesions can be observed in the lungs, with microscopic lesions characterised by type II pneumocyte hypertrophy and hyperplasia, septal infiltration with mononuclear cells and alveolar exudates (Opriessnig et al., 2002).

The replication of BVS occurs in macrophages, with porcine alveolar lung macrophages being the primary target. Attachment to macrophages is heparin mediated and entry into macrophages occurs through acidification, with BVS particles endocytosed into clathrin-coated vesicles. The virus spreads readily through the lympho-haematic route, resulting in viraemia lasting 1-4 weeks depending on age (Van Der Linden et al., 2003, Díaz et al., 2012). BVS is known as highly contagious, with 10 or fewer virions required to cause infection (Yoon et al., 1999). BVS is transmitted through the respiratory route, with high viral load detected in the oral fluid from one-week post-inoculation, lasting (intermittently) 4-5 weeks (Prickett et al., 2008, Kittawornrat et al., 2010). BVS can also be transmitted through semen and, as such, is considered a sexually transmitted pathogen (Swenson et al., 1994). The highest viral load is found in the lungs, where the virus can be isolated as soon as one day after the initial exposure (Halbur et al., 1996). High viral load can also be found in blood, as well as the lymphoid organs such as the tonsils, lymph nodes, and spleen (Sur et al., 1993, Lamontagne et al., 2004).

1.9. PRDC clinical outcomes

There are numerous factors that dictate the clinical outcome of an infection by a single pathogen, with the ability of a pathogen to be virulent also dependent on host susceptibility. Casadevall and Pirofski (2018) proposed the MISTEACHING framework listing 11 factors that determine the susceptibility of a host to a pathogen (Casadevall and Pirofski, 2018). This has recently been extended to include “strain” to become the MISTEACHINGS framework (Langford et al., 2021). The acronym MISTEACHINGS stands for: microbiome, immunity, sex, temperature, environment, age, change, history, inoculum, nutrition, genetics and strain. A summary of the multiple factors that contribute to PRDC clinical outcome is shown in **Figure 1.11** and will be briefly described below.

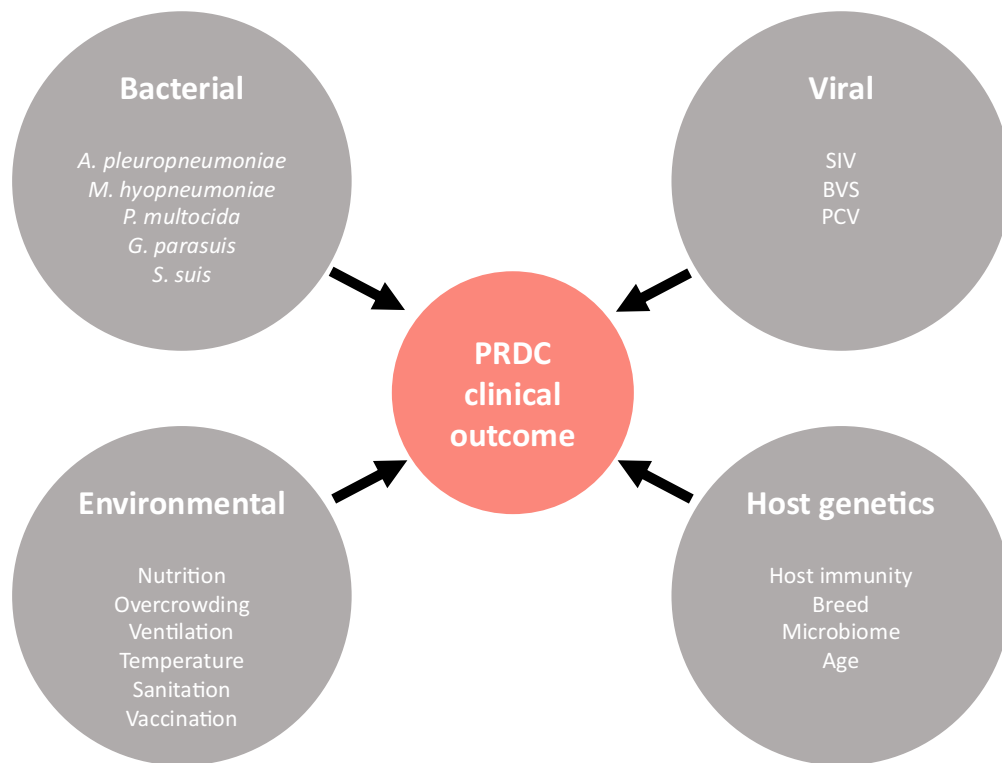


Figure 1.11. Factors influencing porcine respiratory disease complex clinical outcomes. The clinical outcome of infection with a PRDC pathogen involves multiple factors, such as the viral and bacterial pathogen/s present, along with both environmental and host genetic factors. Image adapted from (Saade et al., 2020).

1.10. Pathogen interplay

The pathogens present in PRDC co-infections can influence the clinical outcome of the disease depending on the synergistic, neutral, or antagonistic behaviours between the pathogens involved. In general, bacterial-viral and viral-bacterium co-infections result in a worsened clinical outcome, increased aggravation of pulmonary lesions, a more potent immune response including higher inflammation and increased production of pro-inflammatory cytokines. Additionally, co-viral infections, such as with BVS and SIV or PCV2 and BVS, have been demonstrated to increase the titre of a secondary viral pathogen (Rovira et al., 2002, Kitikoon et al., 2009).

Viral infections also contribute to bacterial infection through the destruction of epithelium and the altering of host immune responses. Following SIV infection, SS colonisation has been shown to be facilitated through loss of ciliated cells, impairing ciliary clearance, and increasing viral production of HA, promoting adherence of SS through their sialic acid capsule (Meng et al., 2015). Furthermore, primary bacterial infections can potentiate the outcome of several viral diseases once the bacterial infection is well established within the respiratory system; for instance, inoculating pigs with MH shortly before PCV2 did not impact the severity of the clinical infection (Sibila et al., 2012), whilst inoculation with MH three weeks before challenge with SIV did potentiate the disease (Deblanc et al., 2013). Additionally, co-infections involving only bacterial pathogens result in higher scoring clinical lesions and altered immune responses such as decreased phagocytosis (Pieters and Maes, 2019,

Gottschalk and Broes, 2019), increased cytotoxicity of immune cells or altered cytokine production favouring bacterial survival (Fourour et al., 2019).

1.10.1. Environmental and host factors

Several other non-pathogen related factors can contribute to the susceptibility of the host, such as those associated with herd management. For example, herd size and stocking density both contribute to the air quality. A limitation on aerial pollutants such as ammonia (>50 mg/L), carbon dioxide, methane, and dust are associated with decreased susceptibility to disease (Done, 1991). In the UK, 40% of UK pork is derived from outdoor breeding herds (AHDB, 2020) and air quality is less of an issue. However, temperature variation maybe more of a stressor in outside herds (Langford et al., 2021). The European farrow-to-finish farming model, where animals are moved between sectors based on their age, is thought to encourage SIV persistence due to regular farrowing allowing the reintroduction of susceptible piglets (Cador et al., 2017). The batch-rearing model where there is independent management of each batch, limits the mixing of animals with different health and immune statuses and is widely used (Cador et al., 2015).

Host factors that contribute to the disease, include the host microbiome. The gut microbiome is a complex population of microorganisms within an animal's digestive tract that plays a vital role in animal health and immunity. Several external factors, both environmental and genetic, influence the host microbiome. Genetically, a reference gene catalogue has been formed for the pig microbiome (Xiao et al., 2016). Microbiome diversity has been strongly linked to host immunological, nutritional, and physiological responses. Strategies to increase animal health through feed include feed supplementation with fermenting bacteria which produce high amounts of lactic acid (>150 mmol/L), and low concentrations of acetic acid (<40 mmol/L) thought to decrease the pH of the feed (pH <4.5) and reduces levels of *Enterobacteriaceae* in the microbiome (Van Winsen et al., 2002). Fungal communities can also be supplemented into feed to increase protein content, antioxidants, anti-inflammatory, anti-obesity and immune system regulation (extensively reviewed (Chuang et al., 2020)).

Several studies suggest that different breeds of swine are more susceptible than others to respiratory diseases. For example, the German Landrace breed is more susceptible to APP infection than the Hampshire breed, determined by a novel respiratory score system (Hoeltig et al., 2009). A finding mirrored with viral pathogens, with the Landrace breed also found to be more likely to show clinical signs and lesions associated with PCV2 infection than other breeds (Opriessnig et al., 2006). Thus, there has been renewed efforts in breeding animals with increased tolerance to infection. In 2018 pigs were genetically edited to remove a portion of the CD163 cell surface receptor, which BVS uses to infect cells, resulting in immunity from the virus, and resistance to infection (Burkard et al., 2018).

Therefore, there is a complex interplay between genetic, environmental, and historical microbiome factors which can contribute to disease susceptibility and clinical outcomes. The coinfections seen in the PRDC

outbreaks have multiple complexities, requiring extensive diagnostic investigations and a multi-disciplinary approach to control outbreaks.

PRDC diagnostics

1.11. Sample types

Although the PRDC relates to infections of the lower respiratory tract, several pathogens involved in the PRDC can cause systemic disease. For example, viral infections can cause viremia - detected in the blood. Therefore, there may be several tissue types that can be investigated depending on the clinical presentations of the animal and the suspected PRDC pathogens at play. The tissue types likely to be sampled for each of the PRDC pathogens are listed in **Table 1.1**.

Oral fluid has been reported to be a robust sample type for pathogens such as APP (Kleinmans et al., 2022), but the use of oral fluid has given inconsistent results when detecting the viral pathogens and MH (Hernandez-Garcia et al., 2017). This may be due to oral fluid being collected through chew ropes, a notoriously 'dirty' sample, as well as sampling several animals within the same pen. Whilst the pooling of samples is a cost-effective surveillance method, the more animals sampled, the increased risk that bacterial and viral loads are diluted - reducing the diagnostic sensitivity (SEN) (Osemeke et al., 2022). Other sample types that can be taken on living animals include nasal swabs, bronchoalveolar lavage fluid (BALF) and blood samples. However, the age of the animal may dictate if an ante-mortem or post-mortem sample is taken, as it is very difficult to take samples from living animals once they reach a certain weight. Although several sample types can be investigated at post-mortem for presence of particular PRDC pathogens, all are detected within the lung tissue.

Table 1.1. Typical tissue samples for diagnostic investigation of each pathogen.

	Tissue	Pathogen							
		APP	MH	PM	SS	GPS	SIV	PCV2	BVS
Ante-mortem	Nasal swab	X	X				X		X
	Oral fluid	X	X				X		X
	BALF		X				X	X	X
	Blood		X				X	X	X
Post-mortem	Lung	X	X	X	X	X	X	X	X
	Tonsils	X	X				X	X	X
	Heart			X	X	X		X	
	Liver				X	X			
	Thymus		X						X
	Spleen				X	X			X
	Kidney				X	X			X
	Intestine								X
	Brain			X	X				
	Synovial fluid			X	X				
	Placenta								X

1.12. Bacterial and viral isolation

Historically bacterial and viral identification through culturing methods was the gold standard for determining the etiological agent of disease. Bacterial cultivation from lung tissue obtained during necropsy is still practised today for APP, SS, GPS, MH, and to a lesser extent PM. However, due to the fastidious nature of MH, the culture and identification of the organism may take up to a month to perform, although recent efforts have been made to optimise the culture media to MH and limit overgrowth of the culture with other bacteria, such as *M. hyorhinis* (Cook et al., 2016). Additionally, for bacteria that are known to be commensal organisms such as GPS, samples should only be taken if there are visible signs of pneumonia and only from the distal region of the lungs, to prevent cross-contamination with non-virulent strains of GPS caused during lung aspiration (Costa-Hurtado et al., 2020). Viral isolation involves using specific cell lines to amplify the virus, and although sensitive, this method can take 1-2 weeks to obtain results. For SIV, common cell lines used include specific-pathogen-free embryonated chicken eggs or Madin-Darby canine kidney cells, whilst pulmonary alveolar macrophages or derived monkey kidney cells are used for BVS. Viral isolation is seldomly practised today due to labour-intensive methods and lengthy incubation times.

1.13. Serological-based diagnostics

Serological testing involves the detection of pathogen-specific antigens or host antibodies (Abs) directed against the pathogen or pathogen-specific antigens. Common serological methods include agglutination, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA) assays – discussed in the sections below.

1.13.1. Agglutination

Agglutination is used to detect the presence of agglutinating Abs that form cross-linkages in the presence of specific antigens forming clumps that are visible under microscopy. Agglutination using anti-serum raised against specific SS serovars can result in cross-reactions due to common epitopes within the capsular polysaccharides, such as that between serotypes 2 and 1/2, and serotypes 1 and 14 (Goyette-Desjardins et al., 2014, Van Calsteren et al., 2016). Conversely, subtyping of SIV isolates can be successfully performed using hemagglutination and neuraminidase inhibition assays with antisera raised against the specific subtypes (Murphy and Webster, 1990).

1.13.2. Immunohistochemistry

Immunohistochemistry is a popular method to detect and localise the presence of an antigen. In situ hybridization uses labelled probes, which anneal to the pathogenic DNA and an Ab to bind the probe and allow conversion of a substrate to determine the presence of the pathogen. For example, MH presence can be detected in lung tissue by fluorescent polyclonal Abs by in situ hybridization or immunohistochemistry using frozen lung slices. Frequently, membrane proteins P36 and P46 are used as genes for MH detection. However,

these techniques are severely hampered by their requirement for tissue fixation, ability to only evaluate small tissue sections at a time, and laborious wash steps (Pfankuche et al., 2018).

1.13.3. Enzyme-linked immunosorbent assay (ELISA)

ELISA is the most commonly used serological test. There are four types of ELISA assays, direct, indirect, sandwich and competitive. The main principle of all ELISA tests is the use of capture Abs to detect a specific antigen. In direct ELISAs, the target antigen is immobilised onto a surface of a 96-well plate and detected with Abs conjugated to an enzyme (enzyme-Abs). The enzyme reacts with a substrate producing a signal, indicating a positive test result. In indirect ELISAs, the antigen is once again immobilised in a 96-well plate. However, two Abs are utilised in the analyte detection, an unlabelled primary Ab which binds to the analyte and a secondary enzyme-Ab that binds to the Fc region of the primary Ab. The advantage of indirect ELISAs is that they produce a higher signal intensity compared to direct ELISAs, due to several enzyme-Ab being able to bind a single primary Ab. In sandwich ELISAs, rather than the antigen being immobilised on the surface, a capture Ab is used. Once the antigen is bound by the capture Ab, the detection is identical to the indirect ELISA, with an antigen-specific primary antibody and the enzyme-Ab as a secondary Ab. The sandwich ELISA is highly specific, as the capture antibody and primary antibody bind to different epitopes on the analyte, reducing the risk of false positives (FP). The principle of competitive ELISAs is based on the pre-incubation of the analyte with a limited number of enzyme-Ab. When the analyte-enzyme-Ab preparation is added to the ELISA plate containing immobilised capture Abs, if a low analyte is present, all the analytes will be linked to the enzyme-Ab and produce a strong signal. If a large amount of analyte is present, the proportion of unlabelled analyte would be far greater than the analyte linked to the enzyme-Ab, and the signal produced will be weak. A major advantage of ELISAs is that they can be used to determine both the presence of a pathogen by using a pathogen-specific antigen as the analyte as well as the vaccination status by detecting the presence of host-Abs as the analyte.

ELISAs are commonly used in PRDC pathogen diagnostics, and a range of commercially available kits are available. The majority of commercial ELISA kits are directed at detecting the presence of pathogen-specific Abs in the sample (Ab-ELISAs). This is due to Ab-ELISAs being useful not only to detect active or relatively recent infections but also to gauge the successful seroconversion of a herd following vaccination. Examples of commercially available Ab-ELISAs that detect the presence of PRDC pathogens include those for MH via indirect or competitive ELISAs using P46 or conserved epitopes of the 74-kDa protein. However, the SEN across the course of an infection can vary widely between commercially available ELISA test, with those directed against Abs to proteins that are expressed strongly and early in the infection preferable (Poeta Silva et al., 2020). Thus, if the target analyte is chosen appropriately, ELISA tests can be highly sensitive. A commercially available Ab-ELISA for BVS uses the N protein as the capture protein, reported to have SEN and SPE as high as 98.8% and 99.9%, respectively (Johnson, 2010). Recently, an Ab-ELISA targeting the BVS M protein has shown a SEN and SPE of 88.8% and 98.57%, respectively (Zhao et al., 2021).

1.13.4. Lateral flow test (LFT)

The introduction of lateral flow test (LFT) strips, which are cheap and quick to use, has shown great promise not only in detecting BVS, but can also indicate the immune status of the animal - therefore guiding vaccination strategies (Wei et al., 2020). Anti-IgG and anti-IgA proteins are conjugated to gold nanoparticles that bind BVS Abs present in the sample. This complex is flowed up the LFT strip where, if anti-BVS IgG Abs are present, they bind to immobilised viral N protein at the test line. The BVS Abs are thus sandwiched between the N protein and the gold, where a purple band will form on the strip, indicating a positive result. 93.2% SEN and 98.7% SEN, compared to ELISA (Lyou et al., 2005), was obtained with LFT.

1.13.5. Limitations of serological testing

With serological testing cross-reactions are frequently reported due to structural similarities between major epitopes of different serovars or serogroups. For example, serological cross-reactions are seen between APP serovars, including serovars 3, 6 and 8 (Mittal et al., 1988), serovars 1 and 9/11 (Mittal, 1990), as well as serovars 4 and 7 (Mittal and Bourdon, 1991). Additionally, cross-reactions with other *Mycoplasma* species e.g., *M. flocculare* and *M. hyorhinis* have been reported to reduce the SPE of serological detection. However, cross-reaction between serological tests for ApxIVA and ApxII produced by "*Actinobacillus porcitonisillarum*" and *Actinobacillus suis* have been reported (Rosendal and Mittal, 1985, Kuhnert et al., 2005).

Serological false negative (FN) results arise from changes in epitopes. For example, APP isolates are known whereby the species-specific ApxIVA antigen is not expressed due to inactivation of the gene by the insertion element ISAp11, resulting in a negative result in antibody-ELISAs targeting this protein (Tegetmeyer et al., 2008). Additionally, isolates can be non-typeable by ELISA, due to alterations in their capsular antigens. For instance, SS isolates are frequently non-typable. Therefore, there is great interest in identifying serotype 2 specific epitopes that can be targeted with ELISA assays, as serotype 2 is associated with virulent SS isolates (Goyette-Desjardins et al., 2019).

Several factors need to be considered when using serological diagnostic methods, such as the history of the herd and the age of the animal being investigated. Whilst Ab detection is useful in naïve herds; it is generally unsuitable for animals with a prior history of infection or who have undergone vaccination. However, being able to differentiate between infected or vaccinated animals (DIVA) is increasingly a priority when designing both vaccinations and diagnostic assays.

Another drawback of Ab-ELISA is that seroconversion occurs at different time frames depending on the pathogen present. For example, seroconversion occurs 7-10 days post-inoculation with BVS (Rotolo et al., 2018). Therefore, early infection frequently results in FN by ELISA. Additionally, the presence of maternally derived Abs may need to be accounted for. For example, PCV2 piglets take 2-4 weeks to seroconvert after maternal Abs have waned (Krejci et al., 2005, Marois et al., 2009). Therefore, piglet colonisation may be difficult to detect with

serological testing. In summary, serological methods have many drawbacks for rapid diagnosis, but in certain circumstances remain a valuable diagnostic method. Such as, with APP-free high-status herds, routine serum analysis by ApxIVA ELISAs may indicate recent sub-clinical infection (Gottschalk, 2015).

1.14. Molecular diagnostics

Molecular diagnostic methods are commonly used to detect pathogenic genetic material within a sample. The detection of regions of DNA or RNA is highly sensitive as a section of DNA is amplified by a polymerase creating millions of copies of the DNA region, even from a single copy. Therefore, molecular diagnostics can be used to detect a pathogen prior to any clinical signs being observed, which is a significant advantage over serological methods (Figure 1.12).

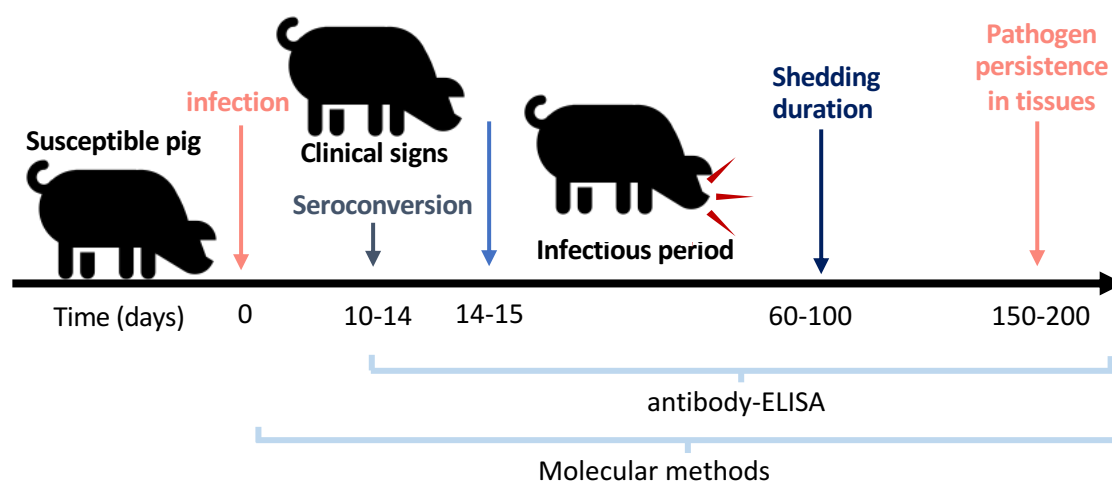


Figure 1.12. The time frame where it is appropriate to implement different diagnostic tools. The use of antibody-ELISA can only occur after seroconversion has occurred, whilst molecular methods can be implemented from the onset of inoculation, given the molecular test has appropriate SEN. Time-frame is representative of BVS infection, image adapted from (Cano, 2013).

Unlike Ab-serological methods, molecular methods can also differentiate an animal that is infected compared to an animal that has been vaccinated. Additionally, the amplification of pathogenic genetic material is highly specific, with much fewer cases of cross-reactions seen than in serological methods. Due to these benefits, molecular detection is now the most used method to determine the infection status of an animal. Diagnosis frequently involves a combination of clinical signs, pathological findings, and appropriate molecular testing – often requiring several molecular assays to be performed until the etiological agent is identified. Although multiplex methods to screen for multiple pathogens in one sample have since gained popularity (Jiang et al., 2010), there is no standardised diagnostic method (serological or molecular) to detect all PRDC pathogens, with the techniques used and assays varying across diagnostic laboratories.

1.14.1. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was invented in 1985 by Kary Mullis (Mullis et al., 1986) and has since revolutionised molecular biology and diagnostics, and is the basis of most prevalent molecular diagnostic methods today. The principle of PCR is the use of two small sections of ssDNA, known as primers, which are complementary to the DNA sequence flanking the region of interest. Specialist equipment is used to heat and cool the reaction multiple times, referred to as cycles. With each cycle the heating of the reaction to high temperatures denatures double-stranded DNA (dsDNA) and reduces secondary structures, the temperature is rapidly decreased which favours the short-primer annealing to complementary sequences. The temperature is then increased to around 72°C where the thermostable DNA polymerase (e.g., Taq polymerase) extends the annealed primers, adding the relative nucleotides to the primers in a 5-3' direction. The result is that each cycle creates precisely double the amount of genetic material that was present in the previous cycle (**Figure 1.13**), assuming 100% efficiency of the primer sets.

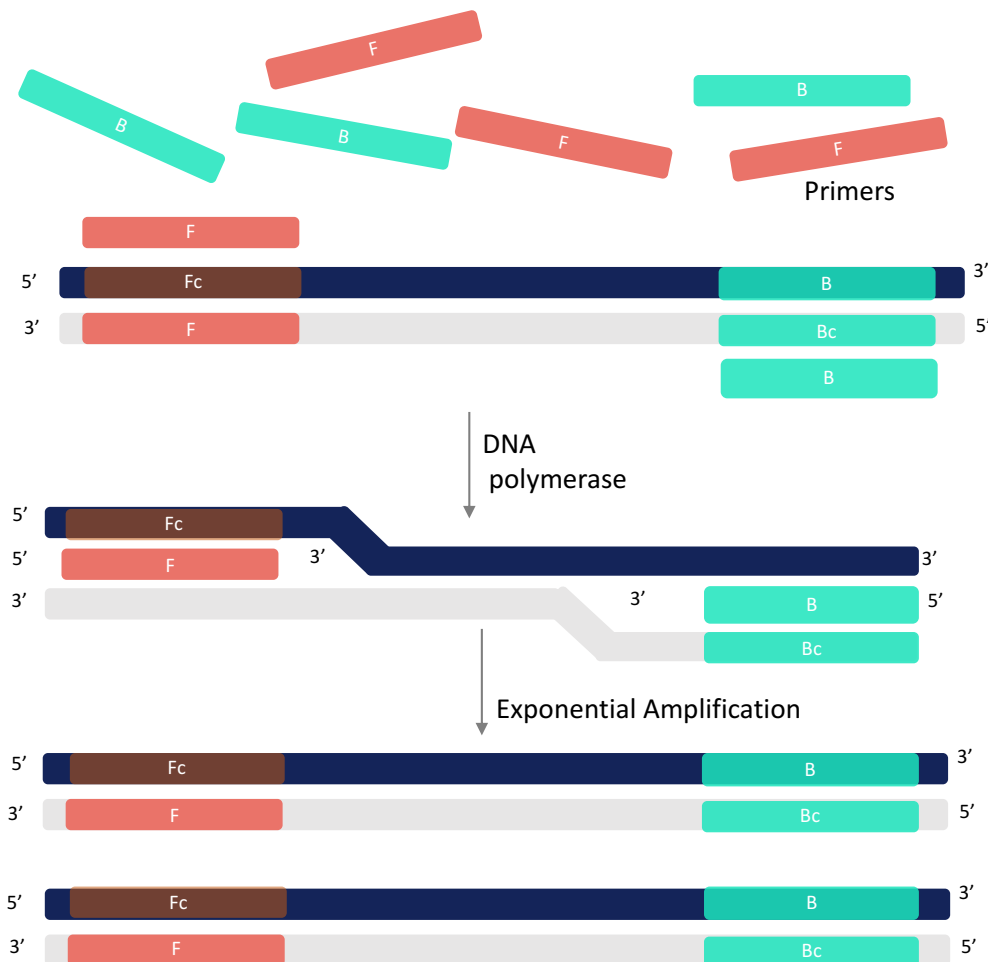


Figure 1.13. Schematic of the polymerase chain reaction (PCR). PCR occurs through thermocycling, during which the sample is heated to a high-temperature sample to denature dsDNA, followed by rapid cooling to favour the annealing of the short oligonucleotide primers to complementary target regions. The temperature is then elevated to the active temperature of the DNA polymerase resulting in primer extension. The heating and cooling steps are then repeated, and exponential amplification occurs.

In quantitative PCR (qPCR), a dye binds to dsDNA, or a specific probe that contains fluorophores that fluoresce when bound to a specific sequence. The number of cycles taken for there to be enough nucleic acid present in the sample to generate a detectable fluorescence signal (usually three standard deviations above the noise) is known as the threshold value or cycle threshold (C_t). The C_t value is important as it directly relates to the amount of starting nucleic acid in the sample. For instance, a lower C_t is indicative of more genetic copies of your target being present than a sample with a higher C_t value. Whilst qPCR has dramatically decreased the detection time compared to conventional PCR, the technique remains inaccessible – requiring complex and expensive machinery to perform the thermocycling. Therefore, performing PCR creates a bottleneck in the diagnosis of PRDC pathogens, as it can only be performed in centralised diagnostic laboratories requiring the transportation of the relevant sample from the farm or abattoir.

1.15. Isothermal amplification

The causative agent of a suspected PRDC infection may take several days to identify correctly. The diagnosis of PRDC pathogens is a key step in facilitating disease management, limiting outbreaks and allowing early treatment – reducing the use of antimicrobials (Fertner et al., 2015). Several other molecular diagnostic techniques have been described that aim to overcome the requirement of thermocycling to achieve nucleic acid amplification. These new techniques allow specific and sensitive amplification at a single temperature and are therefore known as isothermal amplification techniques. The most frequently used isothermal method is loop-mediated isothermal amplification (LAMP).

1.15.1. Loop-mediated isothermal amplification (LAMP)

LAMP was one of the first isothermal amplification techniques to be described (Notomi et al., 2000). LAMP amplification utilises a single polymerase with strand displacement activity, allowing primer integration into dsDNA without requiring the DNA to be heat-denatured. Efficient LAMP amplification requires at least four primers, two forward and two reverse. The outer primer set, known as F3 and B3, are short primer sequences, typically 18-23 base pairs (bp) in length. The inner primer sequences known as, forward inner primer (FIP) and backwards inner primer (BIP) contain two distinct regions, the 3' region, denoted F2 or B2, is complementary to a 18-23 bp region on the target gene, whilst the 5' region, denoted F1c and B1c are reverse-complements of the 20-26 bp region immediately 3' to the F2 or B2 binding region (Eiken Chemical Co.).

Amplification using LAMP is initiated by the complementary binding and extension of the F2 or B2 section of the FIP or BIP primer, respectively. Subsequent displacement of the FIP/BIP produced amplicon occurs by polymerase extension of the F3 or B3 outer primers. Once the amplicon has dissociated from the target sequence, the F1c and B1c regions of the FIP and BIP, now integrated into the amplicon anneal to their complementary regions downstream of the F2 and B3 regions on the amplicon, creating a barbell structure (Notomi et al., 2000). This barbell structure can then be amplified independently of the template DNA, giving rise to the concatenation of amplicon products, which can form one of four distinct structures (Kaur et al., 2020).

Several years after LAMP was first described, two additional primers, known as loop primers, were found to increase the rate of the LAMP reaction (Nagamine et al., 2002). Therefore, LAMP is described as high specific targeting 6-8 regions in each target sequence (**Figure 1.14**).

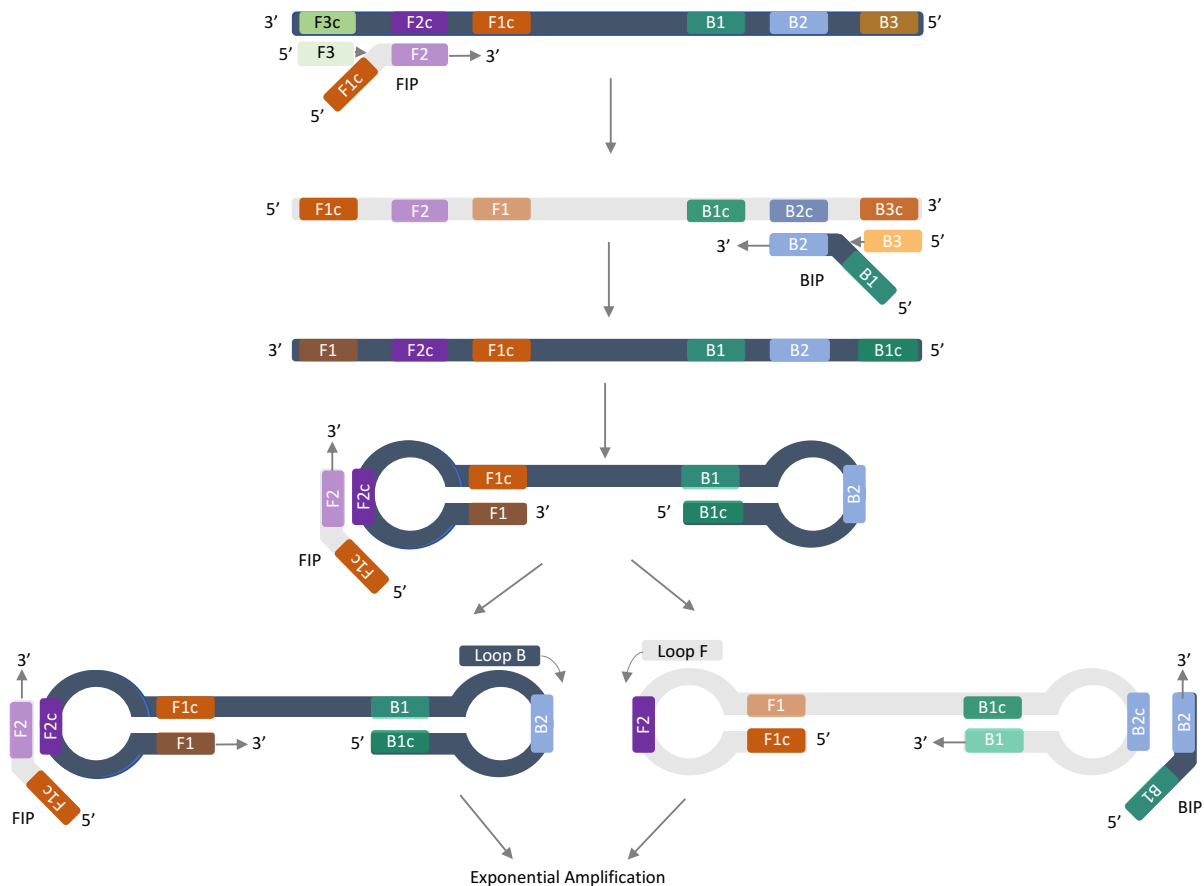


Figure 1.14. Schematic of loop-mediated isothermal amplification (LAMP). The tailed forward inner primer (FIP) and backward inner primer (BIP) containing F2 and F1c, and B2 and B1c regions, respectively, are integrated into the dsDNA through the strand-displacing polymerase, *Bst* DNA polymerase. The *Bst* polymerase extends the FIP and BIP primers through the complementary pairing of their F2 and B2 regions, respectively. The DNA amplicon is displaced from the template strand via the extension of the outer primers F3 and B3. The amplicon originating from the FIP and BIP primers contain the reverse complement regions to the tailed FIP and BIP primers, allowing the amplicon to self-anneal to form dumbbell structures. These amplicon structures can participate further in the LAMP reaction, being amplified with FIP and BIP primers as well as loop forward (LF) and loop backward (LB) primers.

1.16. Point-of-Care (PoC) devices

A Point-of-Care (PoC) diagnostic is a rapid analyte test performed at or near the site of the patient, aiding in the monitoring or diagnosis of a specific disease. The rapid turnaround time of the diagnostic result enables faster disease management or treatment decisions. In 2003, the World Health Organization (WHO) published its requirements that rapid diagnostics for sexually transmitted infections should be affordable, sensitive, specific, user-friendly, rapid, robust, equipment free and deliverable to end-users - abbreviated to ASSURED (Peeling et al., 2006). The ASSURED criteria have since been universally adopted for all PoC diagnostics (Kosack et al., 2017). In 2019, the ASSURED criteria were updated to include real-time connectivity and environmental friendliness to

become REASSURED (Land et al., 2019), mirroring the current market shift away from rapid diagnostic devices for low-income and limited-resource countries towards mass marketability in the global economy.

The global PoC market is growing rapidly; the global COVID-19 pandemic caused an explosion of investment into the market. The PoC market was estimated to be worth USD \$29.5 billion in 2021, and is set to rise to USD \$50.6 billion by 2025 (Business Wire, 2021a). In contrast, the veterinary diagnostic market is estimated to be worth USD \$3.9 billion by 2026 (Business Wire, 2021b). To establish effective prevention and control strategies for the PRDC pathogens, a convenient, sensitive, low-cost, and rapid PoC method would be highly advantageous to the worldwide pig sector, enabling rapid diagnosis, reduced antibiotic use, and improved herd management (e.g., change in vaccines used), with the added value of providing surveillance data.

1.16.1. Lab-on-chip

Although LFT holds the highest market share for PoC platforms, they are currently limited to detecting biological analytes, with no single nucleic acid-based LFT product being brought to market (at the time of writing). Other molecular diagnostic detection methods include fluorescence-based monitoring of reactions. However, these remain constrained to expensive and bulky instruments. Recently, electrochemical detection has proven to satisfy the REASSURED criteria using ion-sensitive field-effect transistors (ISFETs) fabricated onto a complementary metal-oxide-semiconductor (CMOS) (Dinar et al., 2018). This low-cost solution enables the production of a disposable microchip array with thousands of ISFET sensors - each capable of accurately detecting a solution's pH.

The ISFET was first described in 1972 by Piet Berveld to measure the concentration of ions in a solution (Bergveld, 1972). ISFETs are essentially a type of transistor known as a metal oxide semiconductor field effect transistor (MOSFET) comprising (in a simplified form) of a source, a drain, a channel and a gate. In MOSFETs, electrons travel through the channel from the source and exit through the drain, with the flow of electrons controlled by the voltage placed on the metal gate, effectively altering the width of the channel electrons can flow through. For instance, when no voltage is placed on the gate, there is no flow of electrons between the source and the drain. However, when voltage is applied to the gate, holes in the channel substrate are repelled away from the gate, creating what is known as a saturation region. Once the gate voltage is sufficient for the saturation region to be wide enough, electrons can flow between the source and drain (known as the threshold voltage) and the MOSFET is switched on.

In ISFETs, the metal gate typically used in MOSFETS is replaced by an ion-sensitive layer, also known as the sensing layer, which is in contact with the solution. The ion selectivity of the sensing layer can be altered by addition of ion-selective membranes to the gate. Insulators such as silicon nitride, aluminium oxide, silicon dioxide etc. make the sensor sensitive to pH. The ion of interest, in this case, protons, can pass through the ion-selective membrane to bind to the insulator and affect the charge across the gate. Therefore, the solution pH directly impacts the voltage on the gate. Low pH solutions have a greater proton accumulation at the sensing

layer, the resulting positive charge on the gate makes the channel more conductive and the saturation region wider - with higher electron flow and therefore current.

The use of ISFETs in bioanalytical sensors was at first limited, due to ISFET integration with the CMOS production process requiring the design of specific processes to fabricate ISFETs into the CMOS. As described above, the ISFET requires the gate regions, made of insulating materials, to be in direct contact with the solution being analysed. In contrast, the CMOS architecture uses a polysilicon electrode in the gate region to align the source and drain of the transistor. This integration issue was solved in 1999 with the integration of ISFETs into an unmodified CMOS being produced by using a floating gate conduction electrode in which polysilicon is used in conjunction with two metals. The arrangement allowed silicon oxynitride to be used as the CMOS passivation layer as well as the ISFET pH-sensitive material (Bausells et al., 1999).

Today, CMOS-ISFETs are produced using similar materials, an aluminium (Al) gate is sandwiched between silicon dioxide layers (SiO_2) with a silicon nitride (Si_3N_4) pH sensing layer which is in contact with the solution (**Figure 1.15**), resulting in a highly accurate pH measurement. Small microsensors used in diagnostics are often referred to as lab-on-chip devices, which integrate amplification chemistries with a small form factor and SEN of a microsensor.

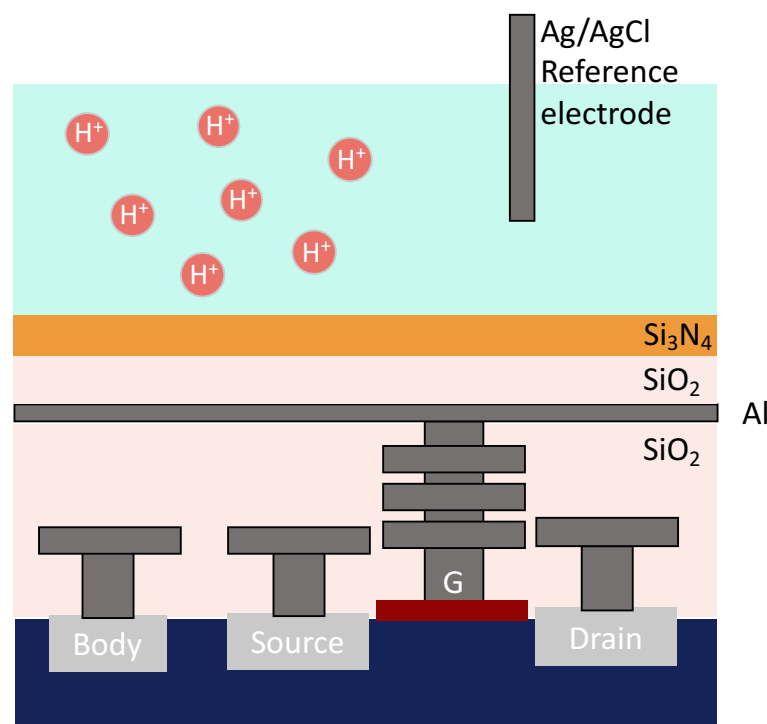


Figure 1.15. Topology of the CMOS-ISFET pH sensor. The CMOS-ISFET platform detects pH of the solution through protonation of the surface in contact with the solution – the pH sensing layer, typically silicon nitride (Si_3N_4), which alters the net charge on the ISFET channel and alters the current flow, which can be detected. Image adapted from (Moser et al., 2019).

The CMOS-ISFET platform has several benefits over other detection techniques. Firstly, they allow sensing in a very small form factor. Secondly, they are incredibly affordable, abiding by Moore's law; as the number of

transistors on a microchip doubles, the cost of the microchip is halved. Thirdly, CMOS-ISFETs do not require post-manufacture surface alterations, ensuring an easily scalable platform for mass production (Toumazou and Georgiou, 2011). Lastly, the technology is energy efficient, with low power requirements, lending itself to PoC applications (Georgiou and Toumazou, 2011).

For amplification events to be detected by the CMOS-ISFET, a change in the pH of the solution needs to occur. During DNA elongation, DNA polymerase adds nucleotides to a growing DNA strand, this occurs through the 3' hydroxyl group of the DNA chain attacking the phosphate of the nucleotide, creating a phosphodiester bond, and releasing inorganic pyrophosphate and a free hydroxyl. Therefore, decreasing the pH of the solution.

The most commonly used isothermal amplification technique for lab-on-chip devices is LAMP, as this results in production of large amounts of amplicon and, therefore a strong pH change. The CMOS-ISFET has been utilised to detect human respiratory pathogens, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and *Aspergillus fumigatus*, with high SEN and SPE (Yu et al., 2020, Rodriguez-Manzano et al., 2021)

1.17. Hypothesis

Using a veterinary directed approach, in collaboration with the fifth largest veterinary company in the world (CEVA) and the largest UK pork producer (BQP, which is owned by Pilgrims the largest pork producer worldwide), I can design and develop a PoC diagnostic device to detect PRDC pathogens.

After consultation with a veterinary focus group, I have identified the most appropriate PRDC pathogens circulating in the UK, which include the bacterial species APP, MH, PM, GPS, and SS, as well as the viral respiratory pathogens SIV, including H1N1 and H2N3 subtypes, PCV2 and BVS-1 and BVS-2. These pathogens will form the basis of two lab-on-chip cartridges, utilising isothermal amplification chemistry and CMOS-ISFET technology. The first cartridge will be able to detect the five bacterial pathogens, and the second will detect the three viral and distinguish relevant subtypes (Figure 1.16).

APP	GPS	MH	PCV2	BVS-1	BVS-2
PM	SS	Ctrl	SIV H1N1 H1N2	SIV H3N2	Ctrl

Figure 1.16. The assay layout of the two cartridges for the detection of the PRDC pathogens. The cartridges are to be split into six wells each, with one cartridge for the detection of the bacterial PRDC pathogens and one cartridge for the detection of the viral bacterial pathogens.

1.18. Aims and objectives

The overarching objective of this Thesis is to develop a PoC diagnostic device for the detection of the five bacterial and three viral pathogens which are commonly associated with the PRDC in the UK.

The objective of this Thesis will be achieved through the completion of several aims:

- Identify optimal primer sets using published (where possible) isothermal assays targeting conserved regions of target genome sequences
- Characterise the performance of different isothermal amplification chemistries with focus on compatibility with the CMOS-ISFET detection platform
- Determine the clinical SEN and SPE of the identified primers using samples of tissue collected from pigs displaying signs of PRDC disease
- Evaluate methods to increase the rapidity of the LAMP biochemistry, with emphasis placed on the production of a LAMP format which is compatible with the lyophilisation process
- Establish a sample preparation method suitable for extraction of nucleic acids rapidly, from solid tissue, namely, lungs
- Assess different LAMP detection methods and their implementation in a PoC setting

Chapter 2: Analytical performance of isothermal techniques

Introduction

Since its universal adaptation in the 1990s, the PCR reaction has remained the gold standard diagnostic technique, owing to unrivalled SEN and SPE. However, the instrumentation capable of performing the precise thermocycling required for PCR is largely still confined to laboratory settings. Recent years have potentiated the gap in the market for PoC PCR machines, and as such several have been developed, including the qPOC (QuantuMDx), Lucerna diagnostic system (PhoenixDx) and NudgeBox (DNA nudge). Alternatively, companies have integrated isothermal amplification techniques into their PoC diagnostic devices. For example, Abbott laboratories and their proprietary nicking enzyme amplification reaction (NEAR), and the UK based Diagnostics for the Real World have implemented their diagnostic devices using nucleic acid sequence-based amplification (NASBA). Isothermal techniques are an attractive alternative to PCR, avoiding the requirement for the thermal melting of DNA, resulting in much less complex and, therefore, less costly machines. Since the early 1990s, several isothermal amplification techniques have been described which are amenable for use at the PoC. Isothermal techniques utilise either several enzymes, such as those used in recombinase polymerase amplification (RPA), helicase dependent amplification (HDA) and NEAR, or a single enzyme, such as polymerase spiral reactions (PSR) and LAMP. The successful development of a PoC device is largely dependent on utilising a robust, sensitive, and specific amplification technique. Each isothermal technique has advantages and disadvantages, which much be considered prior to implementation into a diagnostic test. A major factor, which is often overlooked in academic situations, is the intellectual property (IP) and associated use rights of a technology. Frequently, IP is protected by patents lasting up to 20 years. Once the patent term has elapsed, the IP within it can be used freely; prior to this, firms wishing to implement the technology into a product needed to agree a licencing agreement with the holder of the patent, usually accompanied with a hefty fee. Therefore, the use of technologies which are not currently covered by or where the patent is expiring in the near future can be advantageous when producing affordable products.

An aim of this Chapter was to select an appropriate isothermal chemistry to deploy in our PoC test, and the scientific basis and advantages and disadvantages of potential methods is given below.

2.2. Recombinase polymerase amplification (RPA)

RPA was first published in 2006 and patented by TwistDx in 2003 (Armes and Stemple, 2003, Piepenburg et al., 2006). RPA is capable of amplifying DNA at low temperatures; although the optimal temperature for RPA amplification is 37°C, amplification has been observed as low as 25°C (Crannell et al., 2014, TwistDx Ltd., 2016). The main principle of RPA is to amplify DNA, utilising enzymes of the phage DNA repair pathway. This works via the recombination proteins, UvsX and UvsY. Oligonucleotide primers are coated by UvsX which facilitates the loading of UvsY. In the presence of Watson-Crick complementary base pairing between the primer and the target sequence, UvsY displaces the second strand of DNA, forming a D-loop. This recombination complex between the primer and the target sequence is stabilised by the single-stranded binding protein (SSBP), GP32, and UvsX dissociates from the primer allowing DNA polymerase to extend the primer in 5'-3' direction (**Figure 2.1**). A major

stipulation of RPA achieving amplification is the addition of a crowding agent into the reaction mixture. TwistDx, the manufacturers of RPA, use a 35 kDa poly-ethylene-glycol (PEG35) as a crowding agent; however other molecular weight PEGs have been used (Powell et al., 2018). A major disadvantage of the use of crowding agents is the reaction viscosity, requiring completed RPA reactions to undergo purification prior to electrophoresis to prevent the PEG35 from causing uneven migration of the RPA product. Additionally, in LFT, the PEG35 impedes the capillary action required to flow product up the strip, which can be overcome by an extra dilution step before application to the device.

Since it was first described, RPA has been used to detect numerous veterinary pathogens, including members involved in the PRDC. Typically, RPA amplification is measured using fluorescence, often with an exonuclease III (Exo) probe. These probes are long (45 bp) sequences containing a fluorophore and quencher interspersed by an abasic site, such as a tetrahydrofuran (THF) residue. Once the probe is recombined into a complementary sequence and is thus double-stranded, the DNA repair enzyme, Exo, cleaves the abasic site, separating the fluorophore and quencher, resulting in an increase in fluorescence. Although RPA utilises two primers, similar to PCR, there is limited research into RPA primer design; therefore, with undefined design rules, the manufacturers describe a trial-and-error approach to optimising primer sets. Recently, an automated primer design python script, PrimedRPA, was published (Higgins et al., 2019). However, the uptake of PrimedRPA by RPA users has been limited (accessed 1,855 times as of July 2022). Regardless, several RPA assays have been published for single detection all of the bacterial PRDC pathogens, including APP (Li et al., 2019), MH (Liu et al., 2019), PM (Zhao et al., 2019), GPS (Zhang et al., 2019e) and very recently SS serotype 2 (Jiang et al., 2022), as well as some of the viral targets, SIV (Sun et al., 2018), BVS-2 (Tian et al., 2022, Wang et al., 2017a), and PCV2 (Wang et al., 2016, Yang et al., 2017).

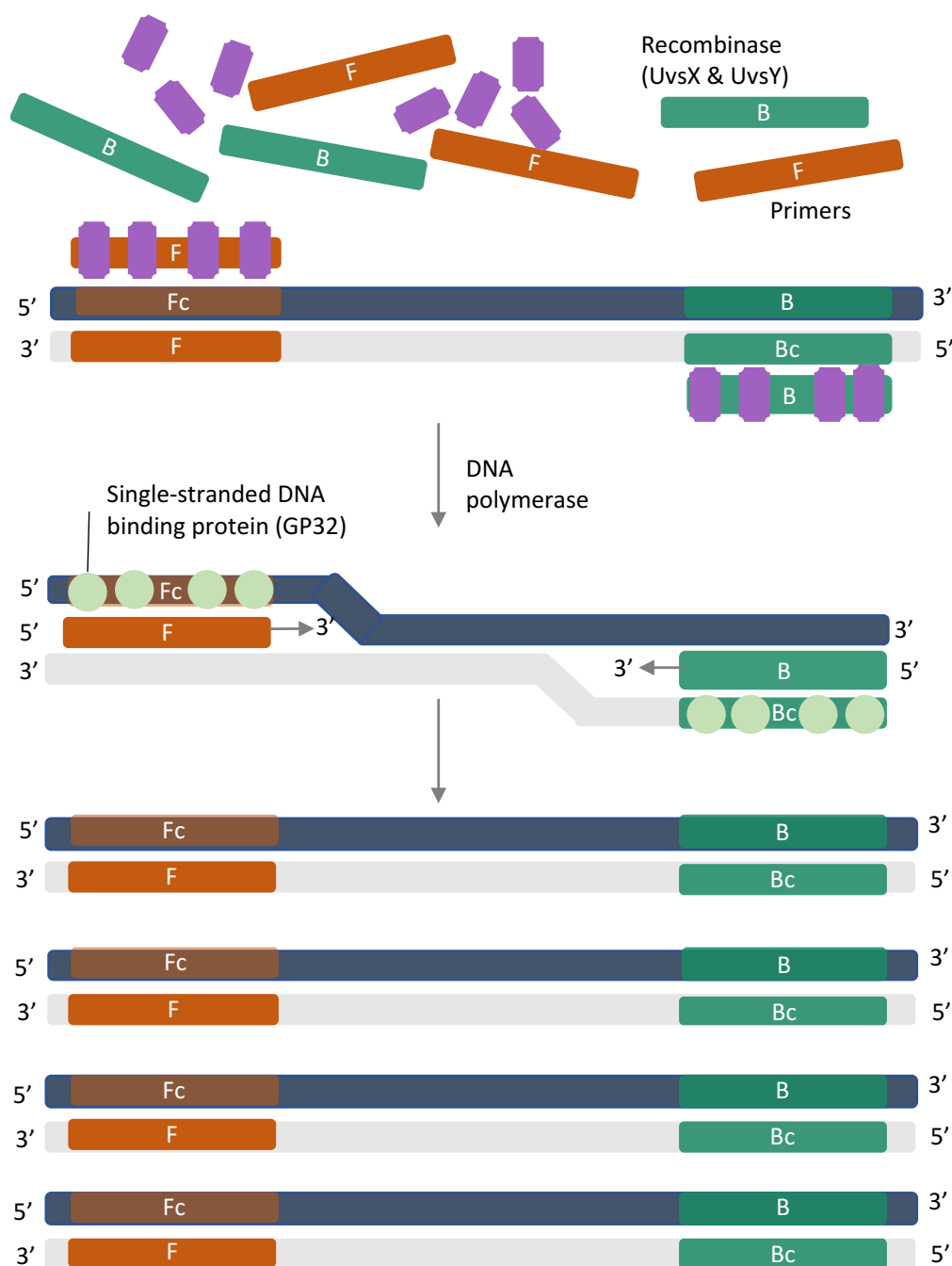


Figure 2.1. Schematic of recombinase polymerase amplification (RPA). Strand invasion occurs via UvsX and UvsY recombining the primers in complementary regions of DNA. The primer-DNA interface is stabilised by the SSBP, GP32, allowing UvsX and UvsY to dissociate from the primer and DNA polymerase to extend the primer along the DNA strand.

2.3. Helicase-dependant amplification (HDA)

HDA was first described in 2004, and subsequently patented by Bio-Helix Corp. in 2007 (Kong et al., 2007). HDA utilises the base-repair pathway in *Escherichia coli*, using the DNA helicase, UvrD, to unwind dsDNA, stimulated by the methyl-directed mismatch repair, MutL protein. The unwound section of DNA requires stabilisation by SSBP, which prevents strand re-association and allows the oligonucleotide primers to hybridise to complementary sequences within the DNA. Once annealed, a DNA polymerase extends each of the two primers

in a 5'-3' direction. The cycle then repeats, resulting in the exponential amplification of the target sequence (Vincent et al., 2004). Originally, the use of *E. coli* proteins limited HDA amplification to 37°C, but the subsequent use of the UvrD homologue from *Thermoanaerobacter tengcongensis*, with the *Bacillus stearothermophilus* strand displacing polymerase (*Bst*), negated the requirement for MutL recruitment of UvrD nor the SSBP accessory proteins (**Figure 2.2**), as well as allowing HDA to be performed at the elevated temperature of 60-65°C, termed the thermostable HDA (tHDA) (An et al., 2005). The use of tHDA has greatly increased the usability of HDA, simplifying the biochemistry to two enzymes rather than four and cutting production costs. In addition, the elevated temperature improves reaction kinetics and reduces the rate of FP results (An et al., 2005). For simplicity, tHDA will be referred to as HDA henceforth. Like RPA, HDA requires only two primers that are longer in length to that used in PCR. Consequently, HDA reactions do suffer from increased rates of primer dimerization, where self-amplification can outcompete target amplification in HDA. In order to minimise the production of non-specific amplicon, HDA uses lower primer concentrations of 75-100 µM compared to PCR (0.1-1 µM) (MyBioSource). Whilst no HDA assays have been described for PRDC pathogens, primer sequences for the detection of porcine adenovirus have been submitted for patent protection, with HDA being mentioned as one embodiment of the IP. However, it is unclear if the primers were validated using HDA (Ping et al., 2017). Although lacking in popularity in the porcine diagnostic field, HDA has given rise to several commercial diagnostic assays, such as the Solana panels, formally AmpliVue (Quidel) (Barreda-García et al., 2018).

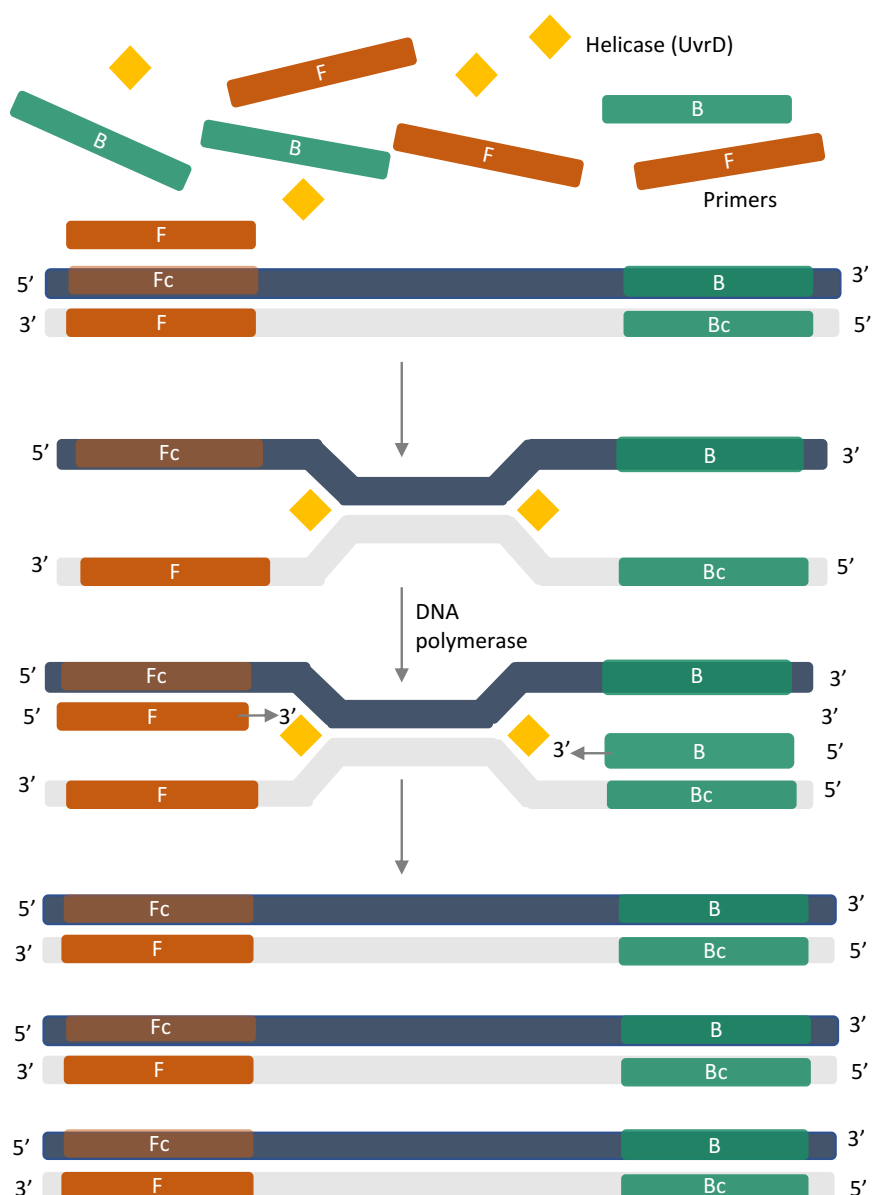


Figure 2.2. Schematic of helicase-dependent amplification (HDA). During HDA amplification, the DNA helicase, UvrD, facilitates the local unwinding of dsDNA, allowing the *Bst* polymerase to extend complimentary bound primers, and exponential amplification occurs.

2.4. Polymerase spiral reaction (PSR)

Polymerase spiral reaction (PSR) is a relatively new isothermal amplification method, first published in 2015 (Liu et al., 2015b). During PSR amplification the additive betaine is used to destabilise the DNA helix at an incubation temperature of 61-65°C. The 3' end of the PSR primer hybridizes to regions of homology in the DNA sequence, and the strand displacing DNA polymerase, *Bst*, initiates extension of the 3' end of the primer. The two primers used in PSR consist of two distinct regions, the 3' region is complementary to the flanks of the region of interest, whilst the 5' region contains a foreign sequence which will not interact with any other DNA that might be present in the sample. However, the 5' region of the forward and back primers are complementary to one another, such that when the primers are incorporated into the amplicon, they can then self-anneal, creating a circle (**Figure**

2.3). Much the same as in LAMP, the PSR amplicon now acts as a priming site for subsequent PSR amplification and the self-concatenation of the amplicon forms a spiral structure (Liu et al., 2015b).

Whilst PSR is the most recent isothermal technology used in this Chapter, very recent studies have described primer sequences for amplifying two porcine pathogens, one primer set for the detection of APP (Sarkar et al., 2022) and another primer set for the detection of the emerging swine respiratory pathogen, causing catastrophic losses in the swine industry, African swine fever (ASF) virus (Woźniakowski et al., 2017).

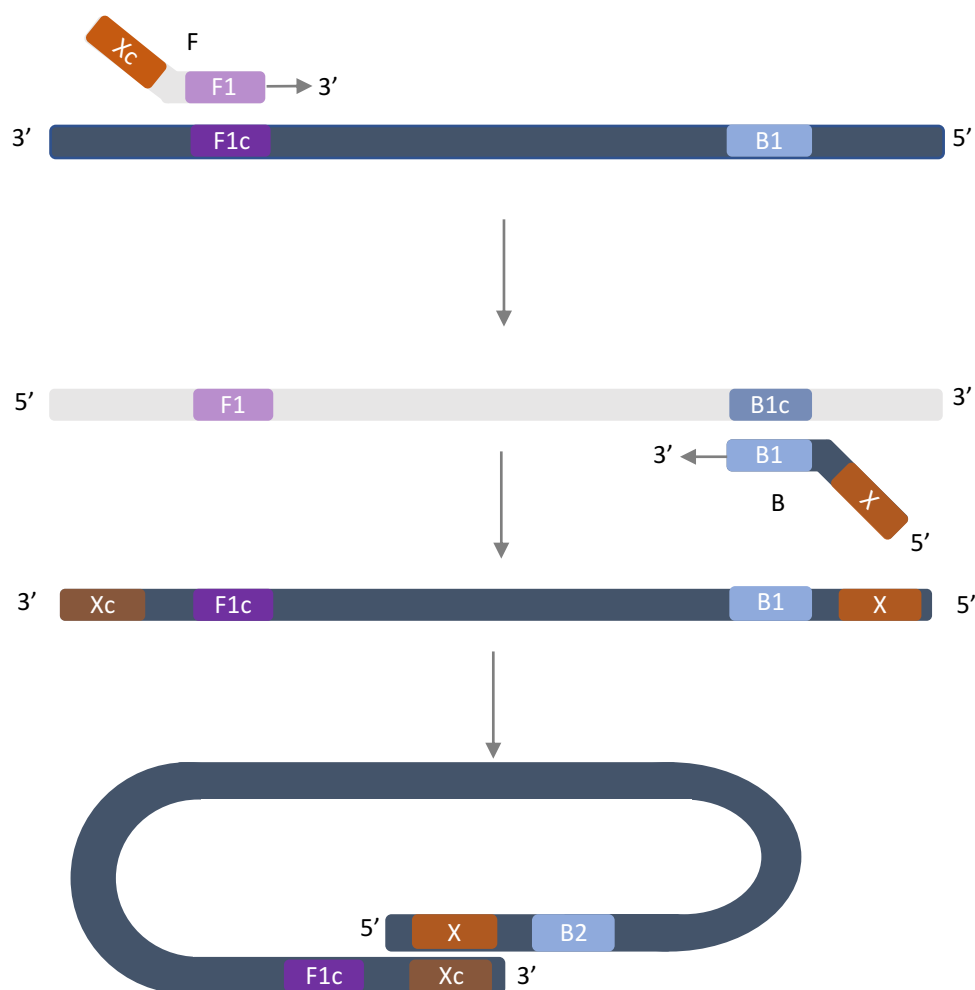


Figure 2.3. Graphical depiction of polymerase spiral reaction (PSR). Amplification with PSR occurs using two tailed primers, a forward (F) and back (B), with each primer tail containing the reverse complement of a 3' region of the target sequence. The primers anneal to homologous sequences via the strand displacing polymerase, *Bst*, displacing downstream DNA, whilst extending the primers. Once the primer is extended past the 3' complement sequence, the amplicon can self-anneal, creating a spiral-shaped amplicon structure.

2.5. Loop-mediated isothermal amplification (LAMP)

LAMP was first published by Notomi et al. in 2000 (Notomi *et al.*, 2000) and was pre-emptively patented by Eiken Chemical Co Ltd. in 1999 (Notomi and Hase, 1999). The LAMP reaction requires four primers (F3, B3, FIP and BIP) and two optional loop primers (LF and LB). Collectively the LAMP primers target 6-8 regions of DNA, with FIP and BIP each targeting two regions (F2 and F1c, B2 and B1c, respectively). The LAMP amplification process was

detailed in **Chapter 1, Section 1.14.1**. The exact spacing between the six target regions (F3, F2, F1c, B1c, B2 and B3) has been stipulated to be 120-180 bp between F2 and B2, 0-20 bp between F2 and F3 and B2 and B3, and 40-60 bp between regions of the FIP and BIP primers, F1c and F2, and, B1c and B2 (Parida et al., 2008). Thus, LAMP primer design is often complex and requires an automated programme (such as PrimerExplorer). For the design of loop primers, target regions of DNA require high conservation, as SNPs within priming regions can more than double the reaction time (Wang, 2016). Primer Explorer does not allow simultaneous design. Therefore, the binding locations of the four core primers must be re-run in the primer software to identify appropriate locations for the loop primers.

Arguably, LAMP is the most widely adopted isothermal amplification chemistry, with 4,176 publications returned in PubMed using the search term 'loop mediated isothermal amplification' (National Center for Biotechnology Information), as of the 16th September 2022. Therefore, it comes as no surprise that there have been numerous publications detailing assays detecting all PRDC pathogens of interest, often several targeting the same pathogen.

2.6. Aims

The main Aim of this Chapter was to perform a comparative analysis of the analytical performance of different isothermal amplification techniques. The parameters which I assessed were analytical SEN and SPE using reference strains, along with the rapidity measured as the time to positive (TTP). An emphasis was placed on the feasibility of implementing the amplification chemistry on a lab-on-chip based CMOS-ISFET device, ensuring the PoC device being developed utilises the most appropriate technology.

Methods

2.7. Bacterial isolates

Bacterial strains used in this Chapter include the serovar 8 reference strain of APP, strain 405 (GenBank accession: CP078508), MH reference strain MH232 (GenBank accession: AE017332), PM laboratory isolate MIDG 3290 (not sequenced), SS reference strain P1/7 (GenBank accession: AM946016) and GPS reference strain SVR5 (GenBank accession: NZ_ABKM000000000). Additional bacterial isolates used are listed in **Supplementary Table 1**. The PM laboratory isolate is a clone from the CC13 clade, associated with the majority of swine PM isolates (Hotchkiss et al., 2011).

2.7.1. Bacterial culture conditions

The culture of bacterial isolates occurred at 37°C for 16-18 hours on brain heart infusion (BHI) broth (BD, Cat 2237500), supplemented with 5% agar (Sigma-Aldrich, Cat W201201) and 0.01% nicotinamide adenine dinucleotide (NAD) (MP Biomedicals, Cat 100319) for APP strain 405. SS strain P1/7, PM strain MIDG 3290 and GPS strain SVR5 were cultured on BHI broth (BD) with 5% agar (Sigma-Aldrich) supplemented with 10% defibrinated horse blood (DHB, TCS biosciences, Cat HB035). The MIDG 3290 strain was kindly donated to the Langford isolate collection by Christopher Hodgson (Moredun Research Institute). Genomic DNA (gDNA) from the divergent SS isolates were kindly supplied by Professor Alexander Tucker (University of Cambridge), MH gDNA reference strain MH232 was supplied by Professor Andrew Rycroft (Royal Veterinary College).

2.8. Genetic material

2.8.1. Bacterial genomic DNA isolation

The extraction of bacterial whole gDNA was performed using MP Biomedicals FastDNA Spin Kit (MP Biomedicals, Cat 116540600-CF), following the manufactures protocol. Briefly, a 10 µL loop of bacterial culture grown on plates was resuspended in 200 µL of sterile phosphate-buffered saline (PBS) (Gibco, Cat 20012027) prior to the addition of 600 µL of cell lysis solution for tissues and cells (CLS-TC) (MP Biomedicals, Cat 116540409). The preparation was pipette mixed, transferred to Lysis Matrix A tube (MP Biomedicals, 116910050-CF), and burst vortexed twice for 20 seconds to aid in the chemical lysis of the bacteria. The suspension was pelleted by centrifugation at 15,000 x g for 5 minutes at 4°C with the supernatant was transferred to a new 2 mL tube. 650 µL of homogenised binding matrix buffer (MP Biomedicals, Cat 116540408) was added to the supernatant and mixed by gentle inversion for 5 minutes. The suspension was then pelleted by centrifugation at 14,000 x g for 1 minute and the supernatant was removed and discarded. The pellet was resuspended in 500 µL of the ethanol-based wash solution supplied with the FastDNA Spin Kit, concentrated SEWS-M (MP Biomedicals, Cat 116540405), prior to being transferred to an MP Biomedicals SPIN module (MP Biomedicals, Cat 112080600-CF). The sample was applied to the spin column via centrifugation at 14,000 x g for 1 minute and the flow-through was discarded. For elution of the sample, 150 µL of pre-warmed (to 65°C) DNase-free water (MP Biomedicals,

Cat 116540406), was incubated on the column for 5 minutes at 65°C. The sample was eluted into a separate collection tube via centrifugation at 14,000 x g for 1 minute.

Whole gDNA was examined for genomic integrity via running on a 1.5% agarose gel stained with ethidium bromide (EtBr) for 30 minutes at 110 V hour with products visualised using a Gel Doc Ez imager (Bio-Rad, Cat 1708270). and products quantified with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Cat ND-ONE-W).

2.8.2. Generation of synthetic templates

Synthetic templates were produced by Integrated DNA Technologies (IDT), the full sequences of the DNA templates produced are given in **Supplementary Table 2**. RNA templates were produced from the synthetic constructs for SIV via transcription via the T7 promoter sequence (5'-TAATACGACTCACTATAG-3') placed at the 5' end of the construct. DNA constructs were rehydrated in Tris-EDTA buffer solution (TE, Sigma-Aldrich, Cat 93283) to a concentration of 10 ng/μL and subsequently quantified by a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

2.8.3. T7 transcription

The HiScribe T7 RNA Synthesis kit (New England Biolabs (NEB), Cat E2040) was used for T7 transcription. T7 transcription reactions consisted of 1x reaction buffer, 10 mM of each deoxyribonucleotide (ATP, GTP, UTP and CTP), 5 μL of T7 RNA polymerase, 2.5 μL of RiboLock RNase inhibitor (RI) at 40 U/μL (Thermo Fisher Scientific, Cat EO0381), 15 μL of synthetic DNA at 10 ng/μL for a total reaction volume of 50 μL. T7 transcription reaction time was 2 hours at 37°C. Subsequent DNase treatment was performed with the Invitrogen DNA-free DNA removal kit (Thermo Fisher Scientific, Cat AM1906). Briefly, 10 μL of 10x DNase I buffer, 4 μL TURBO™ DNase and nuclease-free water (Thermo Fisher Scientific, Cat AM9939) were added to the completed transcription mix, for a total volume of 100 μL. The mix was incubated at 37°C for 30 minutes. The DNase was precipitated with the addition of 10 μL of DNase inactivation reagent and incubated at room temperature for two minutes, with occasional mixing. The precipitated DNase was removed from the reaction via centrifugation at 10,000 x g for 2 minutes and the supernatant was transferred to a new microcentrifuge tube. RNA was quantified via a NanoDrop One spectrophotometer (Thermo Fisher Scientific) and diluted in TE (Sigma-Aldrich). Dilutions were tested in the presence and absence of 0.625 U/μL Avian Myeloblastosis Virus reverse transcriptase (AMV RT, Promega, Cat M5108) in LAMP reactions (**Section 2.14.2**) to ensure adequate DNA removal. Dilutions were stored at -80°C until use.

2.8.4. Asymmetric PCR

The synthesis of ssDNA from the dsDNA construct of PCV2 orf1 was performed with asymmetrical PCR, adapted from Venkatesan et al. (Venkatesan et al., 2013). Asymmetrical PCR involves biasing of the PCR primers to favour the production of one strand, and was performed in 15 μL PCR reactions, consisting of 1x DreamTaq Green PCR MasterMix (Thermo Fisher Scientific, Cat K1081), 2.4 μM total primers, at ratios of 10:0, 25:0, 50:0 and 100:0 in

favour of the T7 primer, and 0.01 ng of the synthetic DNA construct. Amplification was performed with 35 cycles of 30 seconds at 95°C, 30 seconds at 45°C and 40 seconds at 72°C with a final extension step of 10 minutes at 72°C.

The PCR product was separated by electrophoresis on a 1% agarose gel stained with EtBr run at 100 V for 30 minutes. The visualised PCR product was visualised using a Gel Doc Ez imager (Bio-Rad) and excised from the gel to undergo gel purification. Gel purification was performed using the QIAquick gel extraction kit (Qiagen, Cat 28706) as detailed in the manufacturer's instructions, and the DNA quantified by a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

2.8.5. Identification of target genes

Target genes were identified via literature searches, and genome sequences were obtained from the NCBI database as well as the Langford laboratories collection of 300+ APP whole-genome sequences. The reference genomes of each bacterial pathogen were searched for the gene of interest, and publicly available sequences from the relevant species were obtained by BLASTn. Conserved regions for primer design were determined in Geneious Prime 2022.2.1 (<https://www.geneious.com>). Multiple alignments of regions of interest were performed with the Geneious aligner, with the following parameters: global alignment with free end gaps and a cost matrix of identity (1.0/0.0), as our sequences were often fewer than 50 and less than 1 kb in length. The MUSCLE aligner (Muscle 3.8.425) with a maximum of four iterations was used when the number of sequences being aligned was greater than 50 sequences. Designed and published primer sets were mapped to the aligned sequences and percentage pairwise identity, and the number of identical sites were determined.

2.9. Analytical sensitivity

The analytical SEN of assays was determined using limit of detection (LoD) experiments. 10-fold dilutions were made of target DNA or RNA, subsequent testing was performed in the appropriate intervals, i.e., two-fold dilutions rather than 10-fold to determine the detection limit. All experiments were performed in triplicates in 5 µL reactions, unless otherwise stated. The detection limit of each assay is reported as the minimal copies of target nucleic acid per µL of the reaction, where all three replicates exhibited amplification.

2.10. Analytical specificity

A panel of 41 isolates from nine bacterial species were used to determine the SPE of the assays, consisting of three *Actinobacillus suis*, three *Actinobacillus equuli*, five APP, four "*Actinobacillus porcitosillarum*", four GPS, three MH, thirteen PM, two *Pasteurella pneumotropica* and four SS isolates (**Supplementary Table 3**). Reactions were run in the presence of 10 ng of gDNA from each isolate in real-time reactions monitored in a CFX Connect Real-Time PCR thermocycler (Bio-Rad, Cat 1855201).

2.11. RPA conditions

2.11.1. RPA primer design

RPA primers were synthesised by Thermo Fisher Scientific at standard de-salted purification. RPA Exo probes were produced by LGC Biosearch Technologies with dual-HPLC purification. Published RPA assays were used for the detection of APP, MH and PM, alongside assays that were designed in this Chapter for the detection of APP, MH, PM and GPS, in accordance with the TwistDx DNA amplification design manual (TwistDx Ltd., 2019). All RPA primers assessed in this Chapter are listed in **Supplementary Table 3**.

2.11.2. Real-time RPA reactions

RPA reactions were performed using TwistAmp Liquid kits (TwistDx, Cat TALQBAS01). Reaction mixtures contained 1x reaction buffer, 1x core protein mix, 1x energy mix, 0.45 μM of each dNTP (Thermo Fisher Scientific, Cat R0181), 14 mM magnesium acetate (MgAc, TwistDx), 0.6 μM of forward and reverse primers. RPA amplification was visualised with the addition of 0.12 μM of Exo probe and 0.5 U/ μL Exo (NEB, Cat M0206L), or via the addition of various concentrations of DNA dyes, stated in individual sections below. Reactions were run in a CFX connect Real-Time PCR thermocycler (Bio-Rad) at 37°C, with fluorescent readings taken every 30 seconds for 20 minutes. A manual mix, by 10 inversions, was performed after the reactions had been incubated for 4 minutes.

2.11.3. End-point detection of RPA reactions

The products generated in RPA amplification were visualised by gel electrophoresis. The completed RPA reactions underwent purification via the QIAquick PCR purification kit (Qiagen, Cat 28106) following the manufacturer's instructions. Sample eluent (10 μL) was combined with 2 μL of 6x loading dye (NEB, Cat B7024S) and run on a 2% agarose gel stained with Ethidium Bromide (EtBr). Electrophoresis of the RPA product was carried out at 110 V for 1 hour with products visualised using a Gel Doc Ez imager (Bio-Rad).

2.11.4. pH-RPA reactions

pH-RPA reactions were performed as real-time RPA reactions, but with the inclusion of a 1x custom buffer, in place of the 1x reaction buffer, containing 80 mM potassium acetate (KAc, Sigma-Aldrich, Cat 71196-100mL) and 2% PEG35 (Sigma-Aldrich, Cat 81310-1KG), adjusted to pH 12 with 5.0 M sodium hydroxide (NaOH, Honeywell, Cat 011-002-00-6). pH was measured with a SI400 pH meter using a MicroFET pH electrode (Sentron, Cat 9270-010).

2.12. HDA conditions

2.12.1. HDA primer design

HDA primers were designed in Primer3 (Koressaar and Remm, 2007), with the inclusion of parameters set out in the manufacturer's instructions. Briefly, the amplicon length was set to 80-120 bp; the melting temperature (T_m) was set to 68°C-75°C with an optimum of 71°C, the primer length set to 24-33 bp, the primer T_m set to 60-74°C, and the primer GC% set to 25-60%. The exact primer 3 parameters used, are listed in **Supplementary Table 4**. All primers used in this Chapter are listed in **Supplementary Table 3**.

2.12.2. Real-time HDA reactions

HDA reactions were performed with ISoAmp II Universal tHDA kit (NEB, Cat H0110S). Reaction mixtures contained IsoAmp 1x annealing buffer II, 4 mM magnesium sulphate ($MgSO_4$, NEB, Cat B1003S), 40 mM sodium chloride (NaCl, Sigma-Aldrich, Cat 71386), dNTP solution (Thermo Fisher Scientific), 0.35 μ L IsoAmp enzyme mixture, and 0.2x EvaGreen (Biotium, Cat 31000) with 0.75 μ M of each primer in a total reaction mixture of 5 μ L. HDA reactions were run in CFX connect Real-Time PCR thermocycler (Bio-Rad) at 65°C, with fluorescent readings being taken every 30 seconds for 80 minutes.

2.12.3. pH-HDA reactions

The pH-HDA reactions were performed as indicated in **Section 2.7.2** with the replacement of the stock IsoAmp 10x annealing buffer II, with either a 10x or 2x chloride or acetate buffer. The acetate buffer contained 1 mg/mL bovine serum albumin (BSA, NEB, Cat B9000S), 10 mM $MgAc$ (Sigma-Aldrich, Cat M5661-50G) and 1 mM Tris-acetate ethylenediaminetetraacetic acid (EDTA) buffer (Sigma-Aldrich, Cat T8280-1L). The chloride buffer contained 4 mM $MgSO_4$ (NEB), 40 mM NaCl (Sigma-Aldrich), 10 mmol/L potassium chloride (KCl, Sigma-Aldrich, Cat P9541) and 20 mmol/L Tris-hydrochloride (Tris-HCl, Sigma-Aldrich, Cat 10812846001), pH adjusted to pH 7.3 with hydrochloric acid (HCl, Sigma-Aldrich, Cat 258148-25ML) prior to use. The buffers were produced devoid of Tris buffer and tested against their Tris containing counterparts. The buffers without Tris were corrected to pH 7-11 with the addition of NaOH (Honeywell). pH was measured with a SI400 pH meter using an MicroFET pH electrode (Sentron).

2.13. PSR conditions

2.13.1. PSR primer design

PSR primers were synthesised by Thermo Fisher Scientific, purified with standard de-salting. PCR primers used for the detection of MH and PM were from previously published sources, with the addition of a 5' sequence originating from the botanic gene, *bla*_{NDM-1} (GenBank accession: 11027496, nucleotide: 32922–32941 and 33115–33097), as previously described (Liu et al., 2015b). All primers used in this Chapter are listed in **Supplementary Table 3**.

2.13.2. PSR reactions

The PSR reactions were prepared according to the original publication describing PSR (Liu et al., 2015b). Reaction mixtures consisted of 1 x Thermopol buffer (NEB, Cat B9004S), 0.8 M betaine (Sigma-Aldrich, Cat B0300-5VL), 6 mM MgSO₄ (NEB), 1.4 mM dNTPs (Thermo Fisher Scientific), 4 µM of each primer, 0.5 µM SYTO 9 (Thermo Fisher Scientific, Cat S34854) and 0.32 U/µL *Bst* large fragment (*Bst* LF) DNA polymerase (NEB, Cat M0275S). PSR reactions were incubated in a CFX connect Real-Time PCR thermocycler (Bio-Rad) at 63°C for 60 minutes, with fluorescence readings taken every 30 seconds.

2.14. LAMP conditions

2.14.1. LAMP primers

LAMP primers were produced by Thermo Fisher Scientific and purified with standard desalting. Where possible, published primer sets were utilised, and where not available, additional LAMP assays were designed as part of this work. All primers used in this Chapter are listed in **Supplementary Table 3**. LAMP primers were designed against <300 bp regions of conservation using Primer Explorer v5. Where published assays did not contain loop primers, the loop primers were designed in Primer Explorer v5 (Eiken Chemical Co.). LAMP primers that were altered from the published sequences are denoted with a .2 suffix and were designed manually using Geneious Prime 2022.2.1. All primer sets were subjected to NUPACK analysis, to model potential secondary structure within interacting primers (Zadeh et al., 2011), with the parameters: Temperature 63°C, number of strand species: 6, maximum complex size:5. Assays where primer sets exhibited a high probability (>0.8) of forming secondary structures between bases, were discounted and subsequently were not ordered.

2.14.2. Real-time LAMP reactions

LAMP reactions were prepared according to the manufacturer's instructions (NEB). LAMP reactions consisted of: 1x isothermal buffer (NEB, Cat B0537S), 6 mM MgSO₄ (NEB), 1.4 mM each dNTP (Thermo Fisher Scientific), 0.5 µg BSA (NEB), 0.8 M betaine (Sigma-Aldrich), 0.32 U/µL *Bst* 2.0 DNA polymerase (NEB, Cat M0537L), 0.25 µM of F3 and B3, 1.0 µM of LF and LB, 2.0 µM of FIP and BIP primers with 1 µL of template. Amplification was visualised with the addition of 0.5 µM SYTO 9 dye (Thermo Fisher Scientific), and for RNA detection reactions were supplemented with 0.625 U/µL of AMV-RT (Promega, Cat M5108), for a total reaction volume of 5 µL. Amplification was performed in a CFX connect Real-Time PCR thermocycler (Bio-Rad), at 63°C with fluorescence readings being taken every 30 seconds for 45 minutes.

2.14.3. End-point detection of LAMP products

LAMP amplification products were separated via electrophoresis. Completed LAMP reactions were diluted 1:10 in nuclease-free water (Thermo Fisher Scientific), and 5-10 µL of the diluted product was combined with 1-2 µL of 6x loading dye (NEB, Cat B7024S) and ran on a 2% agarose gel supplemented with EtBr. Electrophoresis of the LAMP product was carried out at 90 V for 2 hours and visualised with a Gel Doc Ez imager (Bio-Rad).

2.14.4. pH-LAMP reactions

pH-LAMP reactions were setup as real-time LAMP reactions (**Section 2.14.2**), except that SYTO 9 (Thermo Fisher Scientific) dye was omitted and the 1x isothermal buffer was replaced with a 1x custom buffer consisting of: 50 mM KCl (Sigma-Aldrich), 2 mM MgSO_4 (NEB), 10 mM ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$, Sigma-Aldrich, Cat A4418-100G) and 0.1% Tween 20 (PanReac AppliChem, Cat A4974,0250), adjusted to a pH of 8.5-9.0 with 5.0 M NaOH (Honeywell). pH was measured with a SI400 pH meter using an MicroFET pH electrode (Sentron).

Results

2.15. Recombinase polymerase amplification

There are numerous publications describing LAMP assays for the detection of PRDC pathogens, and significantly fewer published assays which utilise RPA. Therefore, RPA primers and Exo probes were designed in this work for four of the five bacterial pathogens involved in the PRDC. Published RPA assays were compared against assays designed in this study to ensure the best performing assay was taken forward.

2.15.1. *A. pleuropneumoniae*

The published RPA primer set (APP_1) targeted a region of the APP species-specific gene, *apx/IVa*, encoding the repeats-in-toxin IV (ApxIV), which is present in all APP strains (Schaller et al., 1999). In some isolates, *apx/IVa* has undergone a partial duplication, denoted as *apx/IVS* where there is an 87% DNA sequence identity among the 3' region of *apx/IVa* (Srijuntongsiri et al., 2022). In isolates which possess *apx/IVS*, such as MIDG 2331 (GenBank accession: LN908249), the partial duplication has been incorrectly annotated in the GenBank entry as hemolysin A.

An additional RPA assay (APP_2) was designed against the *apx/IVa* gene for a comparator against the published APP_1 RPA assay. The APP_2 assay targeted the same region within *apx/IVa* as a previously described PCR assay (Schaller et al., 2001). To ensure an optimal primer set, the APP_2 RPA assay underwent a primer screening using 250 copies/ μ L of APP serovar 8, the most prevalent serovar in the UK (Li et al., 2016), which determined the primer combination of F4R9 produced the most rapid TTP (**Supplementary Figure 1A**). The mapping of F4R9 to the MIDG 2331 genome showed the binding region was within *apx/IVS* (GenBank accession: LN908249, nucleotide: 1131104-1136935). A comparison was then performed in triplicate between the RPA APP assays, using the same template preparation, both achieved a LoD of 200 copies/ μ L RPA reaction. However, the APP_2 assay designed in this Chapter did display a slightly lower TTP at higher template concentrations (**Figure 2.4A**). It is notable that when the APP_1 amplicon was aligned with the reference serovar strains of APP, serovar 9/11 displayed a 17 bp deletion in the probe sequence, 5' to the fluorophore. Additionally, these strains also contained two dual nucleotide polymorphisms located under the fluorophore and quencher (**Figure 2.4B**). Although not confirmed experimentally, there is a high probability that the APP_1 assay would not detect APP isolates containing the same sequence variations as seen in the reference strains for serovar 9/11.

The *apx/IVa* gene contains a repetitive element, which has been hypothesised to be an advantage in providing multiple-target sites for molecular assays (Srijuntongsiri et al., 2022). However, I found that for the APP_2 assay, the repetitive nature of *apx/IVa* resulted in multiple priming sites. For example, the serovar 5b genome (GenBank accession: GCA_000015885.1) contained two alternative priming sites for the APP_2 F4 primer, both within the *apx/IVa* gene and displaying a single SNP at position 2G>A. Whilst the serovar 6 reference strain (Fem ϕ) showed a single alternative priming site with the same 2G>A mismatch and an additional APP_2 probe priming site. An additional APP RPA assay was subsequently designed, denoted APP_3', targeting a region of the *apx/IVa* gene

located towards the 3' end, which is highly conserved and less repetitive. The primer pair which resulted in the lowest TTP in the APP_3' assay was determined to be F3R6 (**Supplementary Figure 1B**). A comparison in the performance of APP_2 against APP_3' was performed using 10 replicates of the reference strain for serovar 8. The APP_3' assay resulted in a higher analytical SEN with a LoD of 10 copies/ μ L, 20x more sensitive than the APP_2 assay. Additionally, the APP_3' assay had a lower TTP, with all amplification detectable in 6 minutes (**Figure 2.4C**).

Both the assays designed in this study were able to detect all 19 reference serovars of APP (data not shown). Additionally, neither assay resulted in any FP amplification events when tested against any of the 36 isolates from eight species closely related to, or found within, the same niche as APP.

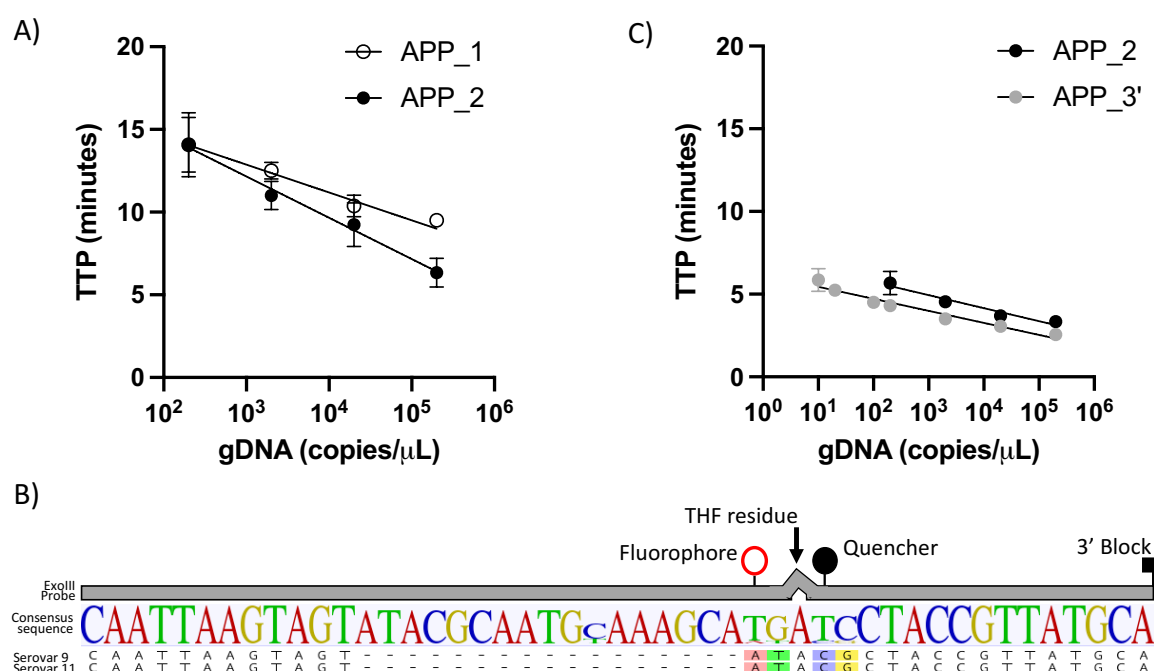


Figure 2.4. Analytical sensitivity of APP-RPA assays. LoD experiment using serovar 8 gDNA, comparing the APP RPA published assay (APP_1) with the APP RPA assay designed in this Chapter (APP_2) (**A**). The probe region of the published APP_1 assay depicts regions of deletions in serovars 9 and 11 (9/11) (**B**). LoD experiment comparing the APP_1 and the 3'APP RPA assay (APP_3') designed in this Chapter (**C**). Data points represent the geometrical mean of three replicates, and error bars the standard deviation (S.D.).

2.15.2. *M. hyopneumoniae*

The published RPA primer set (MH_1) targeted the MH *mhp165* gene (GenBank accession: AAV27748.1). The function of *mhp165* is not yet identified, but this large (6,144 bp) gene is predicted to encode a protein with zinc finger, metalloproteinase, coiled-coil transmembrane domain, secretory signal, and ABC transporter motifs. At the time of writing only 15 MH sequences are available on GenBank. When aligned against the *mhp185* gene only 13 sequences spanned the gene. The MH_1 RPA assay did contain a SNP to the consensus sequences on the reverse primer, however, this was at the 5' end and therefore unlikely to impact amplification efficiency (data not shown).

The MH RPA assay designed in this study targets the P97 cilium adhesin encoded by the 3,327 bp *mhp183* gene (GenBank accession: AAV27763.1). The P97 adhesin is considered a prevalent virulence factor playing a significant role in adherence of MH to ciliated epithelium within the respiratory tract (Clampitt et al., 2021, Hsu et al., 1997, Hsu and Minion, 1998, Zhang et al., 1995). Both the *mhp165* and the *mhp183* genes have been previously targeted in diagnostic assays (Strait et al., 2008).

The *mhp183* gene returned 68 sequences using BLASTn; once aligned 25 sequences covered the gene, showing a hypervariable 3' end (data not shown). RPA primers were designed across a conserved region, and screened against the reference strain, MH232, to establish the most efficient amplification pair, which was determined to be F9R8 (**Supplementary Figure 1C**). When the consensus sequences for the *mhp165* gene was examined, the 134 bp target region of the published assay (MH_1) showed 130 (97%) identical sites and a 99.0% pairwise identity. The consensus sequence for the 116 bp region of the *mhp183* gene targeted in the assay designed in this Chapter (MH_2) displayed 114 (97%) identical sites and 99.8% pairwise identity.

A comparison between the published and designed assay using the reference strain MH232 determined the MH_2 assay was faster and more sensitive compared to the MH_1 assay (**Figure 2.5A**). Therefore, the MH_2 assay was used to perform an extensive LoD using a total of 10 replicates from at least three separate serial dilutions of the MH232 reference strain. The MH_2 assay achieved a SEN of 20 copies/ μ L of the total RPA reactions (**Figure 2.5B**). Furthermore, the MH_2 assay did not result in any amplification when tested with any of the 38 isolates from eight species of bacteria tested.

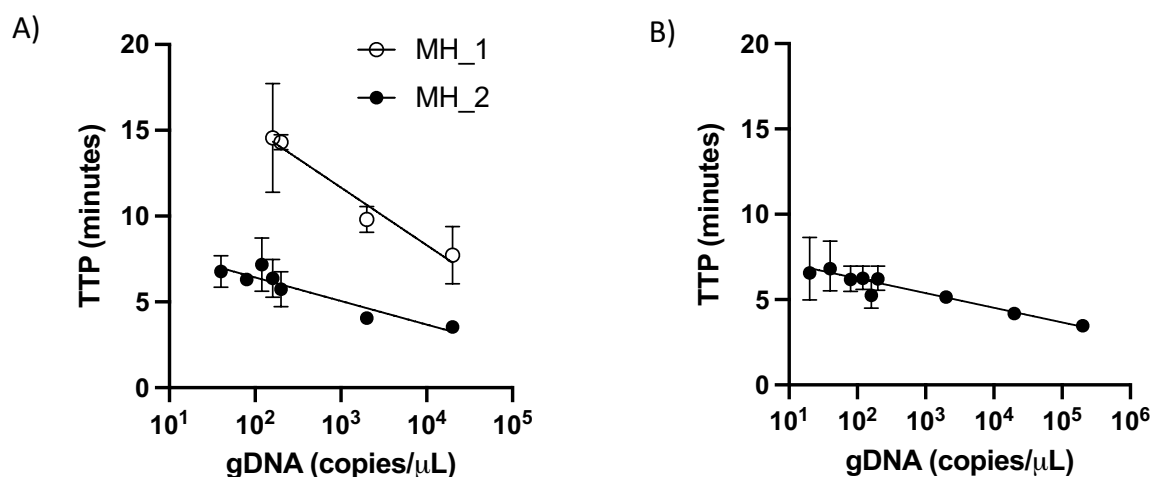


Figure 2.5. Analytical sensitivity of MH-RPA assays. Previously published assay detecting MH (MH_1) compared against the MH RPA assay designed in this Chapter (MH_2) in triplicate against gDNA obtained from the laboratory reference strain MH232 (**A**). The LoD achieved with the MH_2 assay achieved with 10 replicates against gDNA from MH232 (**B**). Data points represent the geometrical mean, and error bars the S.D.

2.15.3. *P. multocida*

Both the published PM RPA assay and that designed in this study target different regions of the *kmt1* gene. The *kmt1* gene is 1,800 bp long, labelled as PM_RS07060, alpha/beta hydrolase on the 'pm 70' reference PM genome

sequence (GenBank accession: NC_002663). A BLASTn search with PM_RS07060 of PM species (Taxid: 747) which includes all subspecies, displayed 136 results. Whilst the same BLASTn search of PM subspecies *multocida* (Taxid: 44283) displayed just 15 results. The alignment of the 136 PM sequences indicated that the published assay amplified a region of high SNP prevalence, with only 29.6% identical sites across the published primers and probe. The RPA assay used in this study was designed into target a region 5' to PM_1 with 86.8% identical sites across the 136 isolates. The designed RPA primers were screened using a PM clone originating a clinical isolate (Hotchkiss et al., 2011), MIDG 3227, in duplicate reactions at 100 copies/ μ L per RPA reaction. The best performing primer pair was F1R5, taking the least time to reach the threshold fluorescence level (**Supplementary Figure 1D**), and are referred to as PM_2.

A comparison of the PM RPA assays was performed using a serial dilution of MIDG 3227, ran in triplicate which showed that PM_2 resulted in more sensitive and rapid amplification (**Figure 2.6A**), and therefore a more detailed LoD was performed with 10-replicates, using at least three different serial dilutions of MIDG 3227. The LoD achieved for PM_2 was 80 copies/ μ L of RPA reaction (**Figure 2.6B**). Additionally, PM_2 was confirmed to be specific, with no amplification when the RPA reactions were run in the presence of gDNA from 28 bacterial isolates, closely related to or commonly found within the same niche as PM.

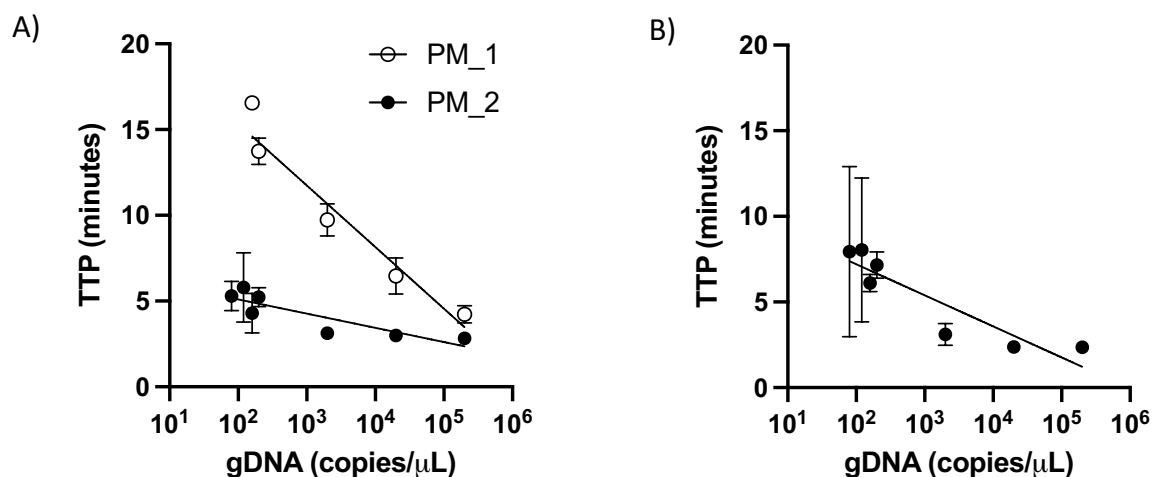


Figure 2.6. Analytical sensitivity of PM-RPA assays. LoD achieved in triplicate against gDNA of PM isolate, MIDG 3227, amplified with PM_1 and PM_2 RPA assays (**A**). PM_2 assay LoD achieved using 10-replicates achieved using three different dilutions of MIDG 3227 (**B**). Data points represent the geometrical mean, and error bars the S.D.

2.15.4. *S. suis*

At the time of writing, there is only one published RPA assay for the detection of SS. However, this assay is specific to the detection of SS serotype 2, using a region of the capsule synthesis gene *cps2J* (Jiang, Zhu and Zhan, 2022). Therefore, an SS assay was designed, targeting the recombination and repair protein, RecN, via the *recN* gene.

The 1,662 bp *recN* gene was chosen as it has been found to have a higher divergence between other *Streptococcus* subspecies and a higher degree of similarity intraspecies than other SS housekeeping genes

(Glazunova et al., 2010). The SS GenBank reference sequence (GenBank accession: NC_012926) was used to identify the *recN* gene (1,614,834 – 1,616,495 bp). A BLASTn search of *recN* identified 134 SS sequences. An alignment of these sequences found that across the 1,662 bp sequence, there are 60.2% identical sites with 96.2% pairwise identity. The central region of the gene was found to have been targeted previously in molecular diagnostic assays (Ishida et al., 2014, Tarini et al., 2019, Tien et al., 2013). Therefore, RPA primers and probes were designed to amplify this region.

The primer screen was performed in duplicate against 80 copies/ μ L of the WT strain P1/7, with the F6R2 primer pair displaying the most rapid TTP result (**Supplementary Figure 1E**). The F6R2 primer pair, denoted SS-RPA was used in a LoD experiment with 10-replicates produced from at least three different serial dilutions of P1/7. The LoD achieved was 10 copies/ μ L RPA reaction (**Figure 2.7**). Furthermore, the SS-RPA assay did not result in any cross-reaction with any of the 37 bacterial isolates commonly found in the same niche as SS.

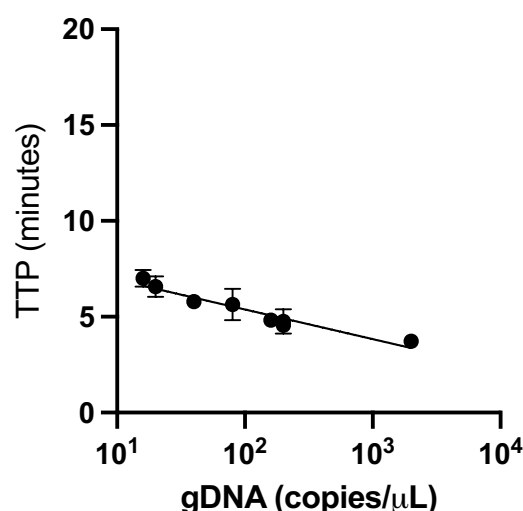


Figure 2.7. Analytical sensitivity of the SS-RPA assay. LoD was achieved with 10-replicates amplifying gDNA obtained from the SS reference strain P1/7. Data points represent the geometrical mean, and error bars the S.D.

2.15.5. pH-RPA

Detection of real-time amplification in RPA requires the addition of a fluorescence-based probe, such as an Exo, formamidopyrimidine DNA glycosylase (Fpg) or endonuclease IV (Nfo) probe, along with the relevant enzyme. During amplification the enzyme acts to separate the fluorophore and quencher of the probe and increase the fluorescence within the reaction. The addition of such a probe creates an additional level of complexity to the assay, which our CMOS-ISFET detection platform does not require. To simplify the RPA detection process, the use of two fluorescent DNA dyes, SYTO 9 and EvaGreen, were compared over a range of concentrations in RPA assays. Both DNA dyes gave strong amplification-like sigmoidal curves in absence of target DNA (data not shown). I eliminated contamination as the cause of this FP signal, as no amplicon-specific peaks were observed in the melt-curve data, nor were specific bands detected when the RPA products were separated by gel electrophoresis (data not shown). The formation of a high degree of primer-dimers in RPA is well documented,

causing FP signals when intercalating fluorescent dyes are used (Piepenburg et al., 2015, Zou et al., 2020). Supported by our finding that primer-noise caused the FP signals as determined by gel electrophoresis and melt-curve analysis of RPA reactions amplified with and without target DNA (**Supplementary Figure 2A** and **Supplementary Figure 2B**, respectively). Furthermore, increasing the temperature of the RPA reactions (37-45°C) did not reduce the primer-dependant noise (**Supplementary Figure 2C**). Thus, to discriminate amplification from noise, the raw fluorescence units (RFU) collected in the absence of target DNA were removed from the RFU of amplifications where the template was present; when this differential was plotted, a sigmoidal amplification curve was obtained across a range of dye concentrations.

The SYTO 9 DNA dye produced 5x less relative signal compared to the EvaGreen dye. However, EvaGreen did show greater inhibition of the RPA reactions compared to SYTO 9 across the range of concentrations used, with the higher EvaGreen concentrations delaying the onset of amplification (**Figure 2.8A** and **Figure 2.8B**). To determine which concentration of which dye resulted in the least inhibitory effect on RPA amplification whilst producing the highest signal:noise ratio, the area under the curve (AUC) for each relative signal plot was divided by the average TTP of that dye concentration. The highest AUC:TTP ratio were obtained when 4 μM of SYTO 9 and 1.5x of EvaGreen were used. Therefore, these concentrations resulted in the highest signal and were the least inhibitory to the RPA reaction (**Figure 2.8C**).

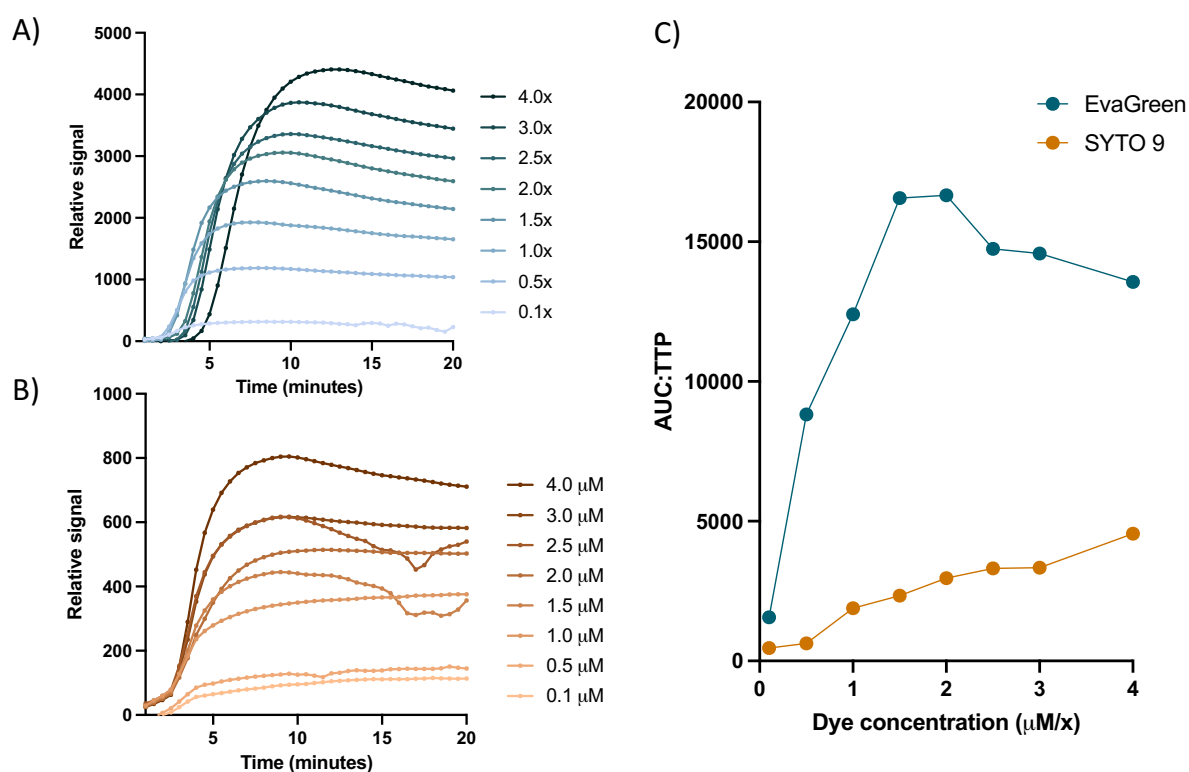


Figure 2.8. Detection of RPA amplification with fluorescence-based dyes. The amplification of 100 copies of gDNA serovar 5b was detected with concentrations of EvaGreen (**A**) and SYTO 9 (**B**) dyes. The area under the curve (AUC) to TTP ratio is plotted against varying concentrations of EvaGreen and SYTO 9 dye (**C**). Data points represent the geometrical mean of three replicates.

The high presence of primer noise in RPA may negatively impact the detection of positive RPA amplification events electrochemically, which is particularly important for the application of RPA on our CMOS-ISFET detection platform. A primer-primer extension event will result in the production of protons, which may be indistinguishable from the protons released during a target amplification event. To assess the viability of using RPA on the CMOS-ISFET platform, I first had to remove the buffering capacity of the RPA reaction, which prevents the detection of proton release. I deduced, through titration of each constituent of the RPA reaction, that the RPA reaction buffer likely consists of 80 mM KAc, 2% PEG35 and 34 mM Tris (data not shown). Thus, a custom reaction buffer was produced that was devoid of Tris. Next, I sought to determine how the pH of the custom reactions buffer affects the amplification in RPA. For the detection on the CMOS-ISFET platform, a higher pH is favourable as pH is not a linear scale. The more alkaline the starting pH, the greater the pH will decrease when protons are released into the solution ($\text{pH} = -\log[\text{H}_3\text{O}^+]$). Therefore, NaOH was titrated into the custom reaction buffer, resulting in a pH ranging from 7.6-12.8. The observed pH of the whole RPA reaction did not vary significantly when the pH of the custom reaction buffer was 7.6-12 (Figure 2.9A), indicating that the RPA reaction constituents supplied by TwistDx still contain a high concentration of Tris, resulting in the high buffering capacity observed here. Subsequently, the custom reaction buffer was altered to give a final pH of the RPA reaction (pH 7.6-9.0). The RPA reactions proved sensitive to fluctuations in pH, with the pH 9.0 RPA reaction being completely inhibited (Figure 2.9B). Therefore, the custom reaction buffer pH 12.6, which equated to a final RPA reaction pH 8.8, was chosen for further experimentation.

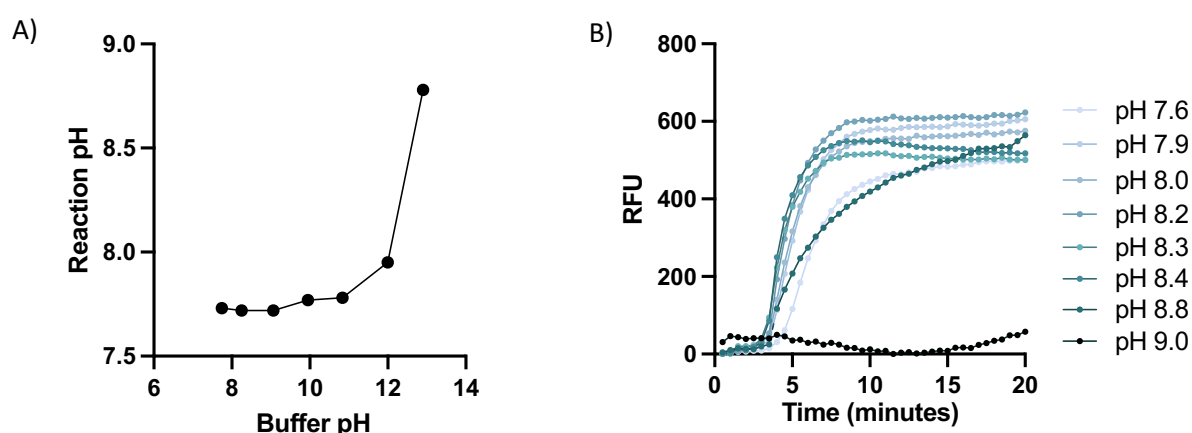


Figure 2.9. Effect of pH on RPA. The buffering capacity of RPA reactions is depicted by the addition of increasing pH of the RPA buffer against the pH of the final RPA reaction (A). The ability of the RPA reaction to amplify 1,000 copies of gDNA obtained from APP serovar 5, using the APP_2 primers and Exo probe with varying RPA reaction pH (B). Data points represent the geometrical mean of three replicates.

In order to determine the compatibility of RPA with the CMOS-ISFET detection platform, I first assessed the ability of a pH change to occur in a minimally buffered RPA reaction. The pH of the RPA solution was measured pre- and post-incubation to calculate the differential pH (ΔpH); the highest ΔpH achieved was 0.8 when target DNA was present and 0.7 in the negative control. However, the pH change was not consistent in replicate experiments. Increasing the RPA incubation temperature to reduce primer noise did not result in any increase

in the achieved Δ pH (**Supplementary Figure 2D**). Taken together, these results indicate that RPA biochemistry is not compatible with CMOS-ISFET-based pH detection.

2.16. Helicase dependant amplification

At the time of writing, there are no publications describing the use of HDA for the detection of any of the PRDC pathogens. Therefore, HDA assays were designed in this work targeting three of the PRDC bacterial pathogens, APP, MH and PM.

2.16.1. *A. pleuropneumoniae*

The design of a HDA assay for APP proved troublesome. Initially, primers were designed against a 1,857 bp region of the species-specific *apxIVA* gene. However, after the primer pairs were screened, it was determined that there was significant amplification in the negative controls, which persisted even when primer pairs were designed against three further target regions of APP: A 534 bp region of the *dsbE*-like gene encoding a disulphide bond formation protein E (DsbE) used previously as a species-specific marker (Chiers et al., 2001). A 1,395 bp region of the *apfB* gene encoding an adhesion protein that is required for bacterial pathogenesis (Zhou et al., 2013). As well as a 1,200 bp region of the *metP*, a metalloprotease identified by Dr Janine Bossé (Imperial College London) to be a good candidate for APP specific molecular assays, as it is highly conserved (>99.7% identity at the nucleotide level) across all APP genomes (personal correspondence). The source of this false amplification was investigated further, and it was found that often the negative controls amplified faster than the positive controls, even when 10,000 copies of gDNA were input, which suggests primer dimerization rather than contamination. As contamination usually results in late or sporadic amplification. Contamination of the assay is also unlikely as when the target was alternated to new genes; the FP amplification did not subside. Additionally, the use of other chemistries at this time showed no contamination. However, for primer-interaction to be confirmed, a melt curve was performed, which did not definitively show that the melting peak had shifted, which is often seen when comparing target amplification compared to primer-dimers (data not shown).

2.16.2. *S. suis*

To perform a comparison of HDA performance against the other isothermal technologies, the development of at least three comparator assays would be ideal. As such, five primer pairs were designed against a 316 bp region of the *recN* gene of SS, which again showed amplification in the absence of target DNA in all but one of the primer pairs (SS_HDA_recN_F4 and R4). The primer pair, which did not display false positive (FP) amplification, targeted a 71 bp section of the *recN* gene (introduced in **Section 2.15.4**), but also displayed the poorest amplification with only 200,000 copies/ μ L reaction amplifying (data not shown).

2.16.3. *M. hyopneumoniae*

Despite the setbacks, two HDA assays were successfully developed for the detection of MH. The MH HDA assay targeted a 630 bp region of the *mhp165* gene, described in **Section 2.15.2**. The screening of five primer pairs

suggested that HDA_MH_2 resulted in the fastest TTP (**Supplementary Figure 3A**). However, FP amplification was determined to occur using this primer pair, thus, the HDA_MH_5 assay was subsequently used. The HDA_MH_5 primer pair amplify a 92 bp region of the *mhp165* gene, which displays 88 identical sites (95.7%) and 98.8% pairwise identity. To determine the performance of the HDA_MH_5 assay, a 10-fold serial dilution was made of the MH reference strain, MH232, which indicated that the HDA_MH_5 was highly sensitive, achieving a LoD of 2 copies/ μ L (**Figure 2.10A**). The SPE of the HDA_MH assay was assessed with 38 strains of bacteria, with MH showing FP amplification in two strains, both belonging to *A. equuli*.

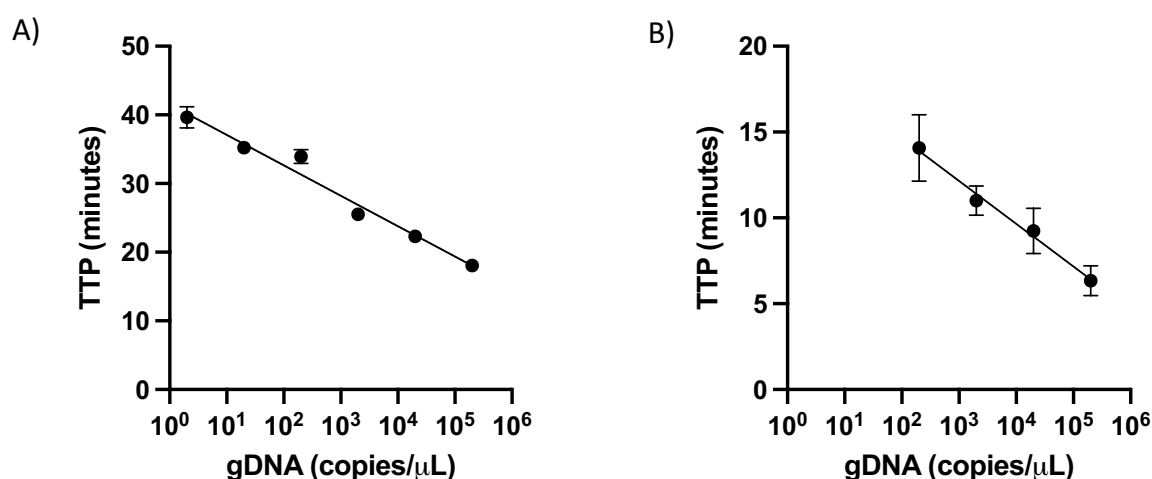


Figure 2.10. The analytical sensitivity of HDA assays. The LoD achieved with MH_5 primer set in 10-fold serial dilutions of gDNA from the reference strain MH232 (**A**) and PM_3 using 10-fold serial dilutions of gDNA obtained from the PM reference strain, MIDG 3290 (**B**). Data points represent the geometrical mean of three-replicates, and error bars the S.D.

2.16.4. *P. multocida*

The PM HDA assay was designed against a 449 bp region of the *kmt1* gene, described in **Section 2.15.3**. The optimal primer pairs were found to be HDA_PM_3, which amplified the 1,000 copies of MIDG 3290 gDNA within 20 minutes of incubation (**Supplementary Figure 3B**). The HDA_PM_3 primers target a 82 bp region of the *kmt1* gene with 99.7% pairwise identity across the 100 PM isolates aligned in BLASTn. In a LoD experiment, the HDA_PM_3 assay detected down to 100 copies/ μ L of a 10-fold serial dilution of the laboratory reference strain, SVR5 (**Figure 2.10B**). With all amplification taking place in under 20 minutes. In order to assess the SPE of the HDA_PM_3 assay, the primer pair was used against 20 isolates closely related to or found within the same niche as PM. The HDA_PM assay showed amplification in nine of these isolates, two belonging to *A. equuli*, two *P. pneumotropica*, two *A. suis*, two "*A. porcitisillarum*" and one APP, suggesting that HDA is prone to FP amplification. Throughout testing, the HDA_PM_3 assay did not result in any false amplification events in the no template controls and as such the FP events were unlikely to be due to contamination. Therefore, this assay was still used in subsequent experiments involving HDA.

2.16.5. pH-HDA

For amplification with HDA to be compatible with the CMOS-ISFET detection platform, the buffering component of the HDA reaction had to be removed. From the literature, two buffers were described, a chloride buffer (Jing et al., 2012) and an acetate buffer (Huimin Kong, 2003). The acetate buffer showed no amplification, whilst the chloride buffer amplified with a lower TTP than the HDA annealing buffer supplied in the Biohelix kit. Subsequently, a version of the chloride buffer was formulated which was devoid of Tris, and NaOH was titrated to produce a range of pHs (8.5-11.9). As seen previously in RPA, the HDA reaction was very resistant to pH change across the range trialled (**Figure 2.11**).

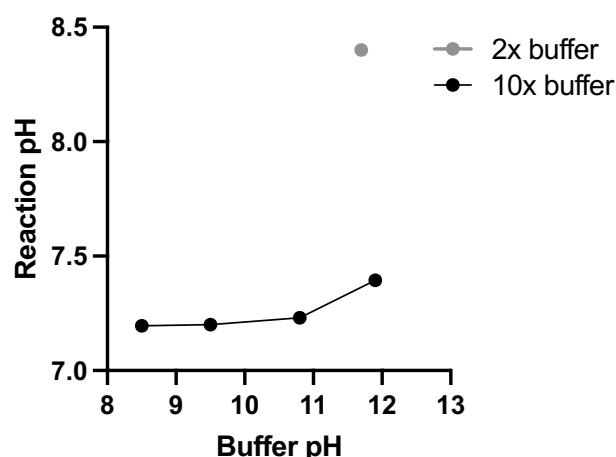


Figure 2.11. The effect of pH on HDA. The increase in the reaction pH in HDA after the addition of 10x reaction buffers, with increasing pH values. Dots represent single data points, with the 2x buffer was only produced at a single pH value.

Only the reactions at the higher end of the pH scale producing amplification in HDA, pH 10.8 and 11.9 (**Figure 2.12A**). The configuration of the reaction mix had to be altered for more buffer to be added; a 2x chloride buffer was produced at pH 11.7, which amplified with a decreased TTP compared to the 10x annealing buffer control (**Figure 2.12B**). The 2x pH 11.7 buffer resulted in an HDA reaction pH of 8.4, compared to the 8.22 annealing buffer control. When determining the differential pH between pre- and post-incubation HDA reactions, the HDA_PM assay resulted in a ΔpH of 0.62. However, the negative template control displayed a decrease in pH of 0.73. Therefore, even in the absence of amplification occurring in the real-time data, the ΔpH produced by HDA reactions was indistinguishable from that in negative template controls compared to target amplification.

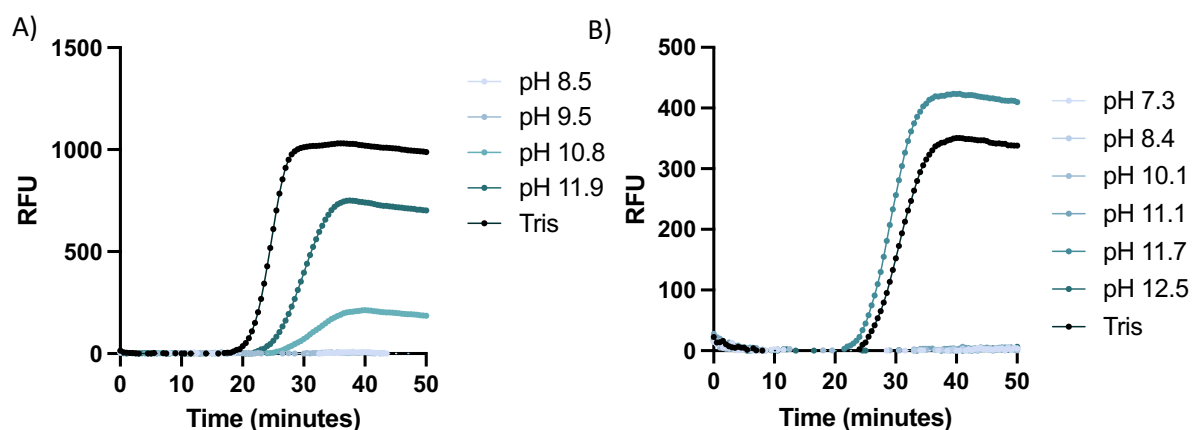


Figure 2.12. The effect pH has on HDA amplification. The amplification with HDA across several different pHs of 10x buffer (A) and 2x buffer (B) using the HDA_PM_3 assay and 200 copies/ μ L of MIDG 3290 gDNA. Data points represent the geometrical mean of three-replicates.

2.17. Polymerase spiral reaction

Few PSR assays have been developed for the detection of the PRDC pathogens, only an assay for the detection of PCV3 (Ji et al., 2019) and, more recently, APP (Sarkar et al., 2022). PCV3 has not been found in the UK, and therefore the PSR-PCV3 assay was not assessed in this work. The published PSR-APP assay was assessed for analytical SEN using 10-fold serial dilutions of gDNA obtained from the serovar 8 reference APP strain. The PSR-APP assay only consistently amplified at the highest concentration tested, 40,000 copies/ μ L of PSR reaction (Figure 2.13).

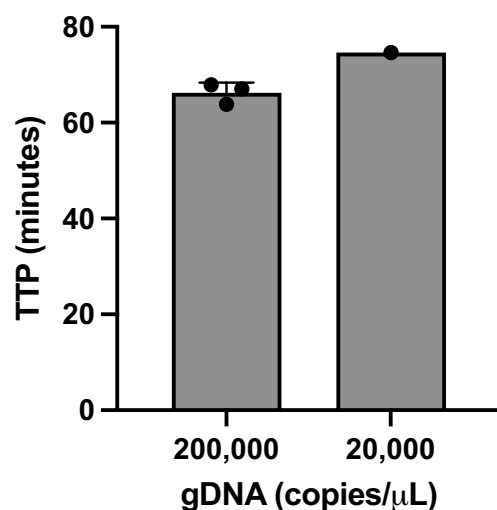


Figure 2.13. APP-PSR amplification. The TTP was obtained when gDNA from the APP reference strain serovar 8 was amplified with the published PRS-APP assay. Columns represent the geometrical mean across three replicates, with data points indicating each replicate and error bars the S.D.

It is typical in PSR to validate the primer sets in PCR prior to their implementation for PSR amplification (Sarkar et al., 2022, Liu et al., 2015b). As such, two PSR assays were designed from published PCR sequences (Scherrer et al., 2016, Strait et al., 2008), with the addition of a 5' unrelated exogenous sequence. The exogenous sequence

was obtained from the original PSR publication, which utilised the *bla*_{NDM-1} botanic gene (Liu et al., 2015b). The PCR assays used targeted a region of the *mhp165* gene of MH and a region of the *toxA* gene of the PM genome. However, neither of these assays produced detectable amplification in PSR (data not shown). Due to time constraints, PSR-based amplification was not assessed further.

2.18. Loop-mediated isothermal amplification

LAMP is the most published isothermal technology, therefore there are several assays already available for the detection of the PRDC pathogens. In this section, I compare at least two published assays for each of the PRDC pathogens to ascertain the optimal target region and primer pair for use in the diagnostic device.

2.18.1. *A. pleuropneumoniae*

The two published LAMP assays for the detection of APP targeted two different genes, with one, denoted (APP_A) targeting a 243 bp region of the *dsbE*-like gene (Ji et al., 2012) and the other (APP_B) a 224 bp region of the *apxIVA* gene (Yang et al., 2009), both of these genes have been previously described in **Section 2.16.1** and **Section 2.15.1**, respectively. The amplified region of APP_A displayed a pairwise identity of 99.3% when aligned across our collection of 300 APP genomes, and the APP_B assay displaying 91.6% pairwise identity. In the original publication of the APP_A assay loop primers were not included in the assay (Ji et al., 2012). However, as loop primers are known to increase reaction kinetics and decrease the TTP (Nagamine et al., 2002), loop primers were designed in this work against the consensus sequence of the APP_A target region (denoted, APP_A+). Subsequent analytical SEN experiments to determine the LoD of each assay showed that the addition of the loop primers not only decreased the TTP by 48.5% but also greatly increased the SEN, with the APP_A+ primer set achieving a LoD of 80 copies/μL, opposed to the original APP_A primer set which only achieved 2,000 copies/μL (**Figure 2.14**). The APP_B primer set also achieved a SEN of 80 copies/μL. However, the APP_A+ remained the most rapid amplifying primer set with a TTP of less than 20 minutes (**Figure 2.14**). The APP_A+ primers were mapped to the consensus sequence of the 300 APP genomes in our database, with F3 and the B2 region of the BIP primer having 96% and 97.4% pairwise identity, respectively, and these were redesigned to include the SNP. The LB primer was also redesigned to match the T_m of the LF primer (Agilent, 2021) (**Table 2.1**).

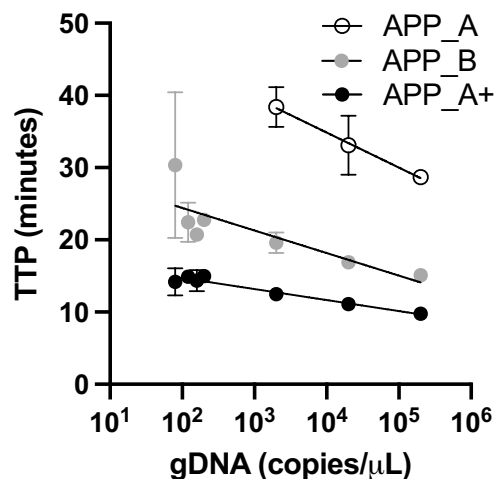


Figure 2.14. Analytical sensitivity of APP-LAMP assays. The LoD performed in triplicate against gDNA obtained from the APP serovar 8 reference strain MIDG 2220. Data points represent the geometrical mean, and error bars the S.D.

The replacement of the APP_A+ primers with the newly designed .2 version, both together or singularly, resulted in a decrease in SEN compared to the original primers when using an isolate containing the SNPs (MIDG 2217, GenBank accession: GB: CP000569) (**Supplementary Figure 4A**).

The APP_A+ primer set was found to have higher SPE for APP, with no FP amplification events occurring when the APP_A+ primer set was used in the presence of gDNA from 36 bacterial isolates closely related to or found in the same niche as APP.

Table 2.1. Primer alterations to the APP_A-LAMP assay.

Primer	Sequence	Length	Pairwise identity (%)	GC %	Rough Tm
F3	CTGAAAGTTTCGTCAGCAC	19	96	47.4	54.5
F3.2	TTTCTAATTCAGACGCTGAAAG	22	100	36.4	54.7
B3	TCTGAAACTGAAAAGTATCCAC	22	100	36.4	54.7
B3.2	ACTGAAGACAAGCGGTAAAA	23	99.9	30.4	53.5
F2	ATTTAATTGATAAACCGGTGTG	23	100	30.4	53.5
B2	AAACTGAAGACAAGCGGTA	19	97.4	42.1	52.4
B2.2	ACTGAAGACAAGCGGTAAAA	20	100	40.0	53.2
F1c	GCTCGAAATCCGGTTATATCC	22	99.9	50.0	60.3
B1c	TCGTTTAAAAGCGTTACAAGAGGA	24	100	37.5	57.6
LF	TTTTTGTTGGTAACGTAC	18	100	33.3	46.9
LB	GACGAACTATTGCAAAAAATCGCG	25	100	40.0	59.7
LB.2	ACTATTGCAAAAAATCGC	18	100	33.0	46.9

2.18.2. *M. hyopneumoniae*

The two published LAMP assays detecting MH compared in this Chapter targeted two different genes in the MH genome. The first, denoted MH_A, amplified a 181 bp section of the 1,017 bp L-lactate dehydrogenase gene (*lctD*) (Liu et al., 2015a) encoding L-lactate dehydrogenase, previously designated as protein P36. Lactate dehydrogenase catalyses the conversion of lactate to pyruvate (Haldimann et al., 1993). The comparator LAMP assay (MH_B) targets a 218 bp region of the *mhp165* gene (Li et al., 2013), described previously in **Section 2.15.2**. The in silico analysis of the genomes available on GenBank were analysed for conservation within the amplified section of each of the published MH LAMP assays. MH_A displayed 99.3.% pairwise identity, with 178 of the 181 bp region of *lctD* sharing identities (98.3%), whilst MH_B displayed 99.0% pairwise identity across the 218 bp region of the *mhp183* gene, with 212 identical sites (97.2%).

The published MH_A assay did not contain loop-primers, thus as above, loop primers were designed against the consensus sequence of the *lctD* gene, and subsequently denoted MH_A+, to differentiate the addition of loop primers from the original MH_A assay. To assess the analytical SEN of these assays, serial dilutions were made of gDNA from the MH reference strain, MH232. The LoD of the MH_A assay was 160 copies/ μ L, which increased to 20 copies/ μ L with the addition of the loop primers. The TTP also increased by 66.0% compared to the original assay. However, the highest SEN was achieved with MH_B which detected down to 8 copies/ μ L in under 20 minutes (**Figure 2.15**). LAMP primer pairs should have similar melting temperatures, with a maximum difference in their respective T_m of 5°C (Agilent, 2021). Therefore, to decrease the TTP of the MH_B assay, the F3 primer and the F2 region of the FIP primer were redesigned. The F3 primer was redesigned to increase the T_m from 53.2°C to 57.1°C, to match the 57.1°C T_m of the opposing B3 primer. The F2 region of the FIP primer was redesigned to increase the inclusivity of the F2 target region from 98.2% pairwise identity to 100% and increase the T_m from 55.3°C to 54°C to match the opposing BIP primer T_m more closely. However, in comparison, the inclusion of the MH_B.2 primers resulted in an increased TTP (**Supplementary Figure 4B**). Therefore, the original MH_B primers were taken forward as the best performing LAMP primers to detect MH. Additionally, no cross-reaction was seen with the MH_B primers when they were used in combination with any of the 38 other pathogens which occupy the same niche as MH.

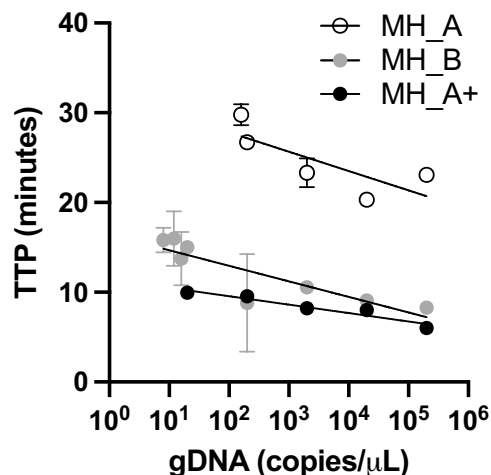


Figure 2.15. Analytical sensitivity of MH-LAMP assays. The LoD achieved with each of the MH assays against gDNA from the reference laboratory strain MH232, performed in triplicate. Data points represent the geometrical mean, and error bars the S.D.

2.18.3. *P. multocida*

The two published LAMP assays to detect the presence of PM both targeted the *kmt1* gene, with one assay, PM_A, targeting a 108 bp region of the 1,800 bp gene (Bhimani et al., 2015), and the other, PM_B, a 204 bp region (Sun et al., 2010). A BLASTn search was performed with the *kmt1* gene which produced 100 sequences, which were subsequently aligned, and the LAMP PM primers were mapped to the consensus sequence. The PM_A target region set showed 99.6% pairwise identity across 103 (95.4%) identical sites. The PM_B primer set was similar, with 99.4% pairwise identity across 193 (94.6%) identical sites. However, when these PM LAMP primer sets were trialled against a PM isolate which is a clinically relevant clone of CC13 (Hotchkiss et al., 2011), MIDG 3290, there was consistently no amplification from the PM_B primer set (data not shown). The sequence data offered no explanation for the lack of detectable amplification when PM_B was used. As the original publication of PM_B did not contain loop primers, these were designed against the consensus sequence of *kmt1*. The inclusion of loop primers in the PM_B assay, denoted PM_B+, produced detectable amplification in the MIDG 3290, however the direct comparison of PM_B amplification with and without loop primers was not assessed. To determine the analytical SEN of PM_A and PM_B+, these LAMP assays were assessed using serial dilutions of MIDG 3290 as template. The PM_A LAMP primers showed high SEN, detecting down to 8 copies/μL, whilst the PM_B+ primers were 50x less sensitive and only detected down to 400 copies/μL (**Figure 2.16**). In case of the eventuality that the loop primers did not rescue the PM_B LAMP amplification, an additional LAMP assay was designed against the consensus sequence of the *kmt1* gene alignment of 100 PM isolates, denoted PM_C. The PM_C LAMP assay targeted a 247 bp region of the *kmt1* gene, with 99.1% pairwise identity 237 (96%) identical sites across the 100 PM *kmt1* sequences. However, the PM_C LAMP assay failed to match the performance of the PM_A LAMP assay, achieving an analytical SEN of 20 copies/μL (**Figure 2.16**).

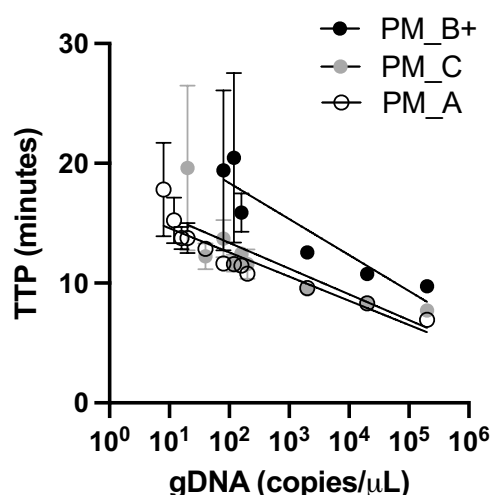


Figure 2.16. Analytical sensitivity of PM-LAMP assays. The LoD achieved using gDNA from the PM reference strain MIDG 3290. Data points represent the geometrical mean of three replicates, and error bars the S.D.

The PM_A assay was therefore determined to be the most sensitive and rapid, however a rising baseline was noted with this assay. An analysis of the primers in NUPACK suggested that the LB primer was interacting with a region of the FIP primer forming a hairpin structure (data not shown). Therefore, the LB primer was redesigned to avoid hairpin formation with FIP. However, neither the redesigned (PM_A.2), nor the complete exclusion of the LB primer prevented the rising baseline from occurring (**Supplementary Figure 4C**). Therefore, the PM_A assay was taken forward as the optimally performing PM assay. To assess the analytical SEN of the PM_A assay, 28 isolates of bacterial species that are commonly found pathogenically and non-pathogenically to inhabit the same niche as PM were tested in LAMP reactions. PM_A did not produce any detectable amplification with any of these 28 bacterial isolates.

2.18.4. *S. suis*

Two published LAMP assays were assessed for their ability to detect the reference strain (a serotype 2 clinical isolate) of SS, P1/7. The first assay, denoted SS_A, targeted a 219 bp region of the 1,662 bp *recN* gene (Arai et al., 2015), and the second, SS_B, targeted a 204 bp region of the 1,347 bp *gdhA* gene (Intorasoot et al., 2016). The *recN* gene was introduced in **Section 2.15.4**, whilst the *gdhA* gene encodes a glutamate dehydrogenase protein and is conserved among SS serotype 2 strains (Okwumabua et al., 2001). A hundred SS sequences were obtained by BLASTn of the relative P1/7 gene used in the LAMP assays and aligned in Geneious Prime. When the SS LAMP primers were mapped to the sequences, SS_A showed 98.6% pairwise identity, whilst SS_B displayed 95.7%. SS_A had 208 (95.0%) identical sites within the target region across the 100 strains, and SS_B only had 117 (85.1%). The primers published in the SS_A assay did not contain loop primers, therefore, loop primers for the SS_A assay were designed in this Chapter. Previously, an increase in SEN and speed was achieved with the inclusion of loop primers. Thus, the SS_A assay with the addition of loop primers was tested and referred to as SS_A. A serial dilution of gDNA obtained from the reference strain, P1/7, showed that the SS_A assay was not

only 15x more sensitive than SS_B, detecting down to 8 copies/ μ L, but also resulted in a 45% decrease in TTP with all amplification with SS_A taking place within 30 minutes (**Figure 2.17**).

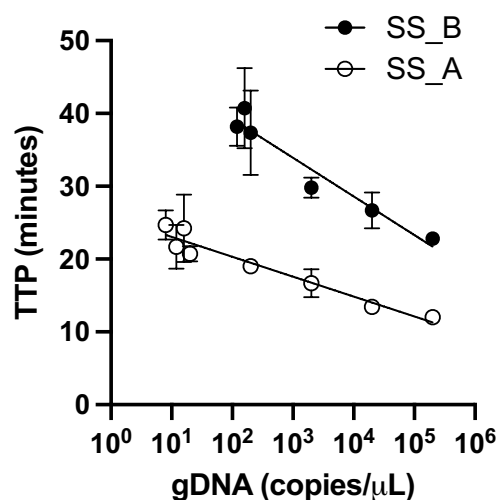


Figure 2.17. Analytical sensitivity of SS-LAMP primers. The LoD achieved against triplicate of gDNA from the laboratory reference strain of SS, P1/7 using the published primer set with designed loop primers (SS_A) and the primer set designed in this Chapter (SS_B). Data points represent the geometrical mean, and error bars the S.D.

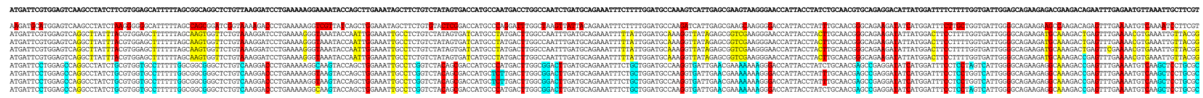
Additionally, the SS_A assay did not display any off-target amplification when tested with 38 isolates related to or known to occupy the same niche as SS.

2.18.4.1. Divergent *S. suis*

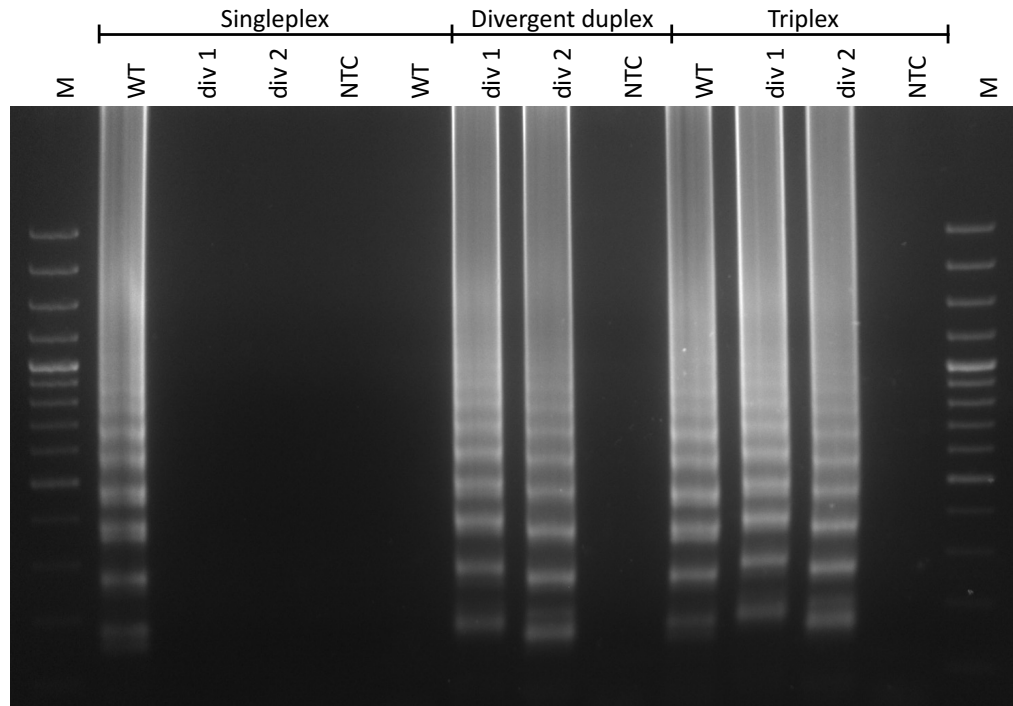
Whilst the SS_A LAMP primers detected the laboratory reference strain P1/7 with high SEN, a divergent clade of SS has been identified from whole genome analysis which has the ability to cause clinical disease (Baig et al., 2015). This divergent clade was not detected by the SS_A primers, not unsurprising as the divergent clade shows considerable genomic variation compared to the classical SS isolates (Baig et al., 2015). Therefore, novel LAMP primers were designed targeting the SS_90588 gene identified by Dr Janine Bossé as one of four genes which are conserved between SS isolates and the divergent clade (personal correspondence), as part of a previous BBSRC LoLa-funded project in the Langford laboratory. A subsequent BLASTn search identified a gene annotated on the P1/7 genome, CAR45278.1, a conserved 305 AA hypothetical protein. A further BLASTp search determined the protein was 100% identical to the DNA winding protein, WhiA (GenBank accession: WP_002937252.1). A sequence alignment of the SS_90588 gene indicated that the divergent clade consisted of two distinct sequence variants, referred to as divergent 1 and divergent 2 (**Figure 2.18A**). In order to detect SS divergent clade isolates, 10 LAMP assays were designed, five for each of the divergent sequence variants (div1A-E and div2A-E). To assess the performance of the divergent primer sets, each were screened against synthetic templates of their respective variant target sequence. The best-performing divergent primer sets were determined to be div1A, div1B, div2A and div2C (data not shown). The detection of the divergent clade requires both divergent 1 and divergent 2 sequence variants to be detected simultaneously. Therefore, the divergent primer pairs were combined into a duplex reaction and screened against both divergent sequences. The duplex

containing divergent primers div1A and div2C (div1A2C) produced the most rapid amplification (**Supplementary Figure 5**). Subsequently, the div1A2C primer sets were assessed in duplex and triplex with the original SS LAMP primers (SS_A) both in real-time and via gel-electrophoresis (**Figure 2.18B** and **Figure 2.18C**, respectively) to demonstrate the lack of cross-reactivity with the inclusion of both primers in a single reaction. Therefore, with the successful implementation of the duplex and triplex assay, I have achieved the first LAMP detection of the divergent clade of SS.

A)



B)



C)

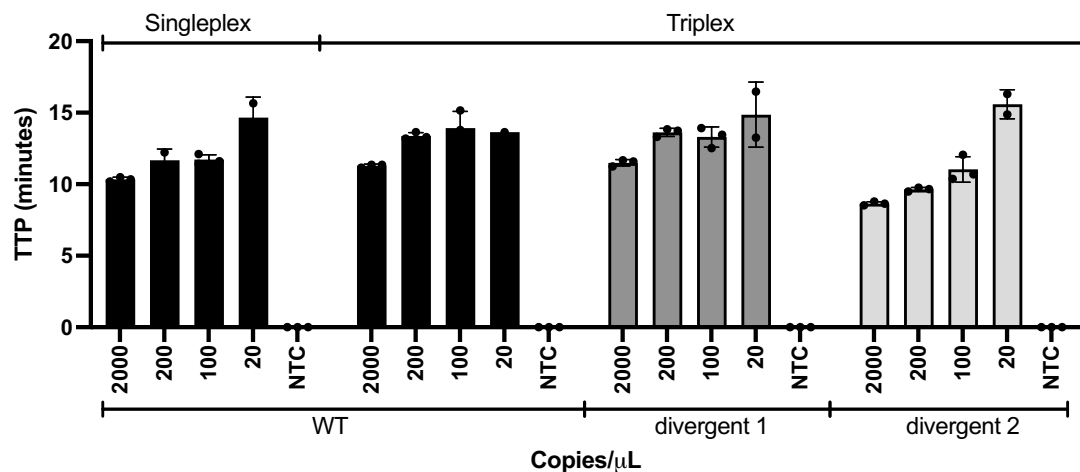


Figure 2.18. Divergent SS-LAMP amplification. The amplification of divergent SS isolates, split into two clades, divergent 1 and divergent 2 based on their respective sequences, with bases differing from the SS consensus sequence highlighted and bases conserved between the divergent 1 and divergent 2 shown in yellow and blue, respectively (A). The LAMP amplification of the SS_A+ single plex assay, the divergent LAMP assays in a duplex and a combination of the three assays in a triplex against each template type, the banding pattern when the reactions were run in gel electrophoresis (B) and the TTP produced in real-time (C). The amplification of gDNA from the WT strep suis (isolate P1/7) with the SS_A+ LAMP primer set, and the triplex SS assay with synthetic DNA representative of sequences found in the divergent 1 clade and divergent 2 clade.

2.18.5. *G. parasuis*

Two LAMP assays have been published for the detection of GPS, both targeting the *infB* gene, denoted GPS_A and GPS_B (Chen et al., 2010, Zhang et al., 2012), respectively. The *infB* gene consists of 2,541 nucleotides and encodes the translation initiation factor IF-2, a housekeeping gene. When the published primers were tested with the GPS laboratory reference strain, SVR5, no amplification occurred (data not shown). GPS sequences were obtained from a BLASTn search of the *infB* gene and aligned in Geneious Prime (n=36), which identified that the GPS_A primers amplified a 169 bp region displaying 98.7% pairwise identity and 157 (92.9%) identical sites. The GPS_B primers target a 244 bp region of the *infB* gene with 98.3% pairwise identity and 226 (92.2%) identical sites. Therefore, the reason why the primers did not amplify the SRV5 strain is unclear. Subsequently, seven LAMP primer sets were designed targeting the *infB* gene, denoted GPS_C-I. An initial primer screen yielded amplification of GPS_C, GPS_E and GPS_F only. The analytical SEN of these primer sets was determined using a serial dilution of SRV5 isolate gDNA. GPS_C achieved a LoD of 2,000 copies/μL, whilst both GPS_E and GPS_F were 100x more sensitive and detected down to 20 copies/μL (**Figure 2.19A**). As GPS_F displayed the most rapid amplification, all occurring in less than 10 minutes, this assay was taken forward into further experiments. As the GPS_F assay was newly designed for this Chapter, a 10-replicate LoD was performed using four different titrations of the GPS laboratory reference strain, SVR5, gDNA. GPS_F achieved a LoD of 20 copies/μL, with only 7 of the 10 replicates amplifying in the presence of 10 copies/μL of SRV5 (**Figure 2.19B**).

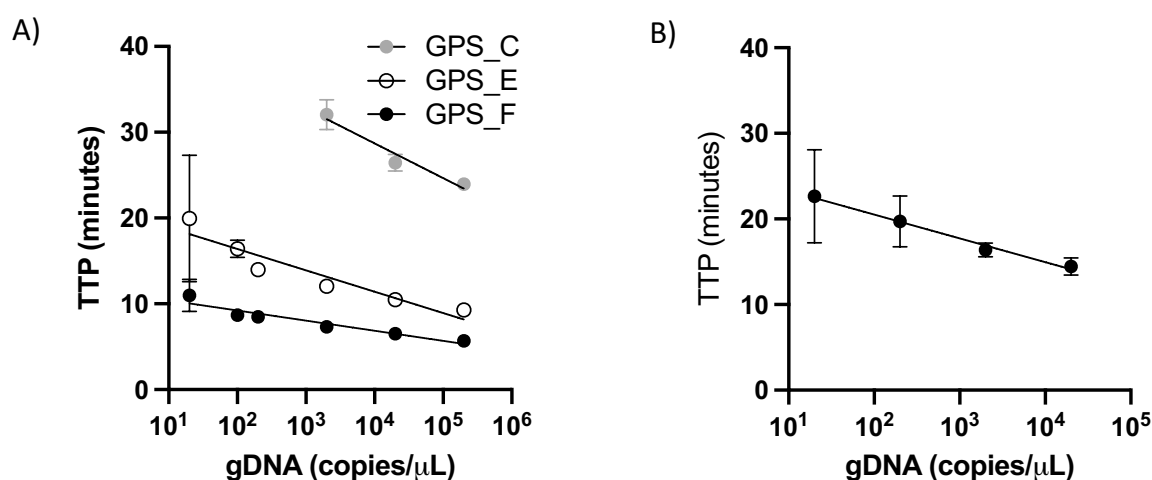


Figure 2.19. Analytical sensitivity of GPS-LAMP assays. The LoD achieved against three-replicates of GPS reference isolate SRV5 gDNA, using the published primer set (SS_A) and the primer set designed in this Chapter (SS_B) (**A**). The LoD achieved with the GPS_F primer set in 10-replicates of SRV5 gDNA (**B**). Data points represent the geometrical mean, and error bars the S.D.

In order to determine the analytical SPE of the GPS_F LAMP assay, the primer sets were tested with 10 ng of gDNA extracted from 38 isolates which occupy the same niche as GPS. Of the 38 isolates, both replicates of nine isolates displayed amplification with the GPS_F primer set, and a single replicate amplified in 18 samples. All FP amplification events occurred after 30 minutes of incubation (**Supplementary Figure 6**). Therefore, whilst GPS_F resulted in the most rapid and sensitive amplification; care must be taken when using this primer set to ensure no false amplification, i.e., not incubating the reaction longer than 30 minutes.

Due to time constraints, this Thesis focuses on the bacterial pathogens involved in the PRDC. However, primer sets were also identified for use against the three major viral pathogens of the PRDC: PCV2, SIV and BVS.

2.18.6. Porcine circovirus 2

The PCV2 genome is very small (1,769 bp) containing overlapping ORFs, therefore, for this Section I will not discuss the amplified region in terms of target genes. Two published assays for PCV2 were selected for testing, upon ordering a synthetic template for the target regions of these assays, no amplification was detected with the published primer sets, nor when the primer sets were used to amplify DNA from a Chinese clinical isolate (data not shown). Upon inspection, the Chinese isolate displayed a poor purity (an A260/280 reading of 2.4 and a A260/A230 reading of 0.28) by Nanodrop, indicating the presence of high amounts of background protein in the sample. The reference genome of PCV2 (NC_005148) underwent a BLASTn search to return 100 genomes of PCV2. The genomes were aligned in Geneious 2.0; several SNPs were found to be present, with the PCV2_A primer set containing three and the PCV2_B primer set containing nine primer-template mismatches compared to the consensus sequence. Overall, the PCV_A primers targeted a 387 bp region, of which a BLASTn search returned 345 (89.1%) identical sites and 99.4% pairwise identity, whilst the PCV_B published assay amplifies a 219 bp region with 149 identical sites (68%) and 97.2% pairwise identity. The high prevalence of SNPs within the target regions of the published LAMP PCV2 assays may explain the lack of amplification using the Chinese clinical isolate. Therefore, three LAMP primer sets, denoted PCV2_C, PCV2_D and PCV2_E were designed against the consensus sequence of the PCV2 genome. An additional primer set was designed as the NUPACK interaction results suggested that there would be primer-interaction in the PCV2_D assay, such that a PCV2_D.2 assay was designed that was devoid of the *in silico* primer-interactions. A primer screen was conducted using the Chinese clinical isolate with all four assays amplifying at the highest concentration tested, the equivalent of 20,000,000 copies/ μ L (**Figure 2.20A**). A further LoD was performed with a synthetic construct of the PCV2 target region for the assays designed in this Chapter. The synthetic construct of PCV2 underwent an asymmetrical PCR to create a ssDNA template, as PCV2 is a ssDNA virus (Meehan et al., 1998). The analytical SEN of PCV2 determined that all assays, apart from PCV2_D.2, detected down to 200 copies/ μ L, whilst the PCV2_D.2 primer set detected down to 2,000 copies/ μ L (**Figure 2.20B**). Further dilutions of the template rendered no increased SEN for any of the PCV2 LAMP primer sets (data not shown). Therefore, the PCV2_D primer set resulted in the most rapid and sensitive amplification of both the Chinese clinical isolate of PCV2 and the ssDNA synthetic template.

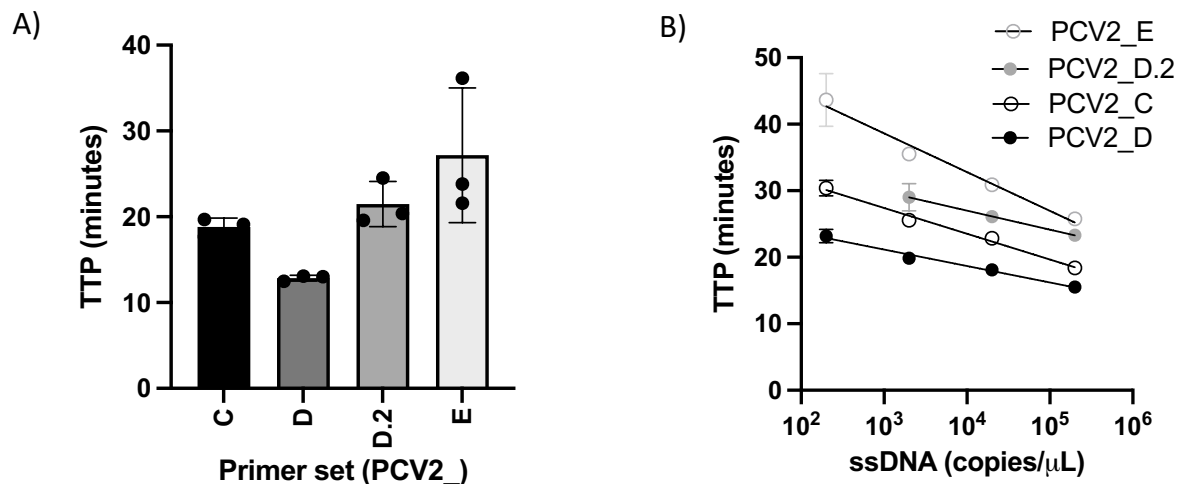


Figure 2.20. Analytical sensitivity of PCV2-LAMP assays. Primer screen of PCV2-LAMP primer against the 20,000,000 copies/μL of PCV2 Chinese isolate (A). LoD experiment performed using 10-fold serial dilutions performed in triplicate using synthetic ssDNA (B). Columns (A) and data points (B) represent the geometrical mean and error bars the S.D.

2.18.7. Swine influenza virus

Numerous assays have been described for the detection of SIV. A consultation with Dr Susannah Williamson (Lead of the APHA UK Pig Expert and Pig Disease Surveillance Groups) steered the focus of the design of the diagnostic assays towards the detection of all SIV serotypes, specifically H1N1 (including the pmd09 variant) and H1N2 subtypes, whilst being able to differentiate the H3N2 subtype. Therefore, the published assays I chose to investigate included a LAMP assay which is H3-specific (SIV_A), targeting a 246 bp region of the HA gene (Gu et al., 2010). An alignment was made of the 1,701 bp HA gene from the H3N2 reference strain (GenBank accession: NC_07366), using 100 isolates obtained from a BLASTn search of influenza A isolates. The alignment indicated that the SIV_A target region displays 99.7% pairwise identity with 173 identical sites (80.8%). The SIV_A assay was also determined to be highly sensitive and rapid, detecting down to 100 copies/μL of H3N2 synthetic RNA within 10 minutes of incubation (**Figure 2.21A**). Additionally, the SIV_A primers were highly specific, displaying no detectable amplification in the presence of the Influenza A H1N1 synthetic template (data not shown).

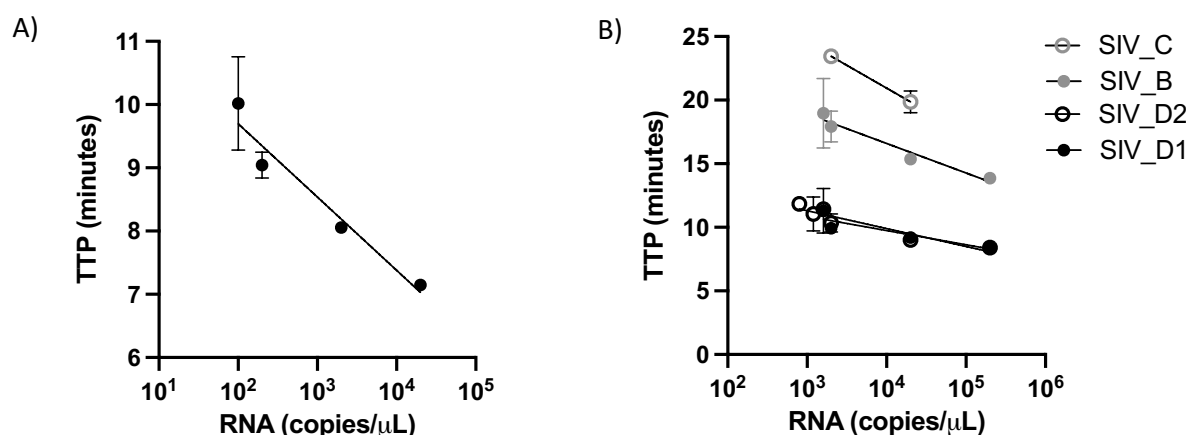


Figure 2.21. Analytical sensitivity of the SIV-LAMP assays. The LoD achieved with the SIV_A LAMP primer sets detecting the HA section of the H3N2 SIV subtype, performed in triplicate with 10-fold serial dilutions of the H3N2 synthetic RNA (A). The LoD achieved with the SIV LAMP primers designed to detect all SIV subtypes by targeting the matrix protein, performed using serial dilutions of transcribed RNA in triplicate (B). Data points represent the geometrical mean, and error bars the S.D.

The detection of all influenza A subtypes (H1-16; N1-9), including all species origins and global distributions, is troublesome due to the high mutation rate prevalent in the influenza A genome (Nobusawa and Sato, 2006). However, regions of the seventh segment of the influenza A genome, which encodes the matrix protein via the (*M*) gene, are well conserved across all subtypes of Influenza A, across viral origins, albeit human, swine or avian (APHA, 2020a). Therefore, three assays were evaluated for the detection SIV via targeting regions of the *M* gene. The three LAMP SIV primer sets were denoted SIV_B, C and D, with SIV_B being designed in this study, whilst SIV_C and SIV_D were previously published (Jung et al., 2015, Nakauchi et al., 2011). A hundred sequences were returned from a BLASTn search of the *M* gene for influenza A virus and were aligned in Geneious prime. It is noteworthy that the resulting consensus sequence returns zero significant matches when a BLASTn search is performed against SIV (Taxid id:12845). However, the region of the *M* gene targeted by all LAMP assays evaluated here has previously been used in qPCR for the detection of all influenza A viruses, regardless of their animal origin (APHA, 2020a). The alignment of the 100 isolates determined that SIV_B targeted a 230 bp region of the *M* gene, with 221 (96.1%) identical sites and 99.9% pairwise identity. The pairwise identity remained the same across the 234 bp region targeted by SIV_C and 219 bp region targeted SIV_D primer sets. However, the identical sites were 226 (96.6%) and 190 (96.9%) for SIV_C and SIV_D, respectively. The published SIV_D primer set contains two sets of outer primers, F3 and B3, each containing two common SNPs to increase sequence coverage. In this Chapter, as I tested a single consensus sequence of SIV RNA, I separated the SIV_D primer sets by their outer primer sets and denoted them as SIV_D1 and SIV_D2.

The SEN of the SIV LAMP primers directed against the *M* gene was determined using synthetically transcribed RNA of the consensus sequence. The SIV_D1 and SIV_D2 primer sets performed very similarly in their reaction time; however, the SIV_D2 primer set was determined to be twice as sensitive as SIV_D1, detecting down to 800 copies/μL, as opposed to the 1,600 copies/μL detected by SIV_D1 (Figure 2.21B). The SIV_B primer set achieved

the same SEN as SIV_D1 (1,600 copies/ μ L), with amplification taking twice as long (**Figure 2.21B**). Meanwhile, the SIV_C primer set proved the least sensitive and the least efficient at amplification, with a detection limit of 2,000 copies/ μ L taking over 20 minutes to reach detectable levels of amplification (**Figure 2.21B**). Therefore, the SIV_A LAMP primer set should be used for the specific detection of the H3N2 subtype of SIV, and the SIV_D2 primer set should be taken forward for the detection of all SIV subtypes. The implementation of both these assays into the diagnostic device will provide sensitive and rapid detection of SIV.

2.18.8. Betaarterivirus suid

After consultation with Dr Susannah Williamson, the detection of both subtypes of BVS, the European type BVS-1 and the North American type BVS-2, both responsible for PRRS, we deemed important for epidemiological awareness that the subtypes remain geographically distinct. A total of seven assays were tested, three for the detection of BVS-1 (BVS_A, BVS_B and BVS_C) and five for the detection of BVS-2 (BVS_D, BVS_E, BVS_F, BVS_G and BVS_H). Of the three LAMP assays for the detection of BVS-1, one was previously published, denoted BVS_A (Rovira et al., 2009). Additionally, two LAMP assays were designed in this work, BVS_B and BVS_C, targeting a region of the nucleocapsid *nsp1* gene. Transcribed RNA was produced covering the target regions of the BVS-1 target regions. The SEN of each of the BVS-1 LAMP assays was determined with the transcribed RNA, with BVS_A and BVS_C producing the most sensitive amplification detecting down to 200 copies/ μ L, whilst BVS_B detected down to 2,000 copies/ μ L (**Figure 2.22A**). BVS_A not only resulted in the sensitive detection of BVS-1, but also produced the most rapid amplification, with the majority of amplification events detectable in less than 30 minutes of incubation (**Figure 2.22A**).

For BVS-2 detection, four LAMP assays were compared, three of which were previously published with BVS_D targeting the matrix protein gene, *M* gene (Rovira et al., 2009), BVS_E targeting the *nsp2* gene (Zhang et al., 2011) and BVS_F also targeted a region of the *M* gene (Qin et al., 2009). Two further LAMP assays, BVS_G and BVS_H, were designed in this work targeting the *nsp2* gene encoding BVS-2 nucleocapsid protein. Once again, transcribed RNA was produced on a single transcript to directly compare the SEN of the LAMP assays. BVS_H achieved the highest SEN and the lowest TTP detecting down to 20 copies/ μ L in under 40 minutes, compared to BVS_D which detected 16,000 copies/ μ L, BVS_E which detected 2,000 copies/ μ L, BVS_F detected only the highest copy number tested, 200,000 copies/ μ L and BVS_G which detected 20,000 copies/ μ L (**Figure 2.22B**).

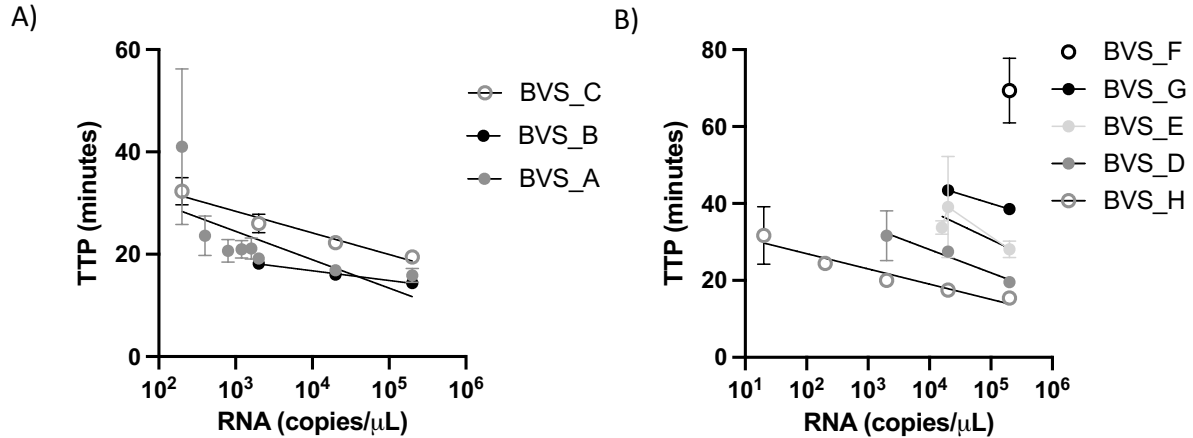


Figure 2.22. Analytical sensitivity of BVS-LAMP assays. The LoD achieved with BVS LAMP primers targeting BVS-1, using serial dilutions of BVS-1 transcribed RNA (A). The LoD achieved with BVS LAMP primers targeting BVS-2, using serial dilutions of BVS-2 transcribed RNA (B). Data points represent the geometrical mean of triplicate data, and error bars the S.D.

The optimal primer sets for the detection of BVS, BVS_A for BVS-1 and BVS_H for BVS-2 were mapped to the consensus sequence obtained by BLASTn search of the whole BVS-1 and 2 genomes. The resultant alignment of BVS-1 genomes contained 65 sequences, with BVS_A targeting a 215 bp region spanning the *M* gene encoding the matrix protein and the *nsp1* gene encoding the nucleocapsid protein of BVS-1. Within the target region of the BVS-1 genome, the BVS_A primer region displayed 96.8% pairwise identity across 159 identical sites (74.0%). An alignment of the BVS-2 genome returned 240 sequences, with BVS_H targeting a 198 bp region of the *nsp2* gene, displaying 96.1% pairwise identity across 141 (71.2%) identical sites.

Therefore, semi-rapid, taking less than 40 minutes, LAMP assays have been identified for the identification and differentiation of the viruses responsible for the PRRS.

2.18.9. Internal control

Often for molecular diagnostic tests to be valid, a set of controls are run which confirms the performance of the assay meets the stipulated criteria of the supplier. For a rapid diagnostic test, the most appropriate control would be an extraction control. Therefore, five assays were designed as internal control (IC) for each of the three housekeeping genes of the *Sus scrofa domesticus* genome, the 12S rRNA (12s), mitochondrial DNA D-loop region (*mtD*) and cytochrome B (*cytB*) genes. The most sensitive primer sets for each gene were determined via a primer screen (Supplementary Figure 7). Subsequently, loop primers were designed for the most sensitive primer set for each gene. A LoD experiment was performed with and without the loop primers, differentiated from the original primer sets by the notation '+' (Figure 2.23A and Figure 2.23B, respectively). The inclusion of loop primers decreased the TTP and increased the SEN for all three assays. All primer sets with loop primers achieved a LoD of 50 copies/μL, with the most rapid primer determined to be *cytB*_D+, producing detectable amplification at the lowest copy within 10 minutes (Figure 2.23B).

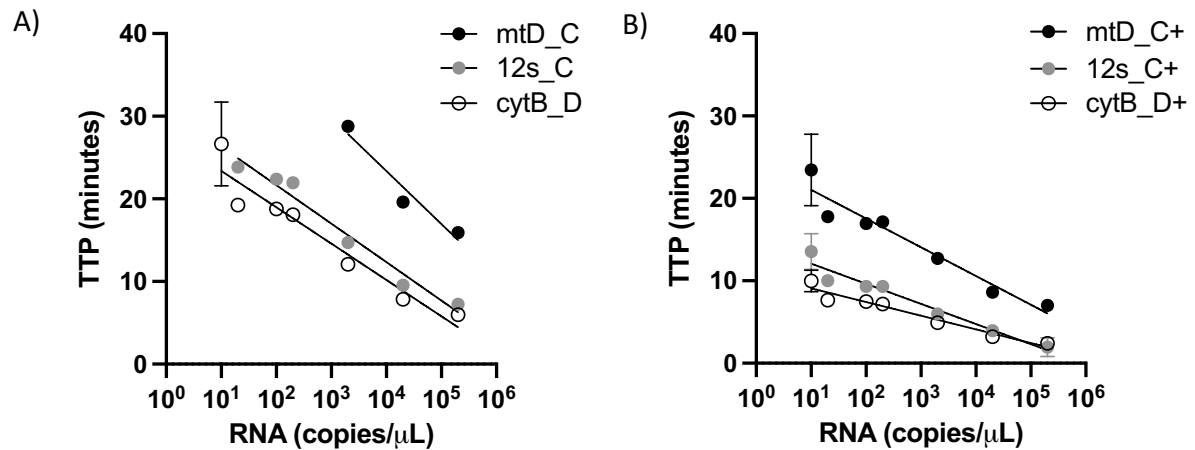


Figure 2.23. Analytical sensitivity of internal control LAMP primers. The LoD achieved using the original IC LAMP primers (A) and the IC primers, with the addition of loop primers (B). All experiments were performed in triplicate using serial dilutions of synthetic DNA produced of target regions of genes of interest.

2.18.10. pH-LAMP

The final diagnostic device will utilise the CMOS-ISFET detection system, detecting the increase in protons liberated into the reaction during amplification. The use of the CMOS-ISFET pH-based method of detecting LAMP amplification has previously been validated within the Pantelis lab group (Malpartida-Cardenas et al., 2019, Moser et al., 2018, Rodriguez-Manzano et al., 2021, Rodriguez-Manzano et al., 2020). Therefore, the LAMP reaction did not require optimisation like the other isothermal methods described in this Chapter. The previously validated pH-LAMP detection method replaces the 10x isothermal buffer supplied with the *Bst* polymerase used in LAMP with a buffer devoid of Tris, and the pH of this custom buffer was increased to a pH of 8.8-9.0 with the addition of NaOH. A LoD experiment was performed with the optimal LAMP primer sets for each of the bacterial PRDC pathogens, comparing the real-time LoD with the decrease in pH measured prior and post-incubation. The results indicated that a pH change of 0.5-1 was detected in all instances where detectable amplification was observed in real-time (Figure 2.24).

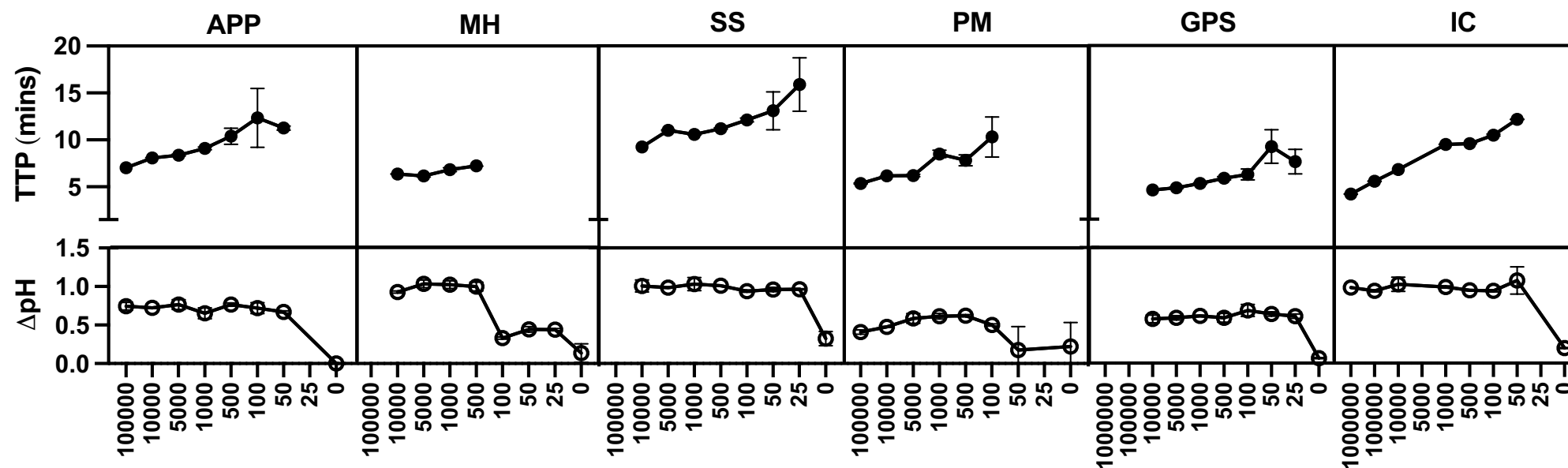


Figure 2.24. pH detection of LAMP amplification. The correlation between real-time LAMP detection (top) and the pH change (Δ pH) determined by the pH pre- and post-incubation of the LAMP reaction (bottom) across multiple LAMP assays for the detection of the main five bacterial PRDC pathogens and the IC. Experiments were performed in duplicate with serial dilutions of gDNA for the bacterial targets and synthetic DNA for the IC. Data points represent the mean for each DNA concentration, and error bars the S.D.

Therefore, there is a direct correlation between the real-time results obtained and the ability to detect a pH change over the course of the LAMP reaction. Additionally, all of the PRDC pathogen primer sets displaying the fastest and most sensitive amplification in this Chapter resulted in a robust pH change and are thus good candidates for further validation and eventual implementation on the CMOS-ISFET device.

A key observation is that LAMP utilises a high concentration of primers compared to other isothermal techniques. LAMP uses a total primer concentration of 6.5 μM , as opposed to 1.32 μM used in RPA and 0.3 μM used in HDA. This may account for why such a robust pH change is observed during LAMP amplification, with a greater concentration of oligonucleotides available to become an amplified product and, thus a greater number of protons produced during amplification. In order to support this hypothesis, the amount of product formed after incubation with each of these isothermal techniques was determined via a fluorometer. The results confirmed that there is vastly more product formed during LAMP amplification ($>400 \text{ ng}/\mu\text{L}$), compared to that formed in an RPA ($-3.55 \text{ ng}/\mu\text{L}$) or HDA reactions ($17.78 \text{ ng}/\mu\text{L}$) (**Figure 2.25**). The negative quantification result achieved in RPA may support the high primer/dimer formation of this technique, as the quantification shown here is the difference between the end-point quantification of the negative and positive. Therefore, the RPA reaction created more double-stranded products in the absence of target DNA than when target DNA was present.

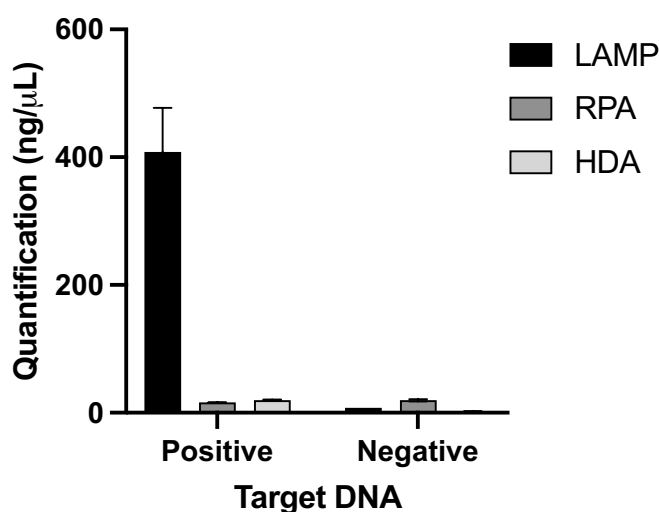


Figure 2.25. Quantification of amplification produced by different isothermal technologies. The quantity of product produced during amplification via LAMP, RPA and HDA with target DNA was present (positive) or absent (negative), as determined by Qubit dsDNA quantification. Columns depict the geometrical average of three replicates, and error bars the S.D.

2.19. Result Summary

Sensitive assays have been identified for the detection of all five bacterial pathogens belonging to the PRDC using LAMP, four with RPA, two with HDA, and one with PSR. The results of the analytical SEN with each of these detection methods are shown in **Table 2.2**.

Table 2.2. The analytical sensitivity achieved across each amplification technique.

Target species	LoD achieved (copies/ μ L)			
	LAMP	RPA	HDA	PSR
APP	80	10	-	40,000
MH	20	20	2	-
PM	8	80	100	-
SS	8	10	-	-
GPS	20	-	-	-

Amplification using LAMP was the only isothermal technique evaluated in this Chapter that is capable of producing a robust pH change and therefore will be compatible with the CMOS-ISFET detection system.

Discussion

In this Chapter I sought to determine the most appropriate isothermal technique for use in our PoC device. Four parameters were investigated, the analytical SEN using LoD experiments, the rapidity the amplification could be detected within (TTP), the analytical SPE using both in silico analysis to ensure inclusivity against a range of isolates and experimentally through a panel of 41 isolates belonging to nine relevant species, and the compatibility of the isothermal techniques with pH-based detection methods.

The analytical SEN of an assay is defined as the amount of an analyte that can consistently be detected and is often reported by the LoD, with a detection certainty of $\geq 95\%$. In this Chapter, I focused on the detection of the PRDC bacterial pathogens using gDNA. Across the four different isothermal amplification techniques, RPA and LAMP achieved the most sensitive amplification, with both techniques achieving identical SEN in the detection of MH, and LAMP being 8x less sensitive than RPA in the detection of APP. However, LAMP displayed 10x increased SEN in the detection of PM and 0.8x more sensitive than RPA in the detection of SS. In comparison, HDA displayed the highest SEN in the detection of MH, being 10x more sensitive than any other amplification technique. Although HDA was the least sensitive when detecting PM, being 12.5x less sensitive than LAMP and 0.8x less sensitive than RPA. Although only one PSR assay produced detectable amplification, the analytical SEN was very poor, only reliably detecting 40,000 copies/ μL , 500x less sensitive than LAMP and 4,000x less sensitive than RPA.

Several factors can influence the reported analytical SEN of an assay, such as the primer set sequence, the template sequence, the template form (i.e., gDNA, plasmid, synthetic), the amplification technique, including the specific reaction components, the reaction volume, and the amplification detection method. Therefore, to ascertain the most sensitive amplification technique, I compared several different techniques, including previously published primer sets and primers designed and screened in this Chapter. Among the published primer sets, the majority had been described for use in LAMP amplification. The most sensitive LAMP assay for the detection of APP was APP_A+, which is the published assay with the addition of loop primers designed in this Chapter. The SEN of the original primer set (without loops) achieved in this study was 2,000 copies/ μL , whilst the original publication reports a 0.2 copy/ μL detection limit (Ji et al., 2012). The other published assay for the detection of APP (APP_B in this Chapter) reported a LoD of 0.32 colony forming units (CFU)/ μL of the serovar 1 type strain (Yang et al., 2009), whilst in this Chapter, I achieved a LoD of 80 copies/ μL with the serovar 8 reference strain. I did not generate CFU equivalent data of the APP serovar 8 strain to be able to translate the CFU to copies, since an advantage of reporting the detection limit as copies is that it is easily normalised across strains, whilst the CFU value will be dependent on several factors, such as the extraction technique performed, the bacterial cultural conditions deployed, and specific strain grown. Several of the published assays reported their SEN as CFU/mL (Sun et al., 2010, Arai et al., 2015, Intorasoot et al., 2016) which requires the reader to assess how much template was added to the reaction as well as the final reaction volume used to determine the CFU/ μL reaction. The reporting of the input quantity of template rather than the final concentration of analyte in the reaction is a common occurrence in diagnostics, especially when reporting clinical SEN where the metric is

usually per mL of biomaterial e.g., blood. However, as input volumes and final reaction volumes can have a large bearing on the reported SEN, it is much more realistic, in this case at least, to report the SEN of the assay in copies/ μ L of reaction.

An additional issue with comparing sensitivities of published assays is the format of the target material. For example, the published MH assay (MH_B) reported a LoD of 10 copies/ μ L, using plasmid DNA, whilst our achieved LoD with the original MH_B primer set was 800 copies/ μ L using gDNA. The use of plasmid DNA is known to be unfavourable for use in testing the SEN of molecular diagnostics due to the ability of the plasmid to supercoil and prevent amplification events (Chen et al., 2007). Therefore, it is recommended that plasmid DNA is first linearised, however, the work by Liu et al. (2011) either failed to perform or describe the linearisation of the plasmid DNA.

Comparatively to LAMP, far fewer RPA assays have been described for the detection of the PRDC pathogens. Therefore, RPA assays were designed in this Chapter for comparison against the performance of other isothermal techniques as well as the identification of the optimal primer set. All the RPA assays designed in this Chapter performed better than the primer sets previously published. Mirroring the results in LAMP, I saw discrepancies between the reported SEN and those achieved in this Chapter. For instance, the published assay targeting APP (APP_1) achieved a SEN of 200 copies/ μ L 10x lower than the SEN reported in the published assay of 536 fg (Li et al., 2019). Whilst reporting the limit as the quantity of DNA in grams provides an easy comparator than when the CFU is reported, genome lengths between isolates still differ and in the original publication of APP_1, the reference isolate used for the SEN experiment is not listed. This exemplifies the difficulties faced when reporting assay SEN in terms of grams. The published RPA assay detecting MH (MH_1) also reports the SEN in terms of femtograms. However, Li et al. (2011) stipulate the type strain of MH used, strain 168, and as such, the reported SEN of 500 fg can be converted to copies as this strain 168 has been genetically characterised (Liu et al., 2011), equating to 20 copies/ μ L of the RPA reaction. However, the reported SEN is 8x higher than the 160 copies/ μ L achieved in this Chapter.

The RPA assay which had the highest reported SEN was PM_1 detecting 2.4 copies/ μ L of PM gDNA, far higher than the 160 copies/ μ L achieved in this Chapter. A surprising result as the published assay was based on LFT detection, which utilises an Nfo probe rather than the Exo probe used in this Chapter, with Exo-based systems reported previously as displaying a higher SEN (Chao et al., 2015).

The least sensitive published assay in this Chapter was the APP_PSR assay, utilising PSR to target APP. I have reported that high quantities of our reference serovar 8 template were required to produce detectable amplification. In the original description of this assay, the LoD for PSR was 5.6 pg/ μ L which equates to 2,184 copies/ μ L of the 2,375,000 bp genome of the serovar 6 reference strain used, ATCC 33590 (Sarkar et al., 2022). Therefore, even in the original publication of this assay, the SEN is much lower than the alternative isothermal chemistries I have explored.

The ability of a PoC test to deliver a result in a timely manner is largely dependent on the biochemistry utilised in the device. Therefore, it is important to consider the rapidity of each of the isothermal chemistries for use in the final PoC device. Upon analysis, RPA resulted in the most rapid amplification, with the TTP for the four best-performing assays taking 10-minutes or less, which is much faster than the recommended incubation time of 20 minutes by the manufacturer (Twist Dx Ltd., 2021). The LAMP assays produced the next most rapid amplification with the TTP for the best-performing bacterial assays taking 30 minutes or less, and the LAMP manufacturer protocol specifies a 30-60 minute incubation (NEB, 2014). The HDA reactions took the most variable amount of time to amplify, with the detection of PM amplifying within 20 minutes, and the detection of MH taking twice as long, although amplification occurred in 40 minutes or less - well within the of 120 minute incubation time recommended by the protocol (NEB, 2022). The large variation between the TTP seen in the HDA assays may be due to the variation in SEN between these assays, with the HDA-PM assay 50x less sensitive than the HDA-MH assay. The PSR assay required the longest incubation time, with amplification only detected after 65 minutes, far greater than the original publication of this primer set which claimed that an incubation of 30 minutes was sufficient (Sarkar et al., 2022). The original PSR protocol and subsequently published assays achieved amplification within 50 minutes (Ji et al., 2019, Liu et al., 2015b, Jangra et al., 2022).

Ascertaining the SPE of an assay is important to guarantee no FP results. FP results within a farm setting could result in very costly unnecessary eradication programmes being initiated. When testing in low prevalence settings, SPE is a hugely important parameter - as the incidence of FP results increases as more true negative (TN) tests are performed (Healy, 2020). A famous example is the original implementation of the CDC 2019 CoV-2 diagnostic kit that showed contamination in one of the three assays used to detect SARS-CoV-2, as well as self-complementation of the primer and probe in one of the other assays (Lee et al., 2021) resulting in a FP rate of 33%, when the test was first implemented (Temple-Raston, 2020).

In this Chapter, amplification with RPA displayed the highest SPE, with no cross-reaction observed when using the panel of bacterial isolates from other species. The majority of the LAMP assays also showed no cross-reactivity. However, the assay for the detection of GPS (GPS_F) did display some cross-reaction occurring if the reaction was incubated longer than 30 minutes. The generation of FP amplification is a well-characterised artefact of LAMP reactions due to the high concentrations of 6-8 sequences increasing the likelihood of primer interactions (Suleman et al., 2016, Rolando et al., 2019). Several strategies have been implemented to limit the incidence of FP amplification in LAMP. This typically includes the incorporation of fluorescence detection systems such as CRISPR-Cas (Trung et al., 2022), fluorescently labelled loop primer with a complementary short oligo that contains a quencher (Ball et al., 2016), or fluorescence resonance energy transfer (FRET) labelling approach on two primers known to only be in close proximity in specific amplicon structures (Hardinge and Murray, 2019). However, these interventions are not suitable for pH detection methods. More appropriate techniques involve modifications to the primer sequences, such as self-avoiding molecular recognition systems (SAMRs), originally used in PCR to prevent primer dimerization, and subsequently implemented in LAMP (Wang et al., 2017b). SAMRs are functional analogues of nucleotide bases that cannot form hydrogen bonds with

themselves, only the natural bases of the template (Hoshika et al., 2010). However, the use of SAMRs was not implemented in this work, as this Chapter demonstrated LAMP primers can be designed that are devoid of FP amplification. Therefore, potentiating the importance of careful consideration during LAMP primer design to avoid sequences prone to hairpin formation, reducing reaction noise and increasing reaction speed and SEN (Meagher et al., 2018).

A major concern is that both HDA assays designed in this Chapter displayed FP results. In the case of the HDA MH assay, two isolates belonging to *A. equuli* showed amplification. The amplification of *A. equuli*, although unusual, should not be of concern as *A. equuli* is an equine pathogen which has only been rarely found to opportunistically cause disease in pigs (Ramos-Vara et al., 2008, Thompson et al., 2010). The HDA MH assay was the most sensitive isothermal assay tested in this Chapter, detecting down to 2 copies/ μ L, which creates the possibility that low-level contamination within the *A. equuli* isolates being tested. However, the HDA assay detecting PM had the lowest SEN of all the assays (100 copies/ μ L), and cross-reaction was more prevalent than it was in MH, with nine of the 20 isolates producing amplification in both replicates, suggesting that the FP amplification is an intrinsic issue with the primer design. It is noteworthy that all FP events occurred in the presence of non-target DNA, whilst in the LoD experiments, no amplification was seen below the LoD, nor in the negative control replicates. The lack of SPE in HDA reactions has been reported frequently, with several techniques developed to aid in the reduction of off-target amplification events. These include the use of the enzyme, RNaseH, to cleave blocked primers only when they are double-stranded and therefore allowing the primer to participate in amplification events only in the presence of the target DNA (Denys, 2014). As well as increasing HDA SEN by using SAMRs, which increase the SEN by decreasing the primer noise (Yang et al., 2015). The inclusion of different crowding agents to mimic the biological environment had an additive effect, increasing the overall speed and SEN of the HDA reaction (Tong et al., 2011). Similarly, the inclusion of the restriction enzyme, Mbol, increased the rapidness of HDA assays by 6 minutes - proposed to increase local helicase activity through mimicking the repair pathway of UvrD (helicase) (Tong et al., 2011). Furthermore, it has been reported that adjusting concentrations of Mg^{2+} ions and dNTPs can increase the signal compared to the noise in HDA and decrease the TTP by up to 25 minutes (Doseeva et al., 2011, Chen et al., 2016a).

In this Chapter, I also described the successful design of an extraction control to provide the reassurance that the extraction process was performed correctly. Typically, a positive IC would also be included in molecular diagnostic kits to determine the reagents were performing as expected, i.e., no deterioration has occurred during storage of the reaction mixture. However, in a single-use scenario, such as a PoC diagnostic test, the extraction control can be utilised to provide an invalid result in the event of poor extraction and the deterioration of the reagents. Although an extraction control does not enable the user to differentiate which eventuality caused the failure, the result remains the same - the test will need to be repeated, including sample preparation. Several FDA-approved rapid diagnostic tests utilise this single control strategy, including Abbotts ID Now COVID-19, Cue's COVID-19 test, Atila BioSystems Inc's iAMP COVID-19 detection kit (U.S. Food & Drug Administration,

2022). Therefore, I envisage the use of a single extraction control will be sufficient to ensure test validity for the end user.

The focus of this Chapter has been to compare different isothermal techniques in the detection of the main bacterial pathogens involved in the PRDC. I have identified sensitive, rapid, and specific LAMP assays for all PRDC pathogens. Additionally, I have developed ultra-rapid and specific RPA assays for four of the five bacterial PRDC pathogens. The overall aim of this Thesis is to create a diagnostic test for the detection of the PRDC pathogens on a lab-on-chip device which utilises pH-dependent detection via a CMOS-ISFET sensor. Therefore, the amplification chemistry chosen must be compatible with a pH change. The CMOS-ISFET sensor has already been extensively validated for LAMP detection, in previous work (Malpartida-Cardenas et al., 2019, Moser et al., 2018, Rodriguez-Manzano et al., 2021, Rodriguez-Manzano et al., 2020). Here, I build upon this work and confirm that a strong pH change is observed in all PRDC bacterial LAMP assays, as well as the extraction control.

Difficulty was encountered in producing robust and reproducible pH changes in RPA and HDA, with the conclusion that either through the high incidence of primer interactions or low production of the overall amplification product, no pH change could be achieved. However, the electrochemical detection of RPA has been previously demonstrated requiring the oxidation of a substrate rather than the direct detection of bi-products of the amplification, often requiring a post-amplification step to be performed i.e., incubation with a substrate. Indirect electrochemical detection methods described for RPA include the use of ferrocene-labelled dNTPs, requiring glycine-HCl and strontium nitrate for post-amplification electrochemical detection (Ortiz et al., 2022). Another method to use a two-step approach using horseradish peroxidase (HRP) labelled primers and the addition of tetramethylbenzidine (TMB) for 5 minutes to measure endpoint oxidation of the precipitated TMB with electrodes (del Río et al., 2017). Alternatively, real-time electrochemical detection has been achieved using a step-loop probe labelled with a redox moiety which is tethered to the electrode surface. Upon hybridization with the probe-target, the redox moiety is held further away from the electrode surface and therefore, the rate of electron transfer decreases, proportional to the amount of target DNA in the solution. Although the step-loop redox probe was shown to be highly sensitive (10 copies/ μ L), the total time to result was 70-75 minutes, which is extraordinarily long for a PoC solution (Khaliliazar et al., 2020). In a similar methodology, a probe labelled with glucose-oxidase (GoX) was used in the detection of RPA amplification. During amplification, the probe was brought into proximity to the electrode via a primer tethered to the electrode surface, and the electrode surface is oxidized by GoX, the rate of which is directly proportional to the proximity of GoX to the electrode surface (Fang et al., 2018). Recently, a label-free RPA based electrochemical sensor has also been described, detecting the binding of DNA to the surface of the electrode where the negatively charged DNA reduces the electron transfer between $K[Fe(CN)_6]^{3-/4-}$ and the sensor (Cui et al., 2022).

To date, nine divergent SS isolates have been described as capable of causing clinical disease (Baig et al., 2015). In this Chapter, I achieve the first detection of divergent strains of SS using LAMP amplification by using a triplex LAMP reaction containing primer sets for the WT SS targeting the *recA* gene and covering all but one of the

divergent strains through the SS_90588 gene. The implementation of the triplex reaction resulted in a 2x decrease in SEN of the WT assay, which is not unsurprising as there are a third less target primers within the LAMP assay when it is in the triplex format. Although not evaluated in this Chapter, an alternative strategy that may have reduced the impact on the WT assay was targeting the same gene region and using degenerative bases. However, as the divergent clade SS are not often isolated, I chose to implement the single-plex SS WT assay in the rest of this Thesis.

Although care was taken to keep parameters between the four isothermal methods as constant as possible, variations between the experiments existed. For the RPA and LAMP experiments assessing the analytical SEN, the templates used for the bacterial gDNA were diluted and run on the same day, ensuring a comparison can be drawn between the analytical SEN of these two amplification chemistries. However, for the HDA and PSR assays, new gDNA was diluted as I found deterioration of the gDNA dilutions, even if they were made in TE and stored at -20°C and undergoing a maximum of one freeze-thaw per aliquot. Therefore, differences in dilutions between HDA and PSR compared to RPA and LAMP may be a factor in differences in the relative performance. Additionally, I reported the SEN of each isothermal amplification chemistry by quantifying gDNA via Nanodrop. Although the Nanodrop has been found to be more reliable in quantification than Qubit, in comparison to qPCR (Nakayama et al., 2016), the most accurate quantification method available currently is digital PCR, ensuring absolute DNA quantification.

In this Chapter, RPA experiments were performed with TwistAmp liquid basic reactions, whilst the publications utilised either the TwistAmp Exo or Nfo kits (lyophilised), or the *ZC BioScience* Exo kit, and may have accounted for differences observed between the original publication data and what I reported in this Chapter. The TwistAmp liquid basic SKU was chosen for this work as it allows increased flexibility over the lyophilised format, such as the addition of alternative buffer, as well as containing an alternative formulation to reduce the instance of primer-dimers compared to the Exo or Nfo SKU (personal correspondence, TwistDx).

Conclusions

In this Chapter, I have investigated the use of several isothermal chemistries in their ability to detect several bacterial pathogens that contribute to the PRDC. The most rapid amplification was achieved with RPA which, overall, displayed a similar SEN and SPE to LAMP. HDA achieved sensitive amplification. However, primer design to prevent FP amplification with HDA proved difficult. The PSR amplification chemistry had very poor SEN, even with the published assay. In determining the amplification chemistry compatibility with CMOS-ISFET detection, only LAMP amplification produced a consistent and high pH change, and therefore the LAMP chemistry remains the most appropriate chemistry to use in conjunction with our PoC device. In summary, LAMP assays for each of the PRDC pathogens of interest have been successfully identified in this Chapter, producing sensitive and specific amplification, and paving the way for implementation onto the CMOS-ISFET detection platform.

Chapter 3: Clinical validation of RPA and LAMP assays

Introduction

The 2019 coronavirus disease 2019 (COVID-19) pandemic resulted in an explosion in the PoC diagnostic market. Similar to the PRDC pathogens, the virus which causes COVID-19, SARS-CoV-2, often results in non-specific symptoms which are difficult to differentiate from other human respiratory viruses (Baj et al., 2020). Therefore, in the attempt to curtail the pandemic, several molecular tests were issued with the U.S. Food and Drug Administration (FDA) emergency use (FEU) status for the rapid diagnosis of SARS-CoV-2. Under FEU, the FDA removed many of the regulatory criteria that would typically be required for a product to be brought to market. One such criteria was the requirement for large clinical sample data sets to be used to calculate the clinical SEN and SPE of the tests (Mitchell et al., 2020). One of the first SARS-CoV-2 rapid diagnostic test to be licenced under FEU was Abbotts ID NOW COVID-19, which uses Abbotts proprietary isothermal amplification chemistry, NEAR. The ID NOW COVID-19 test was promoted heavily by the Trump administration and the White House (**Figure 3.1**). However, subsequent studies found the ID NOW displayed a clinical SEN as low as 44%, leading to several FN results (Basu et al., 2020, Lephart et al., 2021). Thus, potentiating the importance of robust in-depth clinical testing when assessing the performance of PoC devices.

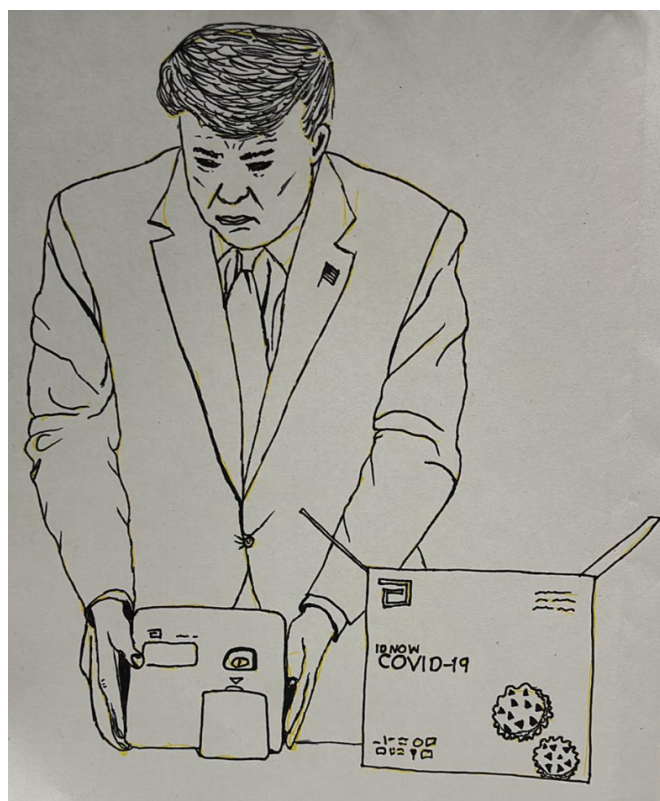


Figure 3.1. Illustration of the Abbott ID NOW COVID-19 test being unveiled by President D.J. Trump. The ID NOW promoted by the White House in 2020, President Trump proudly demonstrates the ‘5-minute test’ by displaying it upside down. Illustration adapted from photographs, medium: ballpoint pen on paper.

The clinical performance of a diagnostic test is an important consideration when ensuring the test is fit for purpose. Correlating the reported clinical SEN of the test to the disease status of the animal can aid in determining the recommended use of the product, i.e., whether the test can be used on symptomatic or

asymptomatic animals. For instance, if the test proves highly sensitive, even when the pathogen is present at low quantities, then the test might be appropriate to determine carrier status. On the other hand, if the test is highly sensitive only when the pathogen is highly abundant, then this test would not be appropriate for use during routine herd surveillance or for use with pooled samples, but instead as a diagnostic tool to confirm the presence of a specific pathogen in combination with the clinical presentation of the animal.

Within the UK and the EU, the sale of veterinary diagnostic tests is not required to meet any criteria set out by a regulatory body. Therefore, unlike human in vitro diagnostics (IVD), there are no requirements placed on manufacturers of these diagnostic tests to report the analytical or clinical SEN or SPE. This lack of transparency in the market may lead to the inappropriate implementation of a test. This once again was the case for the ID NOW COVID-19 test, which was used to screen all staff and visitors to the White House; subsequently shown to be an unsuitable strategy - as the test can only reliably detect symptomatic cases (Tu et al., 2021). Within the swine production system, the introduction of animals into a herd is a high-risk event, often leading to the introduction of disease (Neumann and Hall, 2019). Animals frequently undergo quarantine before joining a herd in lieu of diagnostic testing, which is too costly for the number of animals required to be tested. A recent study on MH surveillance indicated that the MH detection rate of quarantined animals was 13%, at a cost of USD \$5.50/ELISA, whilst the use of PCR increases the detection rate to 75% at a cost of USD \$30/PCR (Clavijo et al., 2021). For animals to be economically tested, pooled samples would be the only viable option, which reduces the SEN of the test, as you are potentially diluting the analyte. Therefore, ascertaining the clinical SEN and SPE of an assay are important parameters when considering the recommended use of the product.

An additional benefit of clinical testing is ensuring the inclusivity of the IVD test. The inclusivity relates to the ability of the assay to detect genetic variants of the pathogen. Often, in silico inclusivity is performed based on the available genomes. However, for several PRDC pathogens, such as MH and APP, the number of publicly available annotated genomes is low, 26 and 74, respectively, as of 2022 (National Center for Biotechnology Information). Whilst reference strains are used frequently, it is not unusual for a limited number of isolates to be available in laboratory collections, and these may be unrepresentative of those currently circulating. Thus, the use of clinical material enables the inclusion of circulating strains which are present at clinically relevant amounts.

In order to determine the clinical SEN and SPE of an IVD assay, a reference method must first be used to characterise a sample as positive or negative for the target pathogen. Typically, the reference method, known as the gold-standard, uses the technique which is most widely adopted in that field. However, the gold-standard technique used can vary greatly between studies. Since the invention of the PCR in 1985 (Mullis et al., 1986), the technique has been widely implemented in molecular diagnostic laboratories. The subsequent addition of fluorescence dyes or probes into PCR has allowed for the real-time monitoring of the amplification reaction enabling the quantification (qPCR) (Holland et al., 1991, Higuchi et al., 1992). qPCR offers several advantages over conventional PCR techniques, such as more rapid time to result, owing to a fluorescent reading being taken

after each amplification cycle, whilst conventional PCR requires the completed reaction to undergo a gel electrophoresis step, to visualise the amplification product. A significant advantage of qPCR over conventional PCR is the ability to obtain a quantitative result based on the cycle in which the qPCR fluorescence surpasses a threshold value known as the cycle threshold (C_t) value of the sample. Therefore, qPCR enables the relative quantification of DNA within a sample. In the context of determining the clinical relevance of samples, the quantification by qPCR allows samples to be characterised by the relative abundance of target DNA contained within the sample and thus can aid in the inference of the dynamics between the pathogens present.

The infection status of an animal is an important parameter to consider, however, the clinical presentation or history of the herd is often unknown to our laboratory at the time of submission for diagnostic evaluation. The Animal and Plant Health Agency (APHA) is a routine diagnostic laboratory which reports quarterly on the prevalence of swine respiratory diseases of samples which are submitted to it for diagnostic investigation (APHA, 2022b). However, no information is provided to identify the sample and thus the prevalence of multiple pathogens occurring in a single animal or on a single farm. Although studies have demonstrated the presence of multiple PRDC pathogens occurring on farms, none of these studies have correlated the quantification of the species along with the clinical relevance (Zhu et al., 2021, Haimi-Hakala et al., 2017, MacInnes et al., 2008).

Previously, I identified primer sets for four of the five bacterial PRDC pathogens; APP, MH, PM and SS for both RPA and LAMP isothermal amplification chemistries (see **Chapter 2**). Although several of the LAMP primer sets originated from published material, few publications have directly compared the performance of LAMP to RPA within a clinical setting. The standard method to determine the performance of an assay is to compare the performance of the isothermal chemistry being explored against the gold-standard qPCR assay. It is not uncommon for isothermal techniques to display a decreased SEN compared to qPCR (Özay and McCalla, 2021), however, isothermal amplification offers rapid amplification, as well as simplified incubation temperatures, which are much more amenable to PoC settings. Therefore, with proper implementation, rapid and affordable diagnostics can be performed on-farm, aiding in the early identification of PRDC pathogens, to ensure the appropriate and timely treatments of these economically important diseases.

3.1. Aims

The aim of this Chapter is to determine the clinical SEN and SPE of RPA and LAMP assays using clinically relevant tissue types. First, I will characterise the presence of different PRDC bacterial pathogens within clinical tissues using qPCR. Next, I will compare the ability of RPA and LAMP assays to detect these pathogens and relate this in terms of SEN (%) and SPE (%).

Methods

3.2. Bacterial strains

Bacterial strains used in this Chapter include APP serovar 8 strain MIDG 2220 (405), PM laboratory strain MIDG 3290, SS reference strain P1/7 and GPS reference strain SVR5. Bacterial culture took place on BHI agar plates consisting of BHI broth (BD, Cat 237500) supplemented with 5% agar (Sigma-Aldrich, Cat 05040), 10% DHB (TCS biosciences, Cat HB035) and 0.01 % NAD (MP Biomedicals, Cat 100319). Bacterial isolates were cultured in a static incubator at 37°C in the presence of 5% CO₂ for 16-18 hours. Whole gDNA of the reference strain, MH232, were kindly supplied by Professor Andrew Rycroft (Royal Veterinary College).

All reference strains used in this Chapter are listed in **Table 3.1**.

Table 3.1. Bacterial reference strains used in Chapter 3.

Pathogen	Reference strain used	Accession	Genome size (bp)
APP	405	ERS155401	2,323,218
MH	MH232	AE017332	892,758
PM	MIDG 3290	-	-
SS	P1/7	AM946016	2,007,491
GPS	SVR5	GCA_000751055.1	2,263,794

3.2.1. Extraction of DNA from bacterial strains

The extraction of whole gDNA from cultured bacteria was performed using the FastDNA Spin Kit (MP Biomedicals, Cat 116540600-CF), following the manufacturer's recommended protocol. Briefly, a 10 µL loop of bacterial culture grown on plates was resuspended in 200 µL of sterile PBS (Gibco, Cat 20012027), prior to the addition of 600 µL of CLS-TC (MP Biomedicals, Cat 116540409). The preparation was pipette mixed, transferred to Lysis matrix A (MP Biomedicals, Cat 116910050-CF), and vortexed twice for 20 seconds to aid in the chemical lysis of the bacteria. The suspension was pelleted by centrifugation at 15,000 x g for 5 minutes at 4°C and the supernatant was transferred to a new 2 mL tube. 650 µL of homogenised binding matrix buffer (MP Biomedicals, Cat 116540408) was added to the supernatant and mixed by gentle inversion for 5 minutes. The suspension was pelleted by centrifugation at 14,000 x g for 1 minute and the supernatant carefully removed and discarded. The pellet was resuspended in 500 µL of concentrated SEWS-M (MP Biomedicals, Cat 116540405) prior to being transferred to the spin module (MP Biomedicals, Cat 112080600-CF). The sample was applied to the spin column via centrifugation at 14,000 x g for 1 minute and the flow-through was discarded. For elution of the sample, 150 µL of pre-warmed (to 65°C) DNase-free water (MP Biomedicals, Cat 116540406), was incubated on the column for 5 minutes at 65°C. The sample was eluted into a separate collection tube via centrifugation at 14,000 x g for 1 minute.

The integrity of extracted gDNA was examined via running on a 1.5% agarose gel containing EtBr for 30 minutes at 110 V and visualised in a Gel Doc Ez imager (Bio-Rad, Cat 1708270). The gDNA was quantified with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Cat ND-ONE-W) and the copy number calculated via the Thermo Fisher Scientific copy number calculator using the genomic sizes listed in **Table 3.1**.

3.3. Clinical samples

This Chapter involved 162 clinical samples obtained from farms across England and Wales (n=125), Portugal (n = 28) and Northern Ireland (n=9), and comprised of 124 lung tissue, 22 liver, 9 oral fluid, 4 heart tissue, 2 synovial fluid, and 1 intestinal sample. The origin of and any known clinical information on the samples can be found in **Supplementary Table 5**.

3.3.1. Extraction of DNA from clinical samples

Solid tissue samples were obtained during necropsies from pigs displaying clinical signs of respiratory infection. For genomic DNA extraction from lung, heart, and liver tissue samples, approximately 50 mg of tissue was excised with a sterile disposable scalpel, with visibly lesioned areas being used where possible. Excised tissue was homogenized in lysing matrix A tube (MP Biomedicals, Cat 116910050-CF) with 360 µL ATL buffer (Qiagen, Cat 19076) using a FastPrep-25 5G (MP Biomedicals, Cat 116005500) set at 6.0 m/s for 60 seconds. Subsequent DNA extraction was performed with a QIAamp DNA Mini Kit (Qiagen Ltd., Cat 51304), and DNA was eluted using 200 µL buffer AE (Qiagen Ltd, Cat 19077).

Oral fluid samples were obtained from farms in England. Each oral fluid sample was extracted from a cotton rope, with each rope used to sample 25 animals at a time, and DNA from each one was individually extracted using the MagMax Express-96 Particle Processor (Thermo Fisher Scientific, Cat 4400074), as previously described (Hernandez-Garcia et al., 2017).

Synovial fluid was obtained from single animals via a needle placed directly into the joint, and 1 mL of the was centrifuged at 12,000 x g for 5 minutes. The pellet was suspended in 360 µL ATL buffer, and DNA extracted with the QIAamp DNA mini kit (Qiagen) as described above for the solid tissues.

3.4. Amplification conditions

3.4.1. qPCR conditions

Published qPCR assays were used to determine the presence of bacterial DNA in clinical tissues, qPCR primers and probes are shown in **Table 3.2**, and were synthesised by IDT. All qPCR assays were prepared with the GoTaq probe qPCR kit (Promega, Cat A6102), consisting of 1x GoTaq probe qPCR master mix, 0.5 µM primers, 0.3 µM probe, unless otherwise stated, and 1 µL of the template, in final reaction volumes of 5 or 10 µL. Real-time results were monitored in a CFX connect Real-Time PCR thermocycler (Bio-Rad, Cat 1845097), with cycling conditions of 95 °C for 2 minutes, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

Table 3.2. qPCR primers and probes used in Chapter 3. qPCR hydrolysis probes referred to as TaqMan probes (Taqprb) containing a 5' carboxyfluorescein (FAM) and 3' Black Hole Quencher 1 (BHQ-1), apart from SS probe which contains in internal, cleavable BHQ-1 and a C6-spacer to block probe extension.

Pathogen	Target gene	Primer ID	Primer sequence (5' – 3')	Conc. (μ M)	Reference
APP	<i>apxIVA</i>	F	GGGGACGTAAC TCGGTGATT	0.5	(Tobias et al., 2012)
		R	GCTCACCACGTTTGCTCAT	0.5	
		Taqprb1	FAM-CGGTGCGGACACCTATATCT-BHQ-1	0.3	
MH	<i>mhp183</i>	F	CCAGAACCAAATTCCTTCGCTG	0.5	(Strait et al., 2008)
		R	ACTGGCTGAAC TTCATCTGGGCTA	0.5	
		Taqprb1	FAM-AGCAGATCTTAGTCAAAGTGCCCGTG-BHQ-1	0.3	
PM	<i>kmt1</i>	F	CAGAGTTTGGTGTGTTGA	0.4	(Settypalli et al., 2016)
		R	CAGACTGACAAGGAAATATAAAC	0.1	
		Taqprb1	FAM -AATC+TGC+TTCCTT+GAC- BHQ-1	0.2	
PM		F2	CACTGGGTAAATAGCGGATAGAGC	0.5	(Wu et al., 2021)
		R2	ACAGAAAAGACAGCAATTTTCGAGC	0.5	
		Taqprb2	FAM-ACAATGGTGGGGCTTTACGCTGA-BHQ-1	0.3	
SS	<i>fbpS</i>	F	TCCRATRCTGCTCTGCCATT	0.5	(Srinivasan et al., 2016)
		R	TGATAGTAGAAGTCCAGCACT	0.5	
		Taqprb1	FAM-AATAGCCC”T” (BHQ-1) GAAAAMCAGCCACWYTTTGARA-C6-spacer	0.3	
GPS	<i>infB</i>	F	CGACTTACTTGAAGCCATTCTTCTT	0.5	(Turni et al., 2010)
		R	CCGCTTGCCATACCCTCTT	0.5	
		Taqprb1	FAM-ATCGGAAGTATTAGAATTAAGTGC-BHQ-1	0.3	

*+ signify locked nucleic acid (LNA) modification to the following base.

"indicates replacement of the interspersed base.

3.4.2. LAMP conditions

LAMP primer sets used in this study are listed in **Table 3.3**. LAMP reactions were performed in 10 μL reaction volumes consisting of 1x isothermal buffer (NEB, Cat B0537), 6 mM MgSO₄ (NEB, Cat B1003S), 1.4 mM dNTPs (Thermo Fisher Scientific, Cat 10297018), 0.5 mg/mL BSA (Sigma-Aldrich, Cat A9418), 0.8 M betaine (Sigma-Aldrich, Cat B0300), 0.5 μM SYTO 9 (Thermo Fisher Scientific, Cat S34854), 0.5 U/μL of *Bst* 2.0 DNA polymerase (NEB, Cat M0537), 0.25 μM F3 and B3, 1 μM LF and LB, 2 μM FIP and BIP. LAMP amplification was monitored in a CFX connect Real-Time PCR thermocycler (Bio-Rad) for 70 cycles of 30 seconds at 63°C, with fluorescence measured each cycle, followed by a melt curve. The melt curve took place after an initial denaturing step of 95°C for 1 minute, followed by melting from 55°C to 95°C in 0.5°C increments, with data acquisition taken at each increment.

Table 3.3. LAMP primers used in Chapter 3.

Pathogen	Target gene	Primer ID	Primer sequence (5' – 3')	Reference
APP	<i>dsbE-like</i>	APP_F3_A	CTGAAAGTTTCGTCAGCAC	(Ji et al., 2012)
		APP_B3_A	TCTGAAACTGAAAAGTATCCAC	
		APP_FIP_A	GCTCGAAATCCGGGTTATATCCT-ATTTAATTGATAAACACGGTGTG	
		APP_BIP_A	TCGTTTAAAAGCGTTACAAGAGGA-AAACTGAAGACAAGCGGTA	This Thesis
		APP_A_LF	TTTTTGTGGTAACGTAC	
		APP_A_LB	ACTATTGCAAAAAATCGC	
MH	<i>mhp165</i>	MH_F3_B	TGTTGAGATCGCATCAAGAA	(Li et al., 2013)
		MH_B3_B	CCTACCAAAACAAAACAAGGTAGT	
		MH_FIP_B	GGCGCTCATGAATATGGTCATCTTTTCTGGATCGGAAATATCCTG	
		MH_BIP_B	TTCCCGTAGTTTTAAAAAATGCGGTTTCCAATCATCCGAATTCCTT	
		MH_LF_B	CCAAACACTAAATTATGCC	
PM	<i>kmt1</i>	PM_F3_A	GGTGCTATCTTGCTTCG	
		PM_B3_A	TCGATCGCTAGCACCACA	

		PM_FIP_A	TGCCGTAGCAGAACTGGACTTTTAGGTGAGCCATGTGAGCT	(Bhimani et al., 2015)
		PM_BIP_A	CACACCGAAGCCAGGACTTTTTTCACCAAATGTACTGGTTGCTTC	
		PM_LF_A	CGACCATCGGTTGCATTTC	
		PM_LB_A	TGGCATTGCATGGCTATCA	
SS	recN	SS_F3_A	TGTCGATGATGTTTTGGACTA	(Arai et al., 2015)
		SS_B3_A	GCTTTCTCCATATACAAGTCTTG	
		SS_FIP_A	AAGCTGAACCTCCAAATCATCTCCTTTTCAGCGAAGAATACAATCTATTGAC	
		SS_BIP_A	TAGAGAAAGAATTGGTTGAACGAGCTTTGCGGATAATATCTTCTAAACAAC	
		SS_LB_A	GGTCAGCTCAGCCAATCACGC	
		SS_LF_A	AGATAAATCATTGCCT	This Thesis
GPS	infB	GPS_F3_F	CTATCCTTGTTCAATCAGGTAC	This Thesis
		GPS_B3_F	TATAACGCCACTTCACGC	
		GPS_FIP_F	CATTTTCATCACGCATTGCACG-AGGCGACATCGTACTTTG	
		GPS_BIP_F	TTCCTGTGGAAGTATTAGGACTTTC-TTTTCTCATCACGCACCA	
		GPS_LB_F	CACGGCCATATTCAAAGCCA	
		GPS_LF_F	GCAGGTGATGAAGCGACAG	

3.4.3. RPA conditions

The RPA primers used in this study are shown in **Table 3.4**. RPA reactions were performed as described in the manual, using TwistAmp liquid reactions (Twist Dx, Cat TALQBAS01). Final RPA reactions components included 1x TwistAmp liquid reaction buffer, 1 x core protein mix, 0.45 mM dNTPs (Thermo Fisher Scientific), 1 x basic energy mix, 0.6 µM of primers, 0.12 µM of probe, 14 mM MgAc (Twist Dx), and 1µL of the template. Real-time RPA reactions were supplemented with 0.02 U/µL of Exo (NEB, Cat M0206) in a final reaction volume of 25 µL. RPA reactions were incubated in a CFX connect Real-Time PCR thermocycler (Bio-Rad) at 37°C for 20 minutes, where applicable fluorescence readings were taken once every 30 seconds for the duration of the incubation period. The reactions were mixed via 10-inversions after 4 minutes of incubation.

Table 3.4. RPA primers used in Chapter 3. THF residue signifies the tetrahydrofuran residue (abasic site), with bases within square brackets show modified oligonucleotides with the moiety shown in curly brackets, e.g., FAM.

Pathogen	Target gene	Primer ID	Primer sequence (5' – 3')	Reference
APP	apxIVA	APP_F2	TAATGGCGGTAATGGCGATGACACCCTCATCGGCG	This Thesis
		APP_R2	CTTGCGCGGTTATCATTATTGGTATCTTCATAAAC	
		APP_Probe2	GTGGCTACGGTGCGGACACC (T [FAM]) [THF] (T [BHQ-1]) ATCTTTAGCAAAGGACACGGACAG	
	3'apxIVA	3'APP-F1	GCGACACAAGAGATATCTCTCCTCCGTGCTTCTGA	This Thesis
		3'APP-R1	GTATTCACACCAAGATCATAAAAATAGAAAATATTC	
		3'APP-Probe1	AAACGTTGGTGAGCACTCAGGTGAGAGAAG (T [FAM]) [THF] G [T (BHQ-1)] TGAGTCGATGGCCGG	
MH	p183 (p97)	MH_F2	GCAAAAGATAGTTCAACTAATCAATATGTAAGT	This Thesis
		MH_R2	ACTTCATCTGGGCTAGCTAAAATTTACGGGGCA	
		MH_Probe2	TCATCTGGGCTAGCTAAAATTTACGGGGCACT (T (BHQ-1) G [THF] C [T (FAM)] AAGATCTGCTTTTA	
PM	kmt1	PM_F2	CACGACGTGCAGAACCAGGTGTTAAAGTCACCATAGG	This Thesis
		PM_R2	GTTTGATTCATCATCTAACCAGCTGAAAGCATTTG	
		PM_Probe2	GCAGAACCAGGTGTTAAAGTCACCATAGGTATGATCCCACTGCAAAGTTT	
SS	recN	SS_F6	CGCTGATTTTACTAAAATAGTCCAAAACATCATCG	This Thesis
		SS_R2	TTGACGGCAATCGTCTCATGCAATTGGAAAGTCGT	
		SS_Probe1	CATCATCGACAGTTCCACCATATTTCTTCG (T [FAM]) [THF] A [T (BHQ-1)] GGTATTGAGCAAATC	

3.5. Data analysis

Fluorescence data obtained during amplification was analysed using CFX Maestro Software 2.3 for CFX Real-Time PCR instruments (Bio-Rad). The software was used to determine C_t values for all amplification using the software's pre-determined threshold. The software establishes the threshold value via the average fluorescence across the first 10 amplification cycles, however in RPA amplification often took place prior to the first 10 cycles (5 minutes of amplification type) being completed. To account for this the threshold value had to be manually adjusted in these instances.

3.6. Characterisation of clinical samples

3.6.1. Relative quantification by qPCR

Standard curves for each of the qPCR assays were produced from 10-fold serial dilutions of gDNA from the appropriate reference strain made in TE buffer and at least 10-replicates were obtained across three different template titrations. An increased number of serial dilutions were made to cover the interval in which less than 100% of the replicates amplified. The LoD was determined to be the lowest copy number where all 10 replicates amplified. The subsequent C_t values obtained were plotted in GraphPad Prism version 9.0 for Mac OS X and the r^2 value calculated from a semi log regression line where X (the copy number of gDNA) is log and Y (C_t value) is linear. Clinical samples were characterised as positive by qPCR if both replicates amplified, the standard curve was used to determine the relative quantification using GraphPad Prism version 9.0 to extrapolate the average C_t value into relative copy numbers with a 95% confidence interval. Clinical samples with a relative quantification below the LoD for that qPCR assay were considered negative.

3.6.2. LAMP and RPA

Clinical samples were considered positive by LAMP and RPA if both replicates exceed the threshold value, and negative if only a single or neither of the replicates reached the threshold value. In LAMP, melt curve analysis data was performed to obtain T_m values using the CFX Maestro Software 2.3, using the pre-programmed parameters.

Clinical SEN and SPE were calculated as percentage agreement between LAMP or RPA and qPCR results. Determined by the number of true positives (TP), FN, TN, and FP, as determined by qPCR. SEN and SPE values were calculated using Excel and a custom Python script (**Supplementary Table 6**) ran in the JupyterLab 3.0.14 environment.

$$SEN (\%) = \frac{TP}{TP + FN} \times 100$$

$$SPE (\%) = \frac{TN}{TN + FP} \times 100$$

3.7. Statistical analysis

Statistical analysis was performed on qPCR results of clinical samples via manual conversion of copy number data to binary presence/absence data. From this data a Spearman's rank-order correlation matrix was produced in GraphPad Prism version 9.0 and in a custom Python script (**Supplementary Table 6**) ran in the JupyterLab 3.0.14 environment. The parameters for this correlation test were a two-tailed test with a 95% confidence interval. From the correlation matrix, a heatmap was created to aid in analysis.

Results

3.8. Performance of qPCR assays

To determine the presence and absence of bacterial pathogens involved in the PRDC, I first evaluated the performance of previously published qPCR assays. The assays were chosen based on the target gene, to mirror that targeted in our isothermal assays, where possible. The relative copy number of each assay was correlated to the C_t of each qPCR assay using a standard curve of serially diluted gDNA from each of the target bacteria. The qPCR assay specific to APP, targeting a 377 bp region of the *apxIVA* gene, could detect down to 10 copies of the serovar 8 APP gDNA (**Figure 3.2A**). The same SEN was obtained in the qPCR assay for the detection of MH, amplifying a 90 bp region of *mhp183*, with the assay reliably able to detect down to 10 copies of the reference strain MH232 (**Figure 3.2B**). The qPCR assay for the detection of PM, amplifying a 113 bp section of the *kmt1* gene (Settypalli et al., 2016), was determined to be 100 copies; however, this assay did suffer from FP amplification in the negative controls. This was not rectified, even when the assay was changed to an alternative qPCR assay which targeted a 95 bp region of the *kmt1* gene (Wu et al., 2021), indicative of an intrinsic contamination issue with low copy numbers of PM. Therefore, the LoD in which the PM qPCR assay described by Settypalli et al. retained its linearity was 100 copies/ μ L or a threshold cycle number of 34.65 (**Figure 3.2C**), and the cut-off for positive amplification with the PM qPCR assay was set to 33 cycles to ensure no FP results. The SS qPCR assay amplified a region of the *fbpS* gene, achieving a LoD of 20 copies of the reference strain P1/7 (**Figure 3.2D**). The GPS qPCR assay showed the lowest LoD, targeting a 74 bp region of the *infB* gene, achieving a LoD of five genomic copies of the reference strain SVR5 (**Figure 3.2E**).

3.9. Characterisation of clinical lung tissue by qPCR

In this Chapter 162 clinical samples were available for investigation, including 124 lung, 22 liver, 9 oral fluid, 4 heart tissue, 2 synovial fluid and 1 intestinal sample. The gDNA extraction of these tissues were predominately performed by Dr Yanwen Li (Imperial College London) and analysed for presence of bacterial DNA as part of this Chapter.

Out of the 162 clinical samples characterised by qPCR, 67 lung samples were subjected to all five bacterial qPCR assays. Of these 67 samples, five were negative for all five pathogens and 62 were positive for at least one pathogen. Of the 62 positive samples, 24 had a single bacterial presence, and the remaining 38 samples displayed co-infection with at least two different bacterial infections (**Figure 3.3A**). APP was the most frequently detected pathogen ($n=18$), followed by MH ($n=5$), and SS ($n=1$). Neither PM, nor GPS were found to be present without other bacterial species (**Figure 3.3B**).

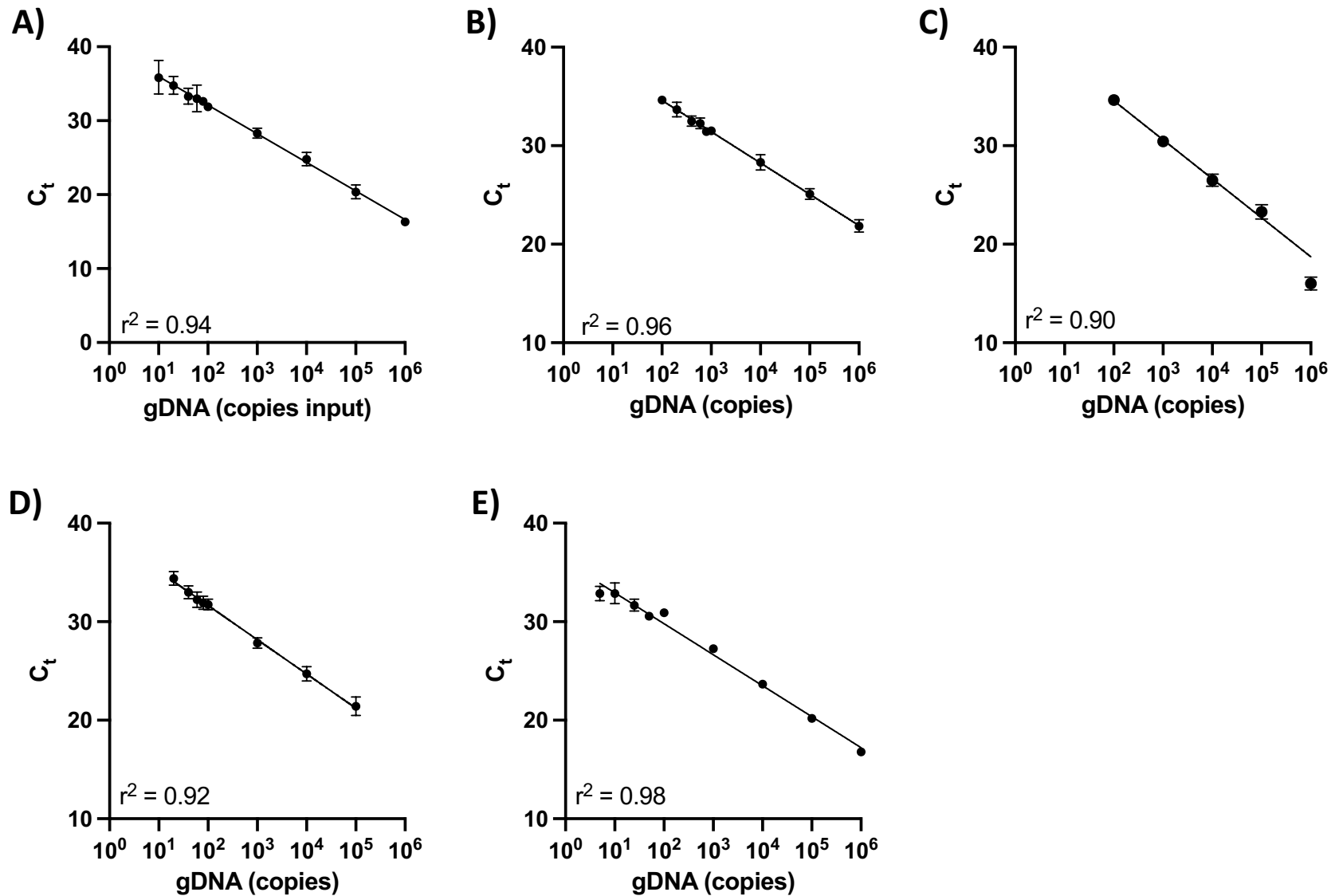


Figure 3.2. Linear regression of five qPCR assays used in this study. The APP qPCR assay against gDNA from APP serovar 8 reference strain MIDG 2220 (405) (A), MH qPCR assay against gDNA from reference strain MH232 (B), GPS qPCR assay against gDNA from the reference strain SRV5 (C), SS qPCR assay against gDNA from the reference strain P1/7 (D), PM qPCR assay against reference strain MIDG 3290 gDNA (E). Linearity of the regression is reported by r^2 values for each plot, calculated in GraphPad Prism 9.0 using at least 10 different data points per gDNA condition.

Co-infection with two pathogens was found in 19 samples, with APP found in all samples displaying dual infection. The presence of APP was most commonly associated with the presence of MH (n=13), followed by GPS (n=3) and SS (n=3). The presence of three bacterial pathogens was found in a total of 19 clinical samples. Co-infection with APP, SS, and GPS were the most common (n=3). Only a single sample was found to be triple-infected with APP and MH, along with either PM, SS or GPS. An individual sample was found not to have a co-infection involving APP. Instead, the presence of MH along with PM and GPS DNA was detected (**Figure 3.3B**). Eight samples displayed the presence of four bacterial pathogens. APP was present in all eight of these samples; in seven of the samples, MH and PM were also present, of which four were found to also contain SS and three were positive for GPS. The last sample containing four bacterial pathogens contained APP along with PM, SS and GPS. Lastly, three samples were found to be co-infected with detectable amounts of all five bacterial pathogen DNA (**Figure 3.3B**).

Overall, APP was a major contributor to bacterial lung samples, present in 54 of the 62 (88.71%) positive lungs, followed by MH (51.61%), SS and GPS (24.19%), and lastly, PM was least abundant (22.58%) (**Table 3.5**).

Table 3.5. The presence of bacterial co-infections. The abundance (n) and contribution (%) of each of the five bacterial pathogens to polymicrobial diseases across the 62 lung tissues.

Pathogen	Contribution to coinfection (n)					Total n (%)
	1 n (%)	2 n (%)	3 n (%)	4 n (%)	5 n (%)	
APP	18 (75.00)	19 (100.00)	7 (87.50)	8 (100.00)	3 (100.00)	55 (88.71)
MH	5 (20.83)	13 (68.42)	3 (37.50)	8 (100.00)	3 (100.00)	32 (51.61)
PM	0 (0.00)	0 (0.00)	2 (25.00)	8 (100.00)	3 (100.00)	14 (22.58)
SS	1 (4.17)	3 (15.79)	4 (50.00)	5 (62.50)	3 (100.00)	15 (24.19)
GPS	0 (0.00)	3 (15.79)	6 (75.00)	3 (37.50)	3 (100.00)	15 (24.19)
Total	24 (38.71)	19 (30.65)	8 (12.90)	8 (12.90)	3 (4.84)	62 (100.00)

To determine if there was a relationship between the abundance and presence of each co-infecting pathogen, I first examined the relative copy numbers of each bacterial species present within the 62 samples which were positive for at least one of the five bacterial pathogens, as determined by qPCR (**Figure 3.4A**). APP showed the highest mean relative copy number, with a mean value of 34,976 copies/ μ L. Whilst MH, PM and GPS displayed similar mean copy numbers of 19,266, 19,457 and 16,890 copies/ μ L, respectively. SS showed a 10-fold lower abundance compared to other species, with a mean copy number of 1,347 copies/ μ L. In order to investigate the relationship between the relative abundance of co-infected pathogens within the lung tissue, the data would require to be normalised against a reference gene to account for tissue input variations. However, this data was not obtained at the time of performing experiments. Additionally, due to the limited number of lung samples, the statistical power was not great enough to be able to draw conclusions between co-infection prevalence and the copy number of pathogens.

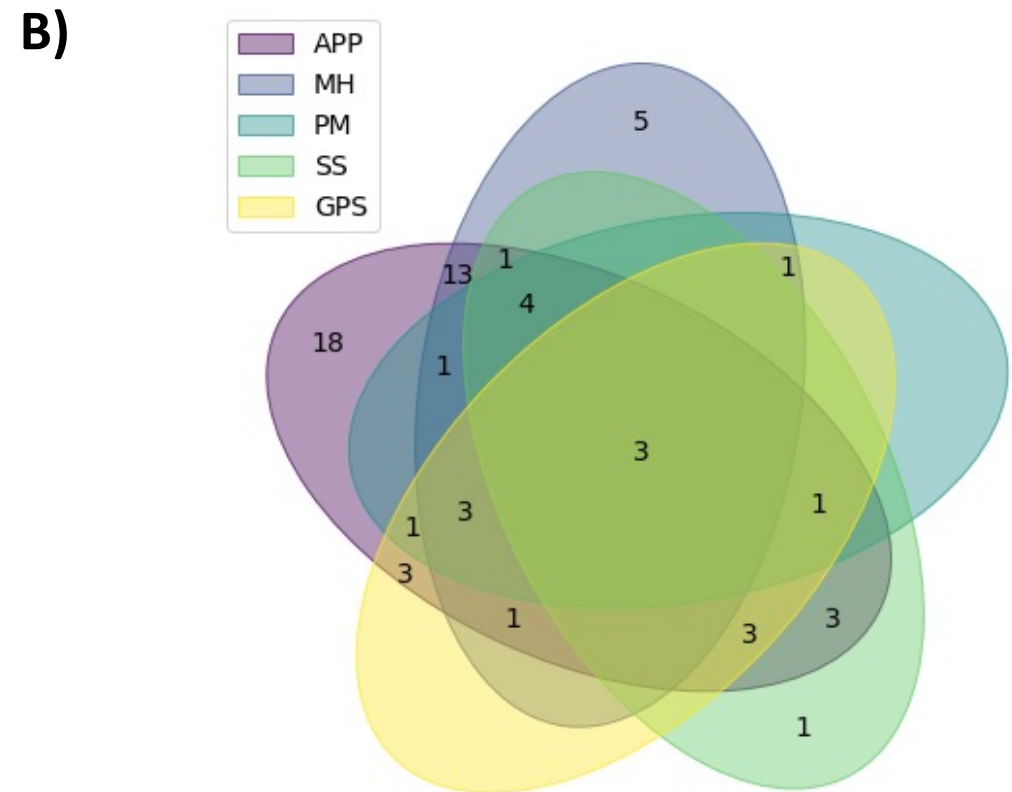
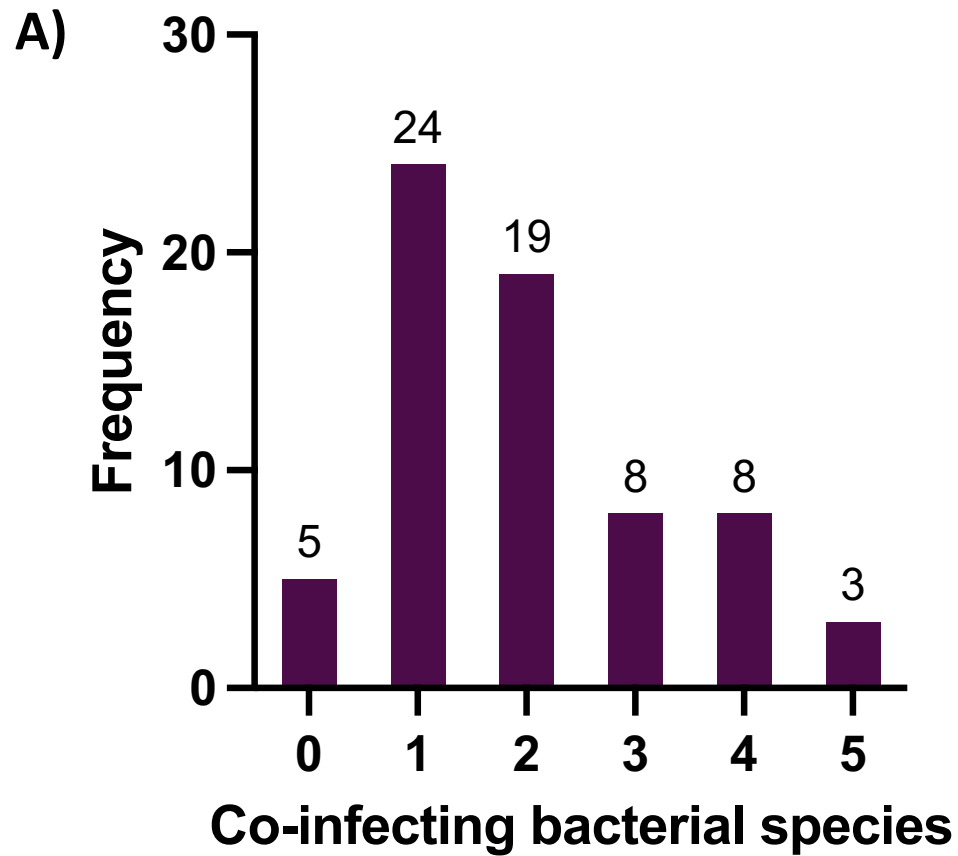


Figure 3.3. The presence of bacterial co-infections across 67 clinical lung samples. The frequency of co-infection found across the 67 lung samples run in each of the five PCR assays for PRDC bacterial pathogens (**A**). The distribution and frequency of the 62 lung samples which were positive for one or more of the bacterial pathogens involved in each co-infection (**B**).

As such, I investigated the correlation between co-infecting pathogens using a correlation matrix for the presence and absence of each pathogen. The Pearson r correlation coefficients showed that although APP and MH are the most common pathogens to be present together, in terms of presence, they display a very weak correlation ($r=-0.02$), **Figure 3.4B**. Suggesting that the presence of either APP or MH does not increase the likelihood of having the other present. All other pathogens showed a positive correlation, with PM and GPS displaying the highest correlation ($r=0.49$).

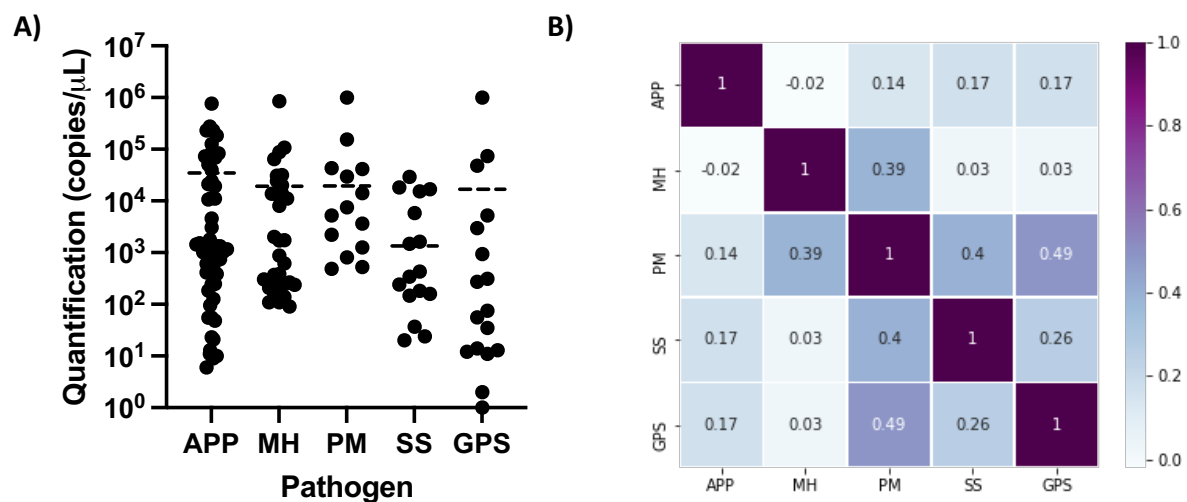


Figure 3.4. The association between bacterial infections. The spread of copy numbers achieved by relative quantification from qPCR amplification of the 62 clinical samples (**A**). A correlation matrix achieved by comparing presence and absence association of bacterial co-infections across the 62 clinical samples, produced using a custom Python script (**B**). Dotted lines depict the mean copy number across each pathogen.

3.10. LAMP and RPA clinical performance for individual bacteria

3.10.1. *A. pleuropneumoniae*

Of the 162 total samples used in this study, 111 were tested for the presence of APP, of which 69 (62.16%) exhibited amplification by qPCR and 42 did not (37.84%). All heart ($n=3$) and oral samples ($n=6$), half the synovial fluid samples tested ($n=2$), and 59 (59.60%) of the lung samples tested ($n=99$) were positive for APP by qPCR. The intestinal sample tested ($n=1$), showed no detectable APP DNA by qPCR.

Over these 111 samples, APP-LAMP achieved a SEN of 76.81% and SPE of 97.62%, with a positive predictive value (PPV) of 98.15% and a negative predictive value (NPV) of 71.93%. A single lung sample resulted in FP amplification, which was not considered positive in qPCR. In comparison, APP-RPA achieved an identical SEN of 76.81% and SPE of 100% (PPV 100%, NPV 72.41%). To aid in the data analysis, the samples were binned into low, medium, and high bacterial loads, as determined by qPCR (**Table 3.6**).

Table 3.6. Comparative performance of APP-LAMP and APP-RPA. The clinical performance of APP-LAMP and APP-RPA compared to qPCR across 111 clinical samples. Samples are grouped into high (H), medium (M), low (L) and negative (N) categories based on their relative copy numbers, as determined by qPCR C_t values in accordance with the APP qPCR assay standard curve.

Cat.	qPCR- C_t	DNA conc. (copies/rxn)	Sample (n)	APP-LAMP						APP-RPA					
				TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)	TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)
H	<27	>2,000	24	23	1	/	/	95.83	/	24	0	/	/	100.0	/
M	26.9-30.9	201-2,000	23	20	3	/	/	86.96	/	22	1	/	/	95.65	/
L	31-37.5	10-200	22	10	12	/	/	45.45	/	7	15	/	/	31.82	/
N	>37.5	<10	42	/	/	41	1	/	97.62	/	/	42	0	/	100.0
Total	/	/	111	53	16	41	1	76.81	97.62	53	16	42	0	76.81	100.0

In the low bacterial load category (below 200 copies/ μ L of the clinical sample) across the 22 clinical samples, APP-LAMP achieved low SEN of 45.45%. Whilst APP-RPA showed slightly decreased SEN, achieving 31.82%. In the medium bacterial load category (201-2,000 copies/ μ L of the clinical sample) across the 23 clinical samples, APP-LAMP failed to detect three of the samples originating from a heart, and two lung samples, achieving a SEN of 86.96%. The samples missed by APP-LAMP were quantified as having a relative bacterial load of 202, 764 and 1,789 copies, as determined by qPCR **Figure 3.5A**). In this category, APP-RPA achieved a much higher SEN of 95.65%, only failing to detect a single heart sample, with a relative quantification of 1,116 copies as determined by qPCR (**Figure 3.5B**). Unsurprisingly, in the high bacterial load category (>2,000 copies/ μ L of the clinical sample), APP-LAMP performed with the highest SEN of 95.83%, failing to detect only a single same lung sample, which had previously been quantified by qPCR as having a bacterial load of 11,455 copies. Within the high bacterial load sample set, APP-RPA achieved a SEN of 100%, detecting all 24 samples.

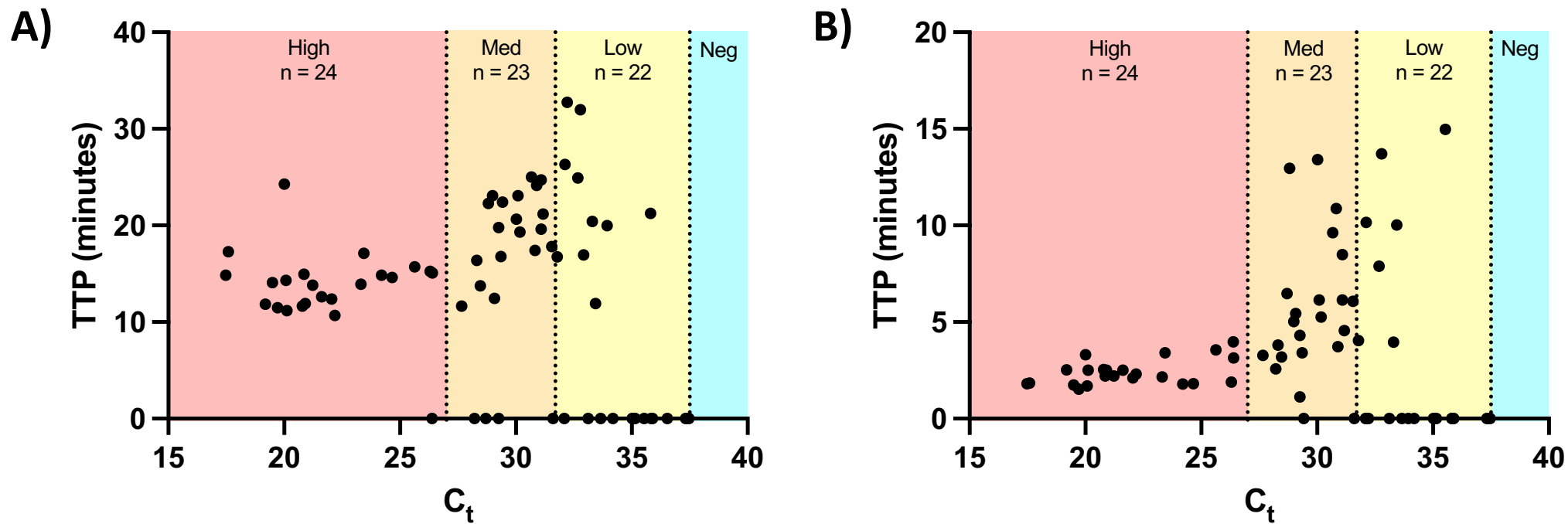


Figure 3.5. The distribution of 69 clinical samples determined APP positive by qPCR. The correlation between the C_t obtained in qPCR and the TTP achieved with APP-LAMP (A) and APP-RPA (B). Each point represents the average TTP and C_t performed on a clinical sample in duplicate. The clinical samples are grouped into high, medium, low, and negative categories, shown in red, orange, yellow and blue, respectively, based upon their relative copy number, as determined by qPCR.

As the APP-RPA assay resulted in multiple priming sites across the *apxIVA* gene, a redesigned RPA assay targeting a 3' region of the *apxIVA* gene (3'APP-RPA) assay was run in a small subset of these clinical samples (n=61). 3'APP-RPA detected 45 of the 51 APP positive samples, as determined by qPCR, with no FP amplification being seen in any of the 10 APP negative samples and achieved a SEN of 84.31% and SPE of 100% (**Figure 3.6**). For a direct comparison, APP-LAMP achieved a SEN and SPE of 77.08 and 100%, respectively in paired samples (n=61) (**Table 3.7**).

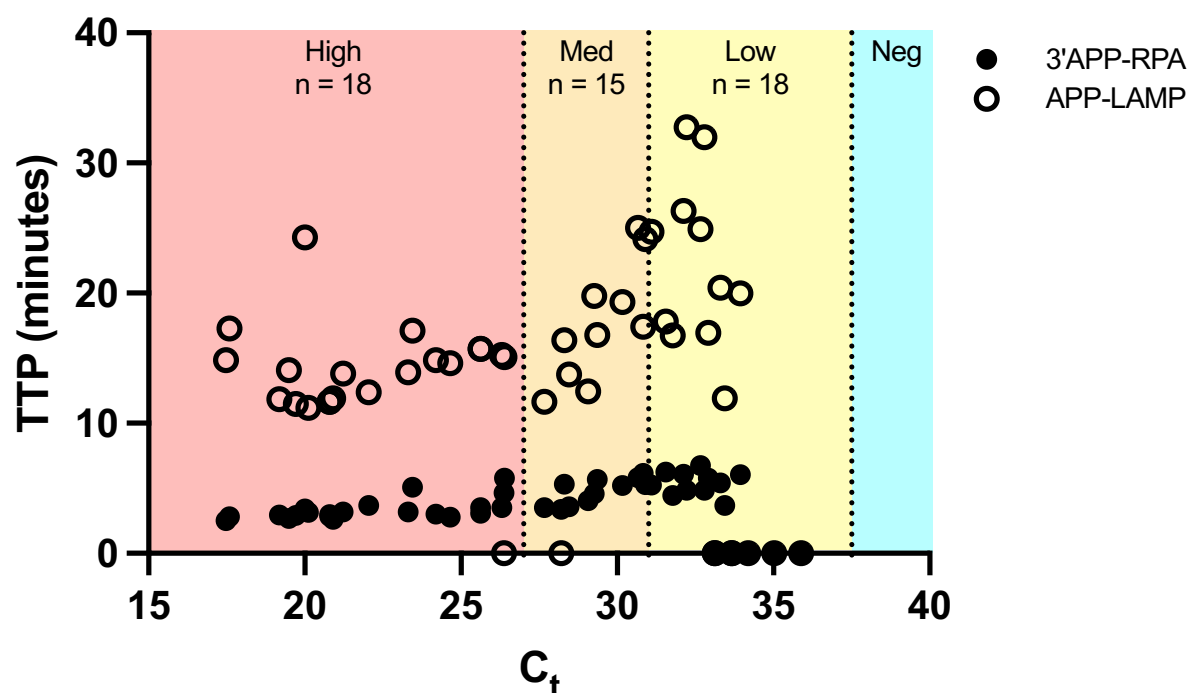


Figure 3.6. The distribution of 51 clinical samples determined APP positive by qPCR. The correlation between the C_t obtained in qPCR and the TTP achieved with 3'APP-RPA and APP-LAMP. Each point represents the average TTP and C_t performed on a clinical sample in duplicate. The clinical samples are grouped into high, medium, low, and negative categories, shown in red, orange, yellow and blue, respectively, based upon their relative copy number as determined by qPCR.

Table 3.7. Comparative performance of APP-LAMP and 3'APP-RPA. The clinical performance of APP-LAMP and 3'APP-RPA compared to qPCR across 57 clinical samples. Samples are grouped into high (H), medium (M), low (L) and negative (N) categories based on their relative copy numbers, as determined by qPCR C_t values in accordance with the APP qPCR assay standard curve.

Cat.	qPCR- C_t	DNA conc. (Copies/rxn)	Sample (n)	APP-LAMP						3'APP-RPA					
				TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)	TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)
H	<27	>2,000	18	17	1	/	/	94.44	/	18	0	/	/	100.0	/
M	26.9-30.9	201-2,000	15	14	1	/	/	93.33	/	15	0	/	/	100.0	/
L	31-37.5	10-200	18	10	8	/	/	55.6	/	10	8	/	/	55.6	/
N	>37.5	<10	10	/	/	10	0	/	100.0	/	/	10	0	/	100.0
Total	/	/	61	37	10	10	0	80.39	100.0	45	6	10	0	84.31	100.0

3.10.2. *M. hyopneumoniae*

Of the 162 total clinical samples, 113 were tested by qPCR for the presence of MH DNA, 38 were positive (33.63%), and 75 (66.37%) were negative. MH DNA was not detected in any of the heart (n=4), synovial fluid (n=2), or intestinal (n=1) tissues tested. Of the 38 positive samples, 36 originated from the lung out of the 100% lung samples (36%) tested. The remaining two positives were both extracted from the six total oral fluid samples tested (33.33%). Across these 113 clinical samples, MH-LAMP achieved a SEN of 71.05%, with 100% SPE, a PPV and NPV of 100 and 87.21%, respectively. To analyse the data more closely, the samples were characterised as being either of low, medium, or high bacterial burden, determined by their relative quantification by qPCR. With the low bacterial burden (10-200 copies/ μ L in the clinical sample), both MH-LAMP and MH-RPA achieved identical SEN of 28.57%, detecting only 2 out of the 7 clinical samples. In MH-LAMP, two lung samples were detected containing 140 and 161 copies, whilst in MH-RPA, an oral sample containing 102 copies and a lung sample containing 140 copies were detected (**Table 3.8**).

Table 3.8. Comparative performance of MH-LAMP and MH-RPA. The clinical performance of MH-LAMP and MH-RPA compared to qPCR across 123 clinical samples. Samples are grouped into high (H), medium (M), low (L) and negative (N) categories based on their relative copy numbers, as determined by qPCR C_t values in accordance with the MH qPCR assay standard curve.

Cat.	qPCR- C_t	DNA conc. (Copies/rxn)	Sample (n)	MH-LAMP						MH-RPA					
				TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)	TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)
H	<29.12	>2,000	18	18	0	/	/	100.0	/	17	1	/	/	94.44	/
M	29.12-32.6	201-2,000	13	7	6	/	/	53.85	/	11	2	/	/	84.62	/
L	32.5-37.4	10-200	7	2	5	/	/	28.57	/	2	5	/	/	28.57	/
N	>37.4	<10	75	/	/	75	0	/	100.0	/	/	75	0	/	100.0
Total	/	/	113	27	11	75	0	71.05	100.0	30	8	75	0	78.95	100.0

Of the 13 clinical samples assigned to the medium bacterial burden category (201-2,000 copies/ μ L in the clinical sample), MH-LAMP achieved a SEN of 53.85%, failing to detect six lung samples of 209, 246, 250, 265, 304 and 1,191 copies, as determined by qPCR (**Figure 3.7A**). Whilst MH-RPA achieved a SEN of 84.62%, failing to detect two lung samples which consisted of 265 and 304 copies, as determined by qPCR (**Figure 3.7B**). The high bacterial burden category (>2,000 copies/ μ L in the clinical sample) was the only category which saw LAMP outperform RPA, with the MH-LAMP assay amplifying all 18 of the positive samples, with a SEN of 100%. In comparison, MH-RPA failed to detect a single lung sample, and therefore achieving a SEN of 94.44%. The sample which failed to be detected by MH-RPA was determined to contain 10,723 copies by qPCR.

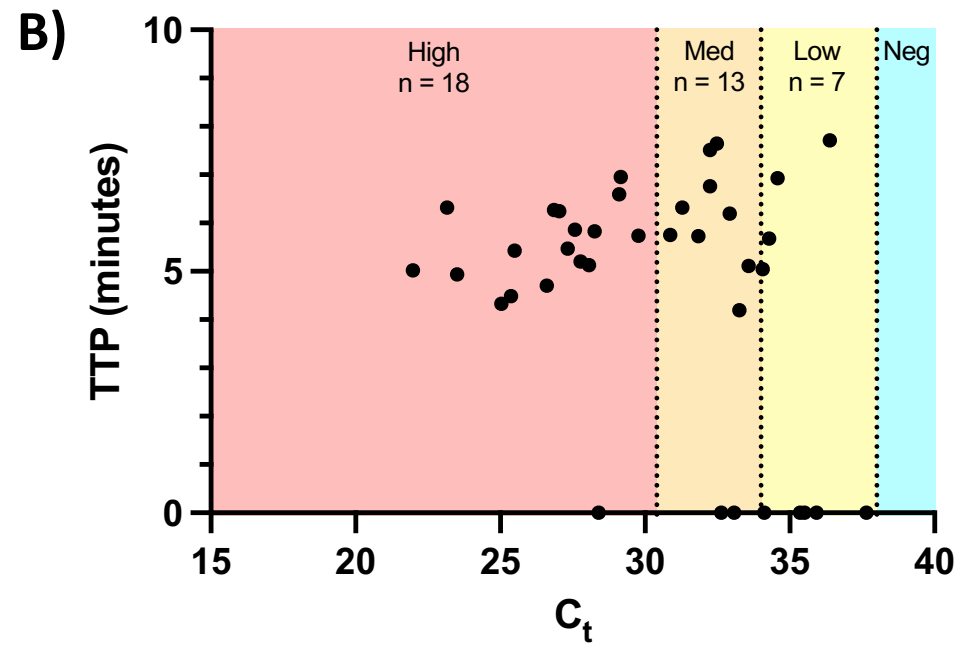
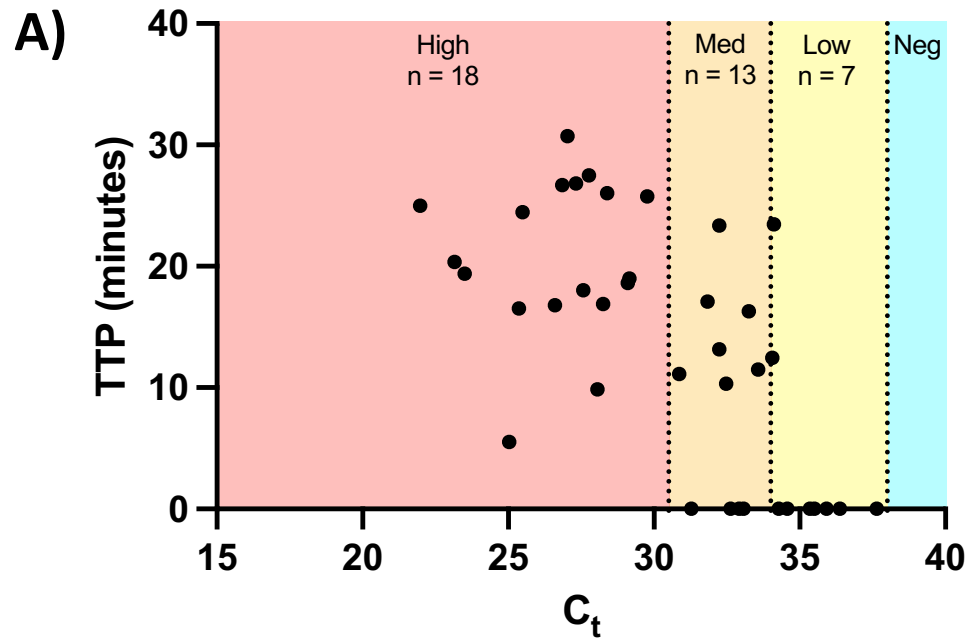


Figure 3.7. The distribution of 38 clinical samples determined MH positive by qPCR. The correlation between the C_t obtained in qPCR and the TTP achieved with MH-LAMP **(A)** and MH-RPA **(B)**. Each point represents the average TTP and C_t performed on a clinical sample in duplicate. The clinical samples are grouped into high, medium, low, and negative categories, shown in red, orange, yellow and blue, respectively, based upon their relative copy number as determined by qPCR.

3.10.3. *P. multocida*

Of the 162 total clinical samples, 92 were tested for the presence of PM DNA by qPCR, consisting of 89 samples originating from the lung, two from the heart and one synovial fluid sample. PM-qPCR determined that 15 samples were positive for PM, all positive samples originated from the lung (16.85%). PM was not present at detectable levels in any other tissue type tested. Of the 15 PM-positive lung tissues, PM-LAMP detected 12 samples, achieving a SEN of 80.00%. All samples that PM-LAMP failed to detect contained more than 800 copies, as quantified by qPCR. Several (n=5) FP PM-LAMP reactions were observed, with the majority (80%) occurring after 30 minutes of incubation. These FP amplification events were determined to be a result of primer dimerization, with melt-curve analysis showing non-specific T_m of 86-87°C, several degrees higher than the T_m range (84.5-85°C) achieved from specific amplicon (in the presence of target DNA). Therefore, a 30 minute incubation cut-off time was implemented for PM-LAMP reactions. Within the 30-minute cut-off, one clinical sample still resulted in a FP amplification, with amplification at 17.1 minutes and the non-specific amplicon T_m of 87°C; therefore, the SPE of the PM-LAMP assay was determined to be 98.70%. The PM-RPA assay achieved a SEN of 53.33%, amplifying only 8 of the 15 PM positive lung samples. Additionally, PM-RPA also resulted in unusually high amounts of FP results, with 10 samples deemed negative by qPCR amplifying in PM-RPA; therefore, the PM-RPA assay achieved a SPE of 87.01% (**Table 3.9**).

Table 3.9. Comparative performance of PM-LAMP and PM-RPA. The clinical performance of PM-LAMP and PM-RPA compared to qPCR across 92 clinical samples. Samples are grouped into high (H), medium (M) and negative (N) categories based on their relative copy numbers, as determined by qPCR C_t values in accordance with the PM qPCR assay standard curve.

Cat.	qPCR- C_t	DNA conc. (Copies/rxn)	Sample (n)	PM-LAMP						PM-RPA					
				TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)	TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)
H	<29.48	>2,000	10	10	0	/	/	100.0	/	8	2	/	/	80.00	/
M	29.48-33.00	250-2,000	5	2	3	/	/	40.00	/	0	5	/	/	0.00	/
L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	>33.00	<200	77	/	/	76	1	/	98.70	/	/	67	10	/	87.01
Total	/	/	92	12	3	76	1	80.00	98.70	8	7	67	10	53.33	87.01

Considering the FP observed in the qPCR PM assay, there were no positive clinical samples which could be quantified as containing less than 250 copies by qPCR. Therefore, the medium (250-2,000 copies) and high (>2,000 copies) categories were used to determine the isothermal assay performance in more detail. PM-LAMP achieved a SEN of 40% in the medium bacterial load category, with only two of the five samples detected, which displayed the two highest copy numbers in this category, 814 and 1,256 copies, respectively (**Figure 3.8A**). PM-RPA failed to amplify any of the medium category lung samples. 10 samples were characterised as containing high bacterial burden of PM, with PM-LAMP amplifying all these isolates, thus achieving a SEN of 100%. Whilst PM-RPA amplified only eight of these high copy number samples, a SEN of 80.00%. The two high copy number samples that PM-RPA failed to detect had a relative copy number of 7,517 and 41,550, respectively, as determined by qPCR (**Figure 3.8B**).

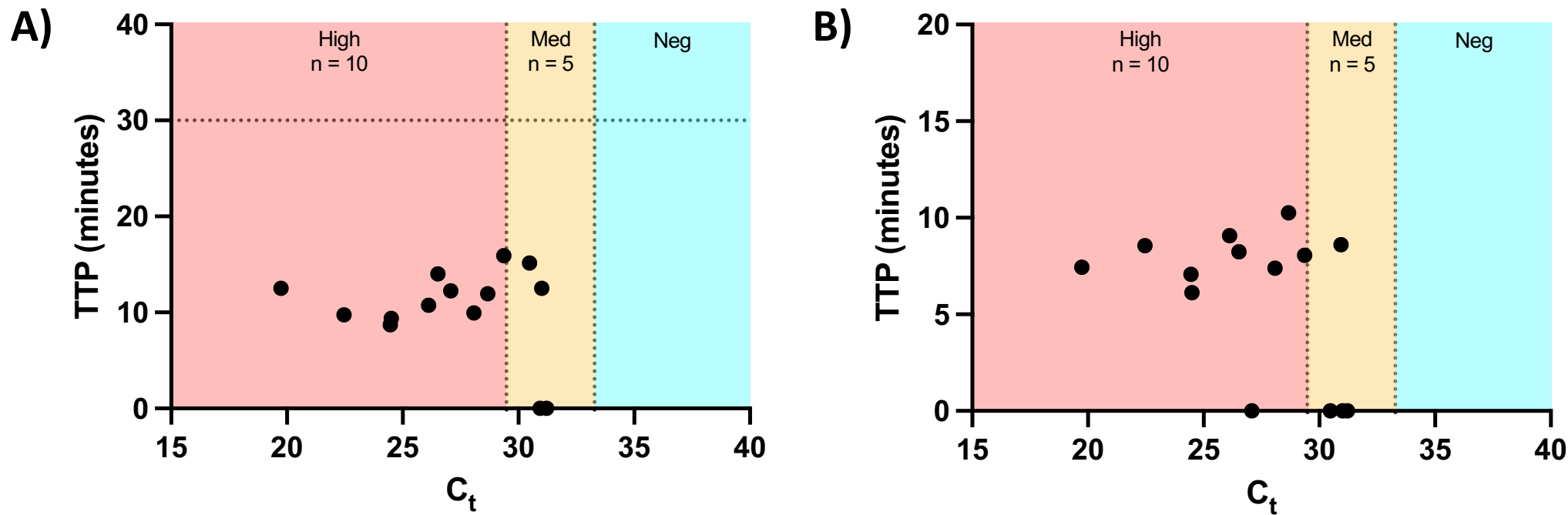


Figure 3.8. The distribution of 15 clinical samples determined PM positive by qPCR. The correlation between the C_t obtained in qPCR and the TTP achieved with PM-LAMP **(A)** and PM-RPA **(B)**. Each point represents the average TTP and C_t performed on a clinical sample in duplicate. The clinical samples are grouped into high, medium, and negative categories, shown in red, orange, and blue, respectively, based upon their relative copy number as determined by qPCR.

3.10.4. *S. suis*

Of the 162 total clinical samples, 121 were tested for the presence of SS by qPCR. 42 of the 121 samples tested were positive (34.71%), and 79 were negative (65.29%) for SS DNA. Of the 42 positive samples, SS was found in tissues originating from the lung (n=30/105, 28.57%), oral fluid (n=9, 100%), joint (n=1, 50%), intestine (n=1, 100%) and heart (n=1/4, 25%). There was no tissue type tested which did not show the presence of SS DNA in at least one of the samples tested. SS-LAMP achieved an overall SEN and SPE of 45.24% and 100%, respectively, across the 121 samples tested. The performance of SS-RPA was similar, achieving a SEN of 40.48% and SPE of 100% (**Table 3.10**).

Table 3.10. Comparative performance of SS-LAMP and SS-RPA. The clinical performance of SS-LAMP and SS-RPA compared to qPCR across 121 clinical samples. Samples are grouped into high (H), medium (M), low (L) and negative (N) categories based on their relative copy numbers, as determined by qPCR C_t values in accordance with the SS qPCR assay standard curve.

Cat.	qPCR- C_t	DNA conc. (Copies/rxn)	Sample (n)	SS-LAMP						SS-RPA					
				TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)	TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)
H	<27.4	>2,000	15	15	0	/	/	100.0	/	14	1	/	/	93.33	/
M	27.4-30.7	201-2,000	8	4	4	/	/	50.00	/	3	5	/	/	37.50	/
L	30.7-34.3	10-200	19	0	19	/	/	0.00	/	0	19	/	/	0.00	/
N	>34.3	<10	79	/	/	79	0	/	100	/	/	79	0	/	100.0
Total	/	/	121	19	23	79	0	45.24	100	17	25	79	0	40.48	100.0

When classifying the positive samples (n=42) as containing low, medium, and high copy numbers of SS, almost half the samples (n=19, 45.24%) contained less than 200 copies of SS per μ L; these 19 low bacterial burden samples contained 18 lung samples and one synovial fluid sample. The SS-LAMP and SS-RPA failed to detect a single low category sample as positive, resulting in a SEN of 0.00% for both. Eight clinical samples were characterised as having a medium copy number of SS, quantified as containing 201-2,000 copies, as determined by qPCR, consisting single samples originating from heart and intestine, and four lung samples. Of these eight samples characterised as containing medium copy numbers of SS, SS-LAMP detected four, a SEN of 50%, with all detectable samples having a relative quantification of >731 copies (**Figure 3.9A**). SS-RPA detected three of the eight medium bacterial copy samples, a SEN of 37.5%. SS-RPA had identical results to SS-LAMP at the low copy numbers, including both isothermal assays failing to detect the heart sample that had a relative quantification of 241 copies. Additionally, to the three samples that SS-LAMP failed to detect, SS-RPA also did not detect an intestinal sample containing 731 copies, as well as a lung sample containing 714 copies of SS (**Figure 3.9B**). Fourteen samples were characterised as having high bacterial copy numbers of SS, >2,000, consisting of nine oral fluid samples and six lung samples. Of the high bacterial copy number samples, SS-LAMP detected all 15, with a SEN of 100%, whilst SS-RPA detected 14, a SEN of 93.33%. The sample that SS-RPA failed to detect was a lung sample characterised as containing 6,113 copies of SS DNA, as determined by qPCR.

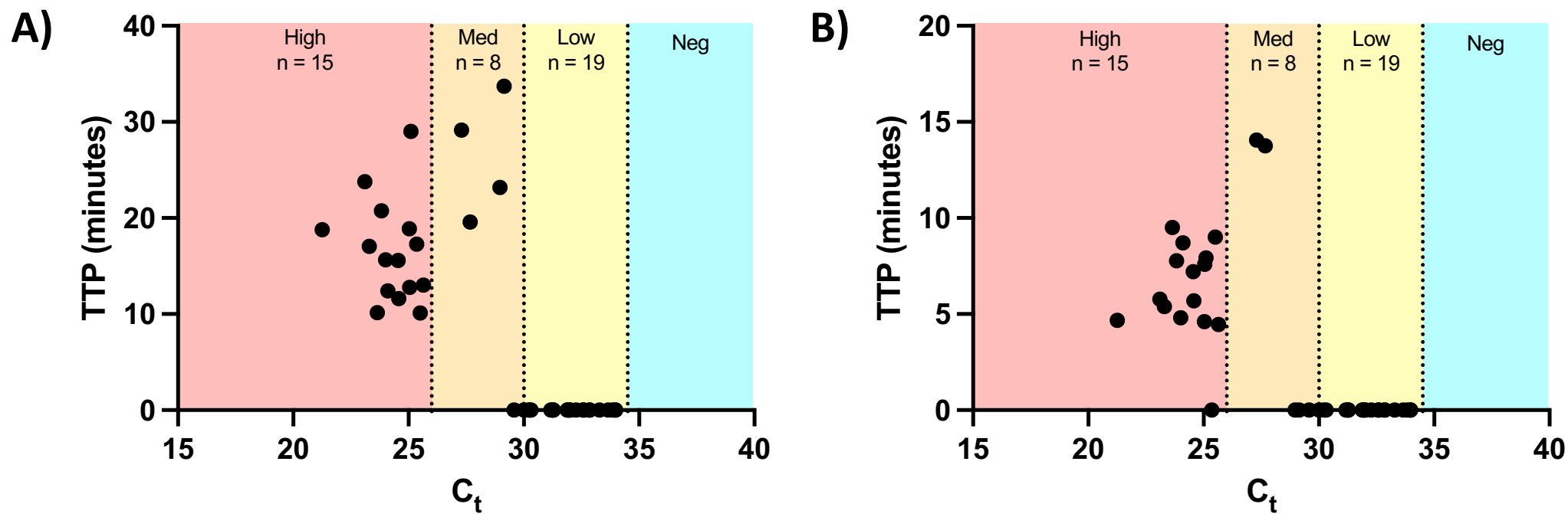


Figure 3.9. The distribution of 42 clinical samples determined SS positive by qPCR. The correlation between the C_t obtained in qPCR and the TTP achieved with SS-LAMP **(A)** and SS-RPA **(B)**. Each point represents the average TTP and C_t performed on a clinical sample in duplicate. The clinical samples are grouped into high, medium, low, and negative categories, shown in red, orange, yellow and blue, respectively, based upon their relative copy number as determined by qPCR.

3.10.5. *G. parasuis*

Out of the 162 clinical samples available, 111 were tested for the presence of GPS DNA by qPCR, with 59 of the samples displaying detectable levels of GPS DNA (53.15%), and the remaining 52 samples displaying undetectable levels (46.84%). All 23 samples originating from liver tissue and all three from oral fluid samples were positive for GPS. One of the two heart tissue samples (50%), and 33 of the 82 lung tissues tested (40.24%) were positive for GPS. The only synovial fluid sample tested was not positive for GPS by qPCR. GPS-LAMP achieved an overall SEN and SPE of 79.66% and 98.08%, respectively, detecting 47 of the 59 positive GPS samples (**Table 3.11**).

Table 3.11. The clinical performance of GPS-LAMP. GPS-LAMP compared to qPCR across 111 clinical samples. Samples are grouped into high (H), medium (M), low (L) and negative (N) categories based on their relative copy numbers, as determined by qPCR C_t values in accordance with the GPS qPCR assay standard curve.

GPS-qPCR				GPS-LAMP					
Cat.	qPCR- C_t	DNA conc. (Copies/rxn)	Samples (n)	TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)
H	<25.55	>2,000	12	12	0	/	/	100.00	/
M	25.55-28	201-2,000	19	17	2	/	/	89.47	/
L	28.01-33.1	10-200	28	18	10	/	/	64.29	/
N	>33.1	<10	52	/	/	51	1	/	98.08
Total	/	/	111	47	12	51	1	79.66	98.08

The 59 clinical samples determined to be positive for GPS DNA, by qPCR, were categorised as containing low, medium, and high bacterial loads based on their qPCR relative copy numbers. The low bacterial load category (<200 copies) contained 28 clinical samples, consisting of 18 lung and 10 liver samples. GPS-LAMP detected 18 of these samples, with a SEN of 64.29%. The low bacterial load samples which GPS-LAMP failed to detect all had a relative quantification of fewer than 50 copies, as determined by qPCR. The medium bacterial load category (201-2,000 copies) contained 19 samples, consisting of seven samples originating from the liver, 10 from the and two oral fluid samples. GPS-LAMP detected 17 of these medium bacterial load samples, a SEN of 89.47%, the two samples within this category that GPS-LAMP failed to detect were both liver samples containing 225 and 419 copies, as determined by qPCR (**Figure 3.10**). Twelve of the clinical samples were determined to be of high bacterial load, containing more than 2,000 copies per μ L, as determined by qPCR. These high bacterial load samples comprised seven originating from lung tissue, three from liver tissue, and one each from oral fluid and heart tissue. GPS-LAMP detected all 12 of these samples, with a SEN of 100%.

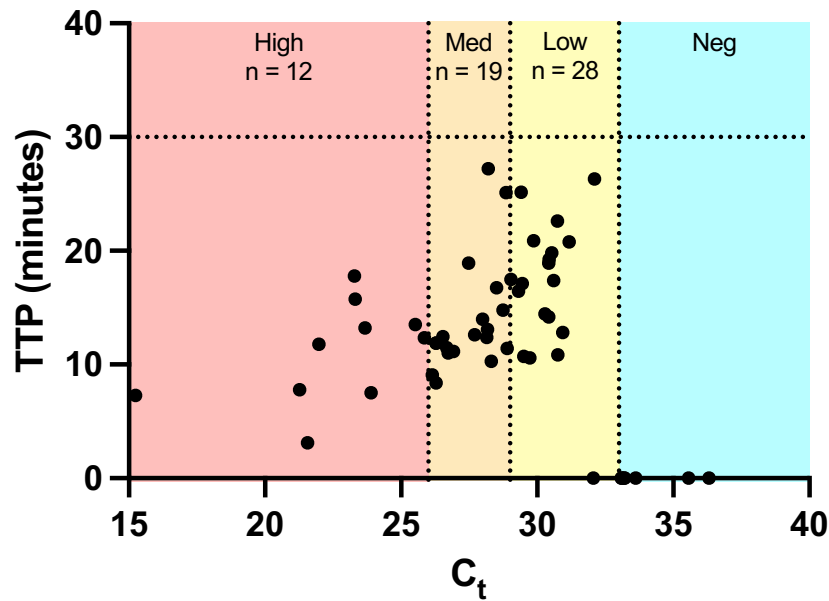


Figure 3.10. The distribution of 60 clinical samples determined GPS positive by qPCR. The correlation between the C_t obtained in qPCR and the TTP achieved with GPS-LAMP. Each point represents the average TTP, and C_t performed on a clinical sample in duplicate. The clinical samples are grouped into high, medium, low, and negative categories, shown in red, orange, yellow and blue, respectively, based upon their relative copy number as determined by qPCR.

3.11. Result summary

To our knowledge, this is the first study to extensively evaluate the comparative clinical performance of RPA and LAMP assays against bacterial agents of the PDRC. Our principal finding is that LAMP and RPA have similar clinical performance in comparison to qPCR. The LAMP assays detecting PM and SS displayed a higher clinical SEN (80.00% and 45.24%, respectively) compared to RPA (53.55% and 40.48%, respectively). Whilst two RPA assay, detecting APP (3') and MH produced higher SEN (84.31% and 78.95%, respectively) than that achieved with the LAMP assays (80.39% and 71.05%, respectively). The last comparator assay showed identical clinical SEN in both RPA and LAMP detection (APP 76.81%). All comparator results between these isothermal methods are listed in **Table 3.12**.

Table 3.12. Direct comparison between LAMP and RPA results.

Target species	Samples (n)	LAMP		RPA	
		SEN (%)	SPE (%)	SEN (%)	SPE (%)
APP	111	76.81	97.62	76.81	100
APP-3'	61	80.39	100	84.31	100
MH	113	71.05	100	78.95	100
PM	92	80.00	98.70	53.33	87.01
SS	121	45.24	100	40.48	100

Discussion

A core element in determining that an IVD test is fit for use is to assess the clinical performance against the gold standard. In this Chapter, I sought to determine the clinical SEN and SPE of our favoured assays (see **Chapter 2**) targeting the five bacterial pathogens involved in the PRDC. Overall, I found that neither isothermal amplification method performed better than the other across the four bacterial target pathogens.

The PRDC is a complex disease with several pathogens which can singularly or in combination contribute to the severity of the disease. In this study, I investigated by qPCR 67 clinical lung samples for the presence of the top five bacterial pathogens that contribute to the PRDC, 62 of which were present for at least one pathogen. To our knowledge, this is the most in-depth analysis of the prevalence of bacterial lung co-infection performed on swine in the UK and, including copy number, worldwide. I found that a major contributor to lung bacterial infections is APP (88.71%), which was seen at the highest prevalence in lungs positive for one and two bacterial species. A previous study into the presence of pig lung bacterial pathogens in Canada also found a high prevalence of APP (78%) (MacInnes et al., 2008), a finding that was mirrored in Finland (80%) (Haimi-Hakala et al., 2017). Recently, a study in China found the overall prevalence of SS in lung samples of asymptomatic pigs was also very similar to our findings, 26.6% and 24.19%, respectively (Zhu et al., 2021). However, the instance of APP in lungs was greatly reduced, with 1.1% of the 376 lung samples tested being positive, whilst our rate of APP incidence was 88.71%. Therefore, whilst the presence of APP may not contribute to the instance of SS being found in the lungs, a correlation might exist between co-infection with SS and APP that contribute to clinical findings. I also found that PM is unlikely to cause disease alone, and contributed least to bacterial coinfections, only present when at least two other pathogens were detected, at a frequency of 22.58%. Similar to our findings that PM had an overall low prevalence, Kim et al. (2019) also found that PM was present in Korean lung tissue at 16.8%, which was most likely to be associated with viral co-infection with PRRSC or PCV2 (61.3% and 37.5%, respectively) (Kim et al., 2019). Overall, our findings demonstrate that multiple bacterial pathogens are often present in a single lung sample. Therefore, there is a need to determine the overall pathogen landscape as it evolves on farm to equip veterinarians with the diagnostic power to rapidly react to PRDC outbreaks.

RPA and LAMP displayed an identical SEN when detecting APP, 76.81%. The breakdown of the samples into high, medium, and low bacterial categories, based on their relative quantification by qPCR, showed that APP-LAMP failed to detect only one sample of high bacterial load and three at medium. This could be due to these samples being particularly inhibitory to the LAMP reaction. However, this seems unlikely given the number of publications performing LAMP directly on crude samples (Papadakis et al., 2022, Kundrod et al., 2022, Wang et al., 2017c, Hayashida et al., 2017). Additionally, the original description of this primer set, without the inclusion of loop primers, found the primers achieved a 100% SEN across 167 tonsil samples compared to PCR (Ji et al., 2012). Therefore, a more likely explanation for FN results of APP-LAMP at higher copy numbers is primer-template discrepancies, as LAMP requires more primers and, therefore, a greater length of the target region to be conserved. Reports, however, on how the efficiency of the LAMP reaction is affected by primer-template mismatches are conflicting. Wang (2016) reported that mismatches of the 3' end of BIP more than doubled the

TTP, this is contrary to Tamanaha et al. (2022) findings that little change (5-10%) in reaction speed for a single mismatch on one of the LAMP primers (Wang, 2016, Tamanaha et al., 2022).

The SEN of RPA increased in a smaller subset of clinical samples used to detect APP with the 3'-RPA assay (84.31%) across 61 clinical samples, as previously reported (Stringer et al., 2021). The 3'-APP RPA assay also had a higher clinical SEN, compared to the original APP-RPA primer set (82.35%) and the APP-LAMP primer set (80.39%), when compared across paired samples. This is due to the original APP-RPA primer set containing multiple priming sites targeting a relatively repetitive region of the *apxIVA* gene, whilst the 3'-APP-RPA primer set was designed against a highly conserved and less repetitive 3' region of the *apxIVA* gene (Srijuntongsiri et al., 2022). Therefore, the increase in clinical SEN compared to the original APP-RPA primer set mirrors the findings of the analytical SEN (described in **Chapter 2**), i.e., is likely a result of less primer being sequestered by alternative primer site binding, and therefore increased amplification efficiency.

In the detection of MH across the 48 samples that were positive for MH by qPCR, MH-RPA displayed a slight increase in SEN (7.9%) to MH-LAMP. In contrast to APP, MH-RPA failed to detect a single sample in the high copy number category, whilst MH-LAMP detected all 18. The inability of MH-RPA to detect high copy number samples suggests that the target region for the MH-RPA assay may contain SNPs or deletions in the target region. It is important to note that the MH-LAMP, MH-RPA, and the qPCR assay target different genes, *mhp165*, *p93* and *mhp183*, respectively. There have previously been reports of published PCR assays failing to reliably detect MH isolates (Strait et al., 2008). Therefore, a qPCR assay was chosen, which targets the P97 cilium adhesin (*mhp183*) known to be a major contributor to host cell adherence (Clampitt et al., 2021, Hsu et al., 1997, Zhang et al., 1995). Furthermore, the qPCR assay chosen in this study has been reported to display high SEN to MH (Strait et al., 2008). A drawback of using an assay which targets different genes than the isothermal assays being assessed is that it is difficult to ascertain if FN are caused by SNPs or genomic deletions. Such is the case with the MH-RPA assay which failed to detect a single high copy number isolate, which may have arisen from genomic alteration within the target region of the *p93* gene. Although whole genome sequencing would have to be undertaken to determine if sequence differences was the cause of the FN in RPA.

In the medium copy number category, MH-LAMP performed much worse than MH-RPA, failing to detect six samples, whilst MH-RPA only failed to detect two. The MH-LAMP assay targets the *mhp165* gene reported to contain numerous SNPs (Strait et al., 2008). However, when the MH-LAMP assay was published, the authors found the primer set had 100% SEN across 78 lung samples (Li et al., 2013). A potential source of the discrepancy between the results achieved in this Chapter and those achieved in the original publication, is that the MH-LAMP assay and the qPCR comparator assay used by Li et al. (2013) both target the same region of the *mhp165* gene (a 55 bp overlap between target regions), which may cause FN from both assays if regions of the gene are disputed in some clinical isolates.

In the low copy number category, both MH-RPA and MH-LAMP performed identically. However, the samples detected were not identical. MH-RPA detected an oral sample with a low copy number (44 copies), whilst the lowest copy number sample MH-LAMP was able to detect was 140 copies (lung). Only a small number of MH-positive clinical samples were characterised as containing low copy number ($n=7$), making it difficult to draw direct comparisons between the isothermal techniques. Additionally, both isothermal MH assays displayed sporadic amplification of these samples, with no apparent trend relating the copy number to TTP, indicative that both assays are near or at their LoD. Therefore, it can be concluded that the clinical SEN of MH-RPA and MH-LAMP are equivalent. Additionally, neither assay displayed FP amplification and thus are highly specific to MH.

Whilst the SS-LAMP displayed a higher SEN than the SS-RPA assay in the detection of SS, 45.24% and 40.48%, respectively, both assays displayed the lowest SEN seen among all the assays. The comparator assay used for SS detection was a qPCR assay targeting the *fbpS* gene, which was published for the detection of human infections (Srinivasan et al., 2016), as SS is considered an important zoonotic agent capable of causing meningitis and sepsis in humans (Gottschalk et al., 2010). SS serotype 2 is the major contributor to human infections and is frequently associated with severe disease in European pigs (Wisselink et al., 2000). At the time of writing, no qPCR assay has been described for the detection of the target gene chosen in this study, *recA*; a housekeeping gene found to have a low number of polymorphic nucleotides (King et al., 2002). Therefore, this is the first example of SS being characterised in swine with this qPCR primer set.

The low SEN of SS-LAMP and SS-RPA may be due to the sampling effect, with the dataset slightly skewed towards the lower copy number isolates ($n=19$), with few assigned to medium copy number ($n=8$). Another possibility is that the *recA* gene is less conserved than previously reported. This is unlikely due to both SS-LAMP and SS-RPA displaying linear trends in the high and medium copy number categories, as expected as C_t increases in qPCR, as does the TTP in the isothermal assays. Neither the SS-RPA nor the SS-LAMP assays were able to detect any of the samples characterised as containing low burdens of SS, as determined by qPCR. In the medium copy number categories, both the SS-RPA assay could not detect clinical samples which displayed a C_t above 28.95, which equates to 1,635 copies, and the SS-LAMP assays could not detect C_t values above 29.57, equating to 342 copies. If the *recA* gene is indeed a poor target, it would be expected that more isolates of the high and medium copy number would be undetected. The in silico inclusivity results from **Chapter 2** also refute the idea that *recA* is poorly conserved, as although only 60.2% pairwise identity was found across the 1,662 bp *recA* gene, the LAMP target region displayed high pairwise identity (98.6%).

A major complexity in assessing assay performance is the relation to clinical disease. The majority of the molecular methods to detect SS in lung samples are PCR-based (Liu et al., 2013), which are non-real-time and therefore have limited ability to quantify the abundance of the bacteria present in the sample. A qPCR assay to detect SS via the 16S rRNA gene has been described but has only been validated on environmental samples (Bonifait et al., 2014, Okwumabua et al., 2003). One study did describe qPCR detection in tonsil samples, finding that qPCR had increased SEN over culture methods (Dekker et al., 2016). However, clinical samples were

obtained from experimentally infected pigs, which are typical of high herd status, and the results are of less clinical relevance. Therefore, there is limited information available to ascertain how the copy numbers determined in this study relate to the clinical manifestations of the disease.

The largest difference between these two isothermal techniques was in the detection of PM where LAMP had an increased SEN of 26.67% over RPA, even though both LAMP and RPA assays targeted the same gene within the PM genome (*kmt1*). Both assays, as well as the qPCR assay, had difficulties with FP results. The qPCR assay consistently produced amplification at C_t 32.5±0.5 in the absence of template, and therefore the cut-off for a positive result by qPCR was a C_t of 33 and below, equating to 250 copies and above. Thus, there was a very small number of samples that were positive by qPCR (n=15). Even with this small sample set, the number of positive samples that were missed by the PM-RPA assay was unexpectedly high, detecting just over half of the positive samples (n=8). PM-LAMP did display FP amplification after 30 minutes of incubation, easily determined by the higher melting temperature of these amplification events (T_m of 87°C, rather than 84.5°C). The published PM-LAMP assay did not report any FP amplification events; however, it should be noted that fluorescent detection was not assessed, and the incubation time was limited to 30 minutes. The FP amplification events may be a result of the continued contamination issues seen with the PM assays, supported by FP events also detected in PM-RPA. However, as RPA uses an Exo probe detection of amplification events, melt curve analysis is not possible. Another possibility contributing to increased FP RPA amplification is that the technique displays higher SEN and is thus more prone to contamination issues. However, the limited number of positive amplification events among qPCR positive samples suggests this is not a viable possibility.

Of the samples that were positive by qPCR, PM-LAMP detected all samples above a C_t of 31.21, equating to 485 copies and above, whilst PM-RPA struggled to detect samples above a C_t of 30.00, quantified as copies and above. A study of dual infection detecting PM via the *kmt1* gene, using a different qPCR assay than used in the present study, but targeting the same gene, determined that at the time of sampling in the acute, subacute, and chronic phases of the disease, the amount of bacterial DNA present was 21, 50 and 180.9 copies, respectively (Dutra et al., 2013). Therefore, our assay performance at these copy numbers would be very poor, being within the medium copy number group, which only displayed a SEN of 40%. This suggests our isothermal assays would only be capable of detecting cases of PM infection where the disease is chronic and at high abundance in the tissue.

The detection of GPS was the only clinical study I undertook which did not have an RPA comparison, whilst a GPS-RPA assay has been previously reported, using spiked synovial fluid samples to determine the clinical SEN of the assay (Zhang et al., 2019e). I did not evaluate this GPS-RPA assay due to time constraints and the limited availability of RPA reagents. Therefore, only the clinical performance of the GPS-LAMP assay was investigated. Similar to the PM-LAMP assay, the GPS-LAMP assay showed non-specific amplification beyond 30 minutes in clinical samples, which could be readily distinguished from specific amplification via melt curve analysis, where the T_m of specific amplification was 83.5°C. Therefore, a cut-off was introduced to prevent FP results from

impacting the SEN of the assay. The GPS-LAMP assay achieved an overall SEN of 79.66% across the 111 samples tested, which was among the highest SEN achieved in the LAMP assays. When divided into low, medium, and high copy number categories, almost half of the GPS-positive samples were assigned to the low category (n=29/60). However, GPS-LAMP was the most sensitive LAMP assay at low copy numbers detecting 64.29% of the positive samples in this category. Clinical manifestation of GPS includes widespread inflammation in tissues, as detected by both molecular and histopathological methods, including liver, spleen, tonsil, lung, synovial, and heart tissue (Oliveira et al., 2001). Therefore, to evaluate clinical testing for GPS, a large range of samples were evaluated, including 82 lung samples, 22 liver, 3 oral fluid, 2 heart and 1 synovial fluid sample. Most of the lung samples were negative for GPS by qPCR (n=49), whilst all liver samples were positive, with the majority being assigned to low (n=10), medium (n=9) copy number categories. Only three were assigned to the high copy number category.

To my knowledge, this is the first study to extensively evaluate the comparative clinical performance of RPA and LAMP assays against bacterial agents of the PDRC. Our principal finding is that LAMP and RPA have similar clinical performance in comparison to qPCR. Two of the assays displayed a higher clinical SEN in LAMP (SS and PM assay), and two displayed a higher SEN in RPA (3'-APP and MH), whilst the last assay showed identical clinical SEN (APP). With the exception of PM, RPA and LAMP performed within 10.2% of one another, with an average difference (excluding PM) of 5.70%. The occurrence of FN results may be due to inhibition of the amplification chemistry by the sample, degradation of the sample, the analyte being at a lower concentration than detectable by the chemistry (below the LOD), or variation in the target sequences leading to inefficient amplification, or a combination of any four of these. Therefore, it is important to perform extensive testing on clinically relevant tissues to ascertain the SEN of the assay. LAMP requires 4-6 regions of homology for primer placement, therefore requiring a 100-200 bp section of highly conserved DNA (Nagamine et al., 2002, Notomi et al., 2000). Additionally, the primer placement in LAMP is highly detailed, with parameters surrounding how each primer can be placed relative to the next, which can be especially difficult in target genes which contain high AT content, such as MH (Minion et al., 2004). Whereas RPA requires three regions (or two if no probe is used), which are relatively shorter and have fewer constraints on the amplicon size, although, like PCR, the shorter the amplicon, the more rapidly the amplification cycles can occur. Additionally, it has been reported that RPA displays high tolerance for mismatches between primer and template, as long as the mismatch does not occur at the 3' end of the primer (Boyle et al., 2013, Daher et al., 2015).

Few other studies have described the relative clinical performance of RPA and LAMP assays. Once such study on the detection of foot and mouth disease virus concluded that RPA exhibited decreased SEN (77%), compared to the LAMP assay (88%), with 43 clinical samples tested (Howson et al., 2017), a clinical SEN which is not dissimilar to the values I report. Therefore, the clinical performance of LAMP and RPA assays appears to be heavily reliant on the specific assay being used, with no clear advantage in terms of clinical SEN of one isothermal amplification technique over the other.

I found all of the published assays tested had a lower clinical SEN than has been previously claimed (**Table 3.13**). Whilst in some assays, loop primers were designed to increase the reaction kinetics, the concentration of the other primers remained the same. Therefore, this is unlikely to result in adverse effects on SEN. However, increasing the total primer in the reaction may result in decreased SPE.

Table 3.13. The clinical performance of published LAMP assays. The clinical performance achieved in this study from previously published LAMP assays, against their reported clinical performance.

	This Chapter			Previously reported						
	SEN (%)	SPE (%)	n	SEN (%)	SPE (%)	Detection method	n	Tissue types	Comparator	Reference
APP*	76.81	97.62	111	100	100	Electrophoresis	167	Tonsil	PCR	(Ji et al., 2012)
MH	71.05	100.00	123	100	100	Colorimetric (SYBR Green I and HNB)	78	Lung	qPCR	(Li et al., 2013)
PM	80.00	98.70	92	Not reported	Not reported	Colorimetric (SYBR Green)	N/A	N/A	PCR	(Bhimani et al., 2015)
SS*	45.24	100.00	121	Not reported	Not repeated	Turbidity	996	Meat	PCR	(Arai et al., 2015)

*Notation signifies deviations from the published assay, by the inclusion of loop primers to increase reaction kinetics.

An important consideration when determining the performance of an assay is the SPE. In contrast to the results in **Chapter 2**, I observed an increase in FP events when using clinical samples. RPA exhibited 100% SPE in all but one assay (PM). The inclusion of the Exo probe in RPA to monitor amplification has been found to increase SPE (TwistDx Ltd., 2019, Zou et al., 2020) but can also be the cause of FP amplification if primer/probe interactions occur. Therefore, the primer/probe combinations must be rigorously screened (TwistDx Ltd., 2019). LAMP exhibited an increased rate of FP in two assays after incubation for greater than 30 minutes. The T_m of these events was consistently higher than the T_m of specific amplification events - reported to occur with *Bst* 2.0 DNA polymerase (Hardinge and Murray, 2020, Rolando et al., 2020). I also encountered one non-specific amplification that occurred within the 30 minute amplification in the PM-LAMP assay; this is likely due to the stochastic nature of LAMP amplification, with the initial amplification event required for self-concatenation of the LAMP reaction taking variable times to occur.

A major advantage of RPA over LAMP is the speed of the amplification, although all of the LAMP primer sets tested contained both loop primers sets, known to increase the reaction time (Nagamine et al., 2002) (see **Chapter 2**). Regardless, in the clinical samples that were positive for both RPA and LAMP (n=122), RPA was often at least twice as fast, 1.51x, as the LAMP reactions. These results mirror what has previously been found in the literature (Diagne et al., 2020, Zou et al., 2020).

Therefore, I conclude that in a clinical application, both LAMP and RPA fulfil the low time-to-result (< 30 minutes) requirement of a rapid diagnostic. However, when assessing the performance of a chosen amplification chemistry, it is important to conduct clinical testing of large sample sizes against a gold-standard technique, not

only to determine clinical SEN but also allows the assessment of inclusivity of the primer set, as well as potential cross-reaction and SPE that can only be determined by large-scale clinical testing.

Our assessment of the qPCR assays used suggests a high SEN, with 5-20 copies per μL of the sample. A major drawback to this study is having limited access to the clinical information relating to the tissue samples. Gaining insight into how the copy number relates to the clinical disease is difficult, as previously published studies often report C_t values (Goecke et al., 2020), which vary based on the assay and PCR reagents used and therefore not easily translational across studies. It is easier to draw comparisons across studies which report the relative copy number found in the clinical tissue; highlighting the need to have a standardised reporting method for clinical data. However, even if all studies reported their results in terms of copy numbers, or CFU per mL, differences still exist based on the efficiency of the sample preparation method chosen. The sample extraction method can be heavily reliant on the sample type, input tissue weight, extraction method and elution volume. Additionally, qPCR does not differentiate between live and dead bacterial cells, and therefore the use of culture still warrants evaluation, especially in bacterial pathogens where susceptibility testing to antibiotics is paramount. It is therefore likely that this PoC diagnostic test will be utilised as a diagnostic aid, to determine the pathogens present but subsequent follow up diagnostic testing will be performed to gain more information such as determining the serotype and antimicrobial sensitivity testing.

To aid in our analysis of the performance of each assay, and in the absence of clinical information, I assigned arbitrary copy number categories based on the relative copy numbers, as determined by qPCR. The parameters of each category, low, medium, and high, were based on the distribution of copy numbers across all sample sets, with the aim to have an equal distribution falling into each category. The copy numbers chosen were <200, 201-2,000 and >2,000, to mirror 10-fold increases to copy numbers, as quantified by qPCR. However, these numbers have no foundations from clinical presentations. Therefore, the performance of each assay may differ to what is obtained if a field trial was carried out. However, this was beyond the scope of this work. Additionally, I envision the PoC diagnostic device being utilised by or under the supervision of veterinarians, and therefore test results will be considered alongside clinical presentation to aid in the diagnosis and inform treatment decisions to combat the PRDC pathogens.

3.12. Conclusions

My findings suggest that co-infection within farm settings, at least in the UK, may be more common than previously thought, potentiating the need for a rapid IVD that can determine the scale of the issue. Our results indicate that a rapid diagnostic would contribute to both the rapid treatment of infection but also in identifying potential shifts in prevalence across the country.

Determining the clinical SEN and SPE remain important factors in ensuring the device is implemented appropriately. The results in this Chapter indicate that neither LAMP nor RPA provide adequate SEN at “low”

bacterial loads. Although the clinical relevance of our arbitrary “low” category remains unknown, the inability to detect low copy numbers would suggest the assays assessed in this Chapter are unsuitable for screening or surveillance situations. This assumes that in sub-clinical cases of infection, the abundance of pathogenic material would be low. Therefore, in the first instance our PoC test would result in high SEN in the cases of active infection where samples have clear clinical signs to aid in the differentiation of pathogens, like the recommended use for Abbotts ID Now.

Chapter 4: Optimisation of LAMP reactions

Introduction

Current diagnostic practices require samples suspected to contain PRDC pathogens to be collected from farms or abattoirs and sent to centralised laboratories for testing. Overall, the identification of pathogens present in a sample can take from days to weeks. The availability of a PoC device to detect the PRDC pathogens would offer significant improvements over the current diagnostic process, enabling veterinarians to diagnose the causative agent rapidly and, if necessary, administer appropriate antibiotics. A key factor in the creation of a PoC device is the ability to deliver a result in a timely manner. Therefore, the focus of this Chapter is to optimise the LAMP biochemistry to minimise the reaction time, and to produce LAMP reactions that are more amenable to on-farm PoC use.

Amplification with LAMP requires the use of a strand-displacing DNA polymerase to separate downstream dsDNA without the requirement of high temperatures (Gill and Ghaemi, 2008, Zhao et al., 2015b). Several DNA polymerases exhibiting strong strand displacement activity have been described, including DNA polymerases from *Bacillus stearothermophilus* (*Bst*), *Bacillus smithii* (*Bsm*) and PyroPhage 3173. Most polymerases consist of two enzymatic activities, a 5'-3' polymerase and a 5'-3' exonuclease. The 5'-3' exonuclease activity decreases the rate of errors during DNA replication (Bialek and Grosse, 1993). However, for amplification to occur, the exonuclease moiety is rendered unfunctional either by complete removal or alterations made to the exonuclease domain, denoted as exo^- . The strand-displacing activity of different polymerases can vary widely as can the catalytic efficiency, with both impacting the fidelity and rapidity of the LAMP reactions. Therefore, in this Chapter, several different polymerases were investigated to identify the optimum for use.

Over fifty years ago, reverse transcriptase's (RTs) were first discovered in retroviruses, required to transcribe viral RNA into DNA for integration into the host genome (Temin and Mizutami, 1970, Baltimore, 1970). Many commercially available RTs included in RT-PCR kits are mesostable (i.e., thermolabile) and active at lower reaction temperatures of 37-42°C. Such RT-PCR protocols require a pre-incubation step at 37-42°C for the RNA template to undergo transcription, referred to as two-step protocols. Performing the RT step at 37°C results in a low rate of polymerisation as the RNA adopts secondary structures at low temperatures (Klarmann et al., 1993). Therefore, the production of cDNA at a higher temperature will reduce the degree of RNA secondary structure, thus improving the polymerisation rate and cDNA production. However, it should be noted that the DNA polymerase and RT can compete for the same target and decrease the SEN of the reaction compared to a two-step approach (Sellner et al., 1992, Monazah et al., 2018).

During the past decades, extensive efforts have been made to increase the thermostability of the retroviral RTs (Arezi and Hogrefe, 2009, Baranauskas et al., 2012, Yasukawa et al., 2010, Konishi et al., 2014), whilst the strategies for one-step PCR often centre around the inclusion of a thermostable polymerase with induced or increased RT activity, ensuring the enzymes can withstand repeated thermocycling. The efforts for one-step RT-LAMP have been less extensive, as most strand-displacing DNA polymerases exhibit some degree of RT activity (Shi et al., 2015b). However, the addition of a dedicated RT enzyme has been shown to increase the overall rate

of the LAMP reaction (NEB, 2016). Therefore, the conversion of RNA into cDNA is a major rate-limiting step for the initiation of LAMP amplification. As such, the selection of a DNA polymerase-compatible RT is an essential consideration for the detection of RNA targets in the LAMP reaction.

During cDNA and DNA synthesis, DNA polymerase and RT both display requirements for a metal ion co-factor and dNTPs. For both enzymes, the metal ion co-factor acts in a two-metal ion system, coordinating the free-energy transfer from the 3'-OH primer terminus with the incoming nucleotide binding (Beese and Steitz, 1991, Steitz et al., 1994). Therefore, for both the DNA polymerase and RT, the concentration of metal ion cofactors and free dNTPs relate to the processivity of the LAMP reaction. Typically, commercial reaction buffers are supplied with specific components to ensure the optimal performance of the enzyme. For instance, DNA polymerases requirement for a metal cofactor is often provided in the reaction buffer as MgSO_4 . Other components frequently provided in the reaction buffer include: KAc, which minimises the repulsion between the negative charge of the primer and template. $(\text{NH}_4)_2\text{SO}_4$ increases the SPE of the polymerase by destabilising weak hydrogen bonds between mismatched DNA templates. Previously, chemical additives have been found to enhance the SPE or efficiency of PCR, such as the crowding agent PEG, formamide, which reduces non-specific amplification, and chemical enhancers such as 2-pyrrolidone, very few have been trialled in LAMP (Özay and McCalla, 2021). One such PCR additive, BSA, which has been shown to result in increased PCR yield (Woide et al., 2010, Tarrand et al., 1978), has recently been associated with conflicting results in LAMP. One publication suggested 1 mg/mL of BSA was optimal for LAMP amplification (Lin et al., 2019), whilst another found BSA had a negative effect on the SPE of LAMP (Gao et al., 2019). Therefore, in this Chapter I investigated the impact of chemical additives ($n=14$), previously shown to be beneficial in PCR, on the TTP of LAMP reactions.

Historically, LAMP has a reputation for producing non-specific amplification; however, as shown in **Chapter 2** and **Chapter 3**, this can be minimised or completely prevented with careful primer design. More frequently, the leading cause of FP amplification in LAMP is the carry-over of amplicon from completed into subsequent LAMP reactions, known as contamination. To minimise carry-over contamination events, uracil-DNA glycosylase (UDG) has been used in PCR and LAMP (Longo et al., 1990, Hsieh et al., 2014). UDG is an enzyme that initiates the base-excision repair pathway by cleaving the N-glycosidic bond between the uracil and sugar backbone of DNA (Sidorenko et al., 2008). Thus, by replacing a proportion of the free nucleotides in the LAMP solutions with dUTP nucleotides, the amplicon will contain the relevant amount of dUTP bases instead of dTTP. In subsequent LAMP reactions, UDG cleaves the amplicon at dUTP residues preventing any previous amplicon from being amplifiable by the DNA polymerase. Conventional UDG is heat-inactivated at 95°C for 10 minutes, with some reported activity remaining after inactivation. Therefore, homologues of UDG that are heat-labile, with inactivation occurring at 40-50°C to ensure no UDG activity remains during the LAMP or PCR amplification step (Jaeger et al., 2000, Sobek et al., 1996). Therefore, in this Chapter, I investigated the feasibility of incorporating a UDG step in the LAMP reaction.

The utilisation of LAMP on the CMOS-ISFET system enables a readably scalable platform at an affordable price. Therefore, for the platform to become a viable product to rival the use of PCR, the system must be amenable for use at the PoC. One of the limitations of the LAMP chemistry is that the reaction mix requires a cold chain, therefore not satisfying the ASSURED WHO criteria of being equipment free and deliverable to end users (Land et al., 2019). As such, the development of a LAMP reaction which is stable without reliance on cold-chain storage is paramount to the uptake of the product in settings where electricity is limited, such as on a farm.

Several strategies can be implemented to increase the ambient temperature stability of biological systems, namely dehydration by applying heat to the reaction to evaporate off the water (also known as air-drying) (Hayashida et al., 2015) or by lyophilisation. Both processes require the addition of stabilising molecules, known as excipients, for biological activity to be retained (Crowe et al., 1992). Lyophilisation has become the most common strategy to enable the transportation and distribution of several amplification chemistries, as well as biological samples and pharmaceutical products. The lyophilisation process occurs in three main stages, product freezing, primary drying and secondary drying. The product freezing can occur at different temperatures, with slower cooling resulting in larger ice crystals which allows the formation of channels allowing migration of vapour during the subsequent drying phases; however, slow cooling can also produce a surface skin which inhibits the following drying phases (Adams, 2007). In the primary drying phase, the frozen solute is placed under a high vacuum and kept at a constant temperature during which the water molecules (ice) within the solution convert from a solid to a gas without passing through the liquid state. The majority (95%) of the product is dried during the primary drying phase by sublimation, where unbound water molecules are removed from the reaction. In the secondary drying phase, desorption occurs, where the bound or residual water molecules are removed. Heat is applied to the product, and the pressure is decreased, resulting in bound water molecules disassociating.

Thus, I sought to develop the LAMP reactions in a ready-to-use format that can be deployed without cold-chain storage for use in field conditions. Lyophilised LAMP kits have already been commercialised for the detection of several human pathogens, including tuberculosis and malaria (Human Diagnostics Worldwide). However, to my knowledge, no amplification chemistry has been lyophilised in a format that is compatible with the electrochemical sensing, such as the CMOS-ISFET platform.

4.1. Aims

In this Chapter, my aim was to optimise LAMP reactions to provide decreased TTP and increase SEN in a composition that is suitable for lyophilisation. The optimisation of the LAMP reactions involved the evaluation of alternative DNA polymerases and RTs, and the inclusion of several additives in the LAMP mixture. Additionally, I aimed to investigate the feasibility of including the contamination control enzyme, UDG, with focus on how inclusion of this enzyme effects the reaction speed, and the amount of contamination that can be successfully removed by using the UDG system.

Methods

4.2. Genetic material

The genetic material used in this Chapter includes gDNA extracted from two bacterial isolates, APP reference serovar 8, MIDG 2220 and MH reference strain 232, cultured and extracted as previously described in **Chapter 2: Section 2.1 and 2.2**. For RT-LAMP assays targeting BVS and SIV, RNA transcripts of target genes were transcribed from synthetic DNA sequences as described previously in **Chapter 2: Section 2.2**. The SARS-CoV-2 RT-LAMP reactions contained synthetic RNA construct GenBank accession: MN908947.3 (Twist Bioscience, Cat 102024). The SARS-CoV-2 LAMP reactions contained *N* gene positive control DNA (IDT, Cat 10006625). The FluA and FluB RT-LAMP assays contained synthetic viral RNA from influenza A/H1N1 and influenza B, respectively (Twist Bioscience, Cat 103001 and Cat 103003, respectively). The LAMP assays targeting respiratory syncytial virus (RSV), subtypes a and b, and the assay targeting the human rhinovirus (HRV) contained synthetic viral RNA produced by Dr Kenny Malpartida-Cardenas (Imperial College London). The PM8 LAMP assay, targeting human gDNA (hgDNA), contained male hgDNA obtained commercially (Promega, Cat G1471).

4.3. LAMP amplification

4.3.1. LAMP primers

LAMP primers used in this study were produced by either IDT or Thermo Fisher Scientific at 100 nM scale using standard de-salting purification. All primers used in LAMP reactions were added at total primer concentrations of 0.25 μ M F3, 0.25 μ M B3, 1 μ M LF, 1 μ M FLB, 2 μ M FIP 2 μ M BIP. In instances where more than one sequence is given for a primer, denoted -1 and -2, the total primer concentration was as specified above (e.g., 1 μ M FIP-1 and μ M FIP-2, for a total of 2 μ M FIP primers). All primers used in this Chapter are shown in **Table 4.1**.

Table 4.1. Primers used in Chapter 4.

Target organism	Primer ID	Sequence 5' – 3'	Reference
APP	APP_F3_A	CTGAAAGTTTCGTCAGCAC	(Yang et al., 2009)
	APP_B3_A	TCTGAAACTGAAAAGTATCCAC	
	APP_LF_A*	TTTTTGTGGTAACGTAC	
	APP_LB_A*	GACGAAACTATTGCAAAAAATCGCG	
	APP_FIP_A	GCTCGAAATCCGGGTATATCCT-ATTTAATTGATAAACACGGTGTG	
	APP_BIP_A	TCGTTTAAAAGCGTTACAAGAGGA-AACTGAAGACAAGCGGTA	
MH	MH_F3_B	TGTTGAGATCGCATCAAGAA	(Li et al., 2013)
	MH_B3_B	CCTACCAAACAAAACAAGGTAGT	
	MH_LF_B	CCAAACACTAAATTATGCC	
	MH_LB_B	GGAGGCTTTTAAAAGGG	
	MH_FIP_B	GGCGCTCATGAATATGGTCATCTTTTCTGGATCGGAAATATCCTG	
	MH_BIP_B	TTCCCGTAGTTTTAAAAAATGGCGTTTTCCAATCATCCGAATTCCTT	
SIV	SIV_F3_B	TTTCTATCATCCCGTCAGGC	This Thesis
	SIV_B3_B	CCCATTCCCATTAGGGCA	
	SIV_LF_B	GTTCTTTCTCTGCAAAAACACTTTCC	
	SIV_LB_B	CCAGTGAGCGAGGACTGCA	
	SIV_FIP_B	GCCATTCCATGAGAGCCTCAAGTTTTTCGAGATCGCGCAGAGACT	
	SIV_BIP_B	TTGTGTTTACGCTCACCGTGCTTTTTTTTGGACAAAGCGTCTACG	
BVS	BVS_F3_H	GATGGCCAGCCAGTCAAT	This Thesis

	BVS_B3_H	CCTTGATTAAAGCGGCTCTG	
	BVS_LF_H	CTGGTTTGTGCTGAGCGATGAT	
	BVS_LB_H	CCTCTAGCGACTGAAGATGATGTC	
	BVS_FIP_H	CTTCCCGGTCCCTTGCTCTTTTGTGCCAGATGCTGGGTAAG	
	BVS_BIP_H	AACCCGAGAAGCCCCATTTTTTTAGACACAATTGCCGCTCACT	
SARS-CoV-2	CoV_F3	ACCAATAGCAGTCCAGATGA	(Rodriguez-Manzano et al., 2021)
	CoV_B3	CACGATTGCAGCATTGTTAGC	
	CoV_LF	GGACTGAGATCTTTTATTTTACCGT	
	CoV_LB	ACTGAGGGAGCCTTGAAT ACA	
	CoV_FIP	TCTGGCCCAGTTCCCTAGGTAGTCCAGACA A AATTCGTGGTGG	
	CoV_BIP	GGACTTCCCTATGGTGTAAACAACGGGTGCCAATGTGATCT	
FluA	FluA_F3-1	GACTTGAAGATGTCTTTTGC	(Nakauchi et al., 2011)
	FluA_F3-2	GACTGGAAAGTGTCTTTTGC	
	FluA_B3-1	AGTTTAACTGCTTTGTCCATGT	
	FluA_B3-2	AGTTTAACTGCTCTATCCATGT	
	FluA_LF	GTCTTGTCTTTAGCCA	
	FluA_LB	CAGTGAGCGAGGACTG	
	FluA_FIP-1	TTAGTCAGAGGTGACAGGATTG-CCGATCTTGAGGTTCTC	
	FluA_FIP-2	TTAGTCAGAGGTGACAG G ATTG-CAGATCTTGAGGCTCTC	
FluB	FluA_BIP	TTGT G TTACGCTCACCGTG-TTGGACAAAGCGTCTACG	(Takayama et al., 2019)
	FluB_F3	GCAACCAATGCCAC Y ATA	
	FluB_B3	AATTGCTTTTCTCTCTCAAGA	
	FluB_LF-1	TGAAAGYCTTTTCATAGCAC	
	FluB_LF-2	TGAAAA Y CTTTTCATAGCAC	
	FluB_LB	CAAGAATAAAGACTCACAAC	
	FluB_FIP	TAGTCAAGGGCYCTTTGCCA-CTT Y GAAAGCAGGAATTCTGGA	
	FluB_BIP-1	CAAGACCGCCT A MACAGACTAAA-CATCCTTTTACTTTTCAGGCTCAC	
RSVab	FluB_BIP-2	CAAGACCGCCT A MACAGACTAAA-CATCCTTTTATTCTCAGGCTCAC	(Chen et al., 2021)
	RSVa_F3	AGGAAGAAGTCCTTGCATAT	
	RSVa_B3	GGACTGTACTTTACAAGTGTC	
	RSVa_LB	GATGGTACTGTGATAATGCAGGATC	
	RSVa_FIP	ACAGAGGTGATGTGTGAATTTCCGTACAGCTACCTATCTATGGTG	
	RSVa_BIP	ACAAGGACTGATAGAGGATGGTGCTTGTGGAAAGAAGGATAC	
	RSVb_F3	TCAGTATCCTTCTTTCCACAA	
	RSVb_B3	TGACACTATAGCTCCTAGAGAA	
	RSVb_LB	CAAGTATGATTGCAAAATTATGACATC	
	RSVb_FIP	TCACTTGGTAATGTCAAAGTGTTCAGACACTTGTAAGTACAGTCCA	
HRV	RSVb_BIP	AGTCAGCCTTTGTAACACTGACTGAGCTGCTTATGTCTGTT	(Nakauchi et al., 2019)
	HRV_F3	CYAGCCTGCGTGGCTGCC	
	HRV_B3	GAAACACGGACACCCAAA	
	HRV_LF-1	CAAAACAAGCACACGG	
	HRV_LF-2	CAAGATGAGCACACGC	
	HRV_LB	ACAATCCAGTGTGTAGCTGGTCGTAA	
	HRV_FIP	CATTAGGGGCCGGAGGA-GACAAGGTGTGAAGAGYC	
	HRV_BIP	GGCTAACCTTAAMCCYGCAGC-GTAGTYGGTCCCATCCC	
hgDNA	PM8_F3	GATGCTGGCGCTGAGTAC	(Wormald et al., 2021)
	PM8_B3	GCTAAGCAGTTGGTGGTGC	
	PM8_LF	AGCCTTCTCCATGGTGGTG	
	PM8_LB	GTCATGGGTGTGAACCATGAG	
	PM8_FIP	CTTTTGGCTCCCCCTGCAAAATGGAGTCCACTGGCGTCTT	
	PM8_BIP	TCTGCTGATGCCCCATGTTCCGAGGCATTGCTGATGATCT	

Highlighted bases (bold) have been modified from the published sequence by Dr Kenny Malpartida-Cardenas

*denotes additional primers used in published assays, designed in this Thesis

4.3.2. LAMP amplification conditions

Unless otherwise specified, all LAMP amplification reactions were carried out in 5 µL reactions containing specific primer sets. The LAMP reactions consisted of 1x custom isothermal buffer (without Tris), 0.5 µM SYTO 9 (Thermo

Fisher Scientific, Cat S34854), 1-1.8 mM dNTPs (Thermo Fisher Scientific, Cat R0181), 4-10 mM MgSO₄ (NEB, Cat B1003S), 0-2 mM betaine (Sigma-Aldrich, Cat B0300-5VL), 0-1.4 mM glycerol-free BSA (Sigma-Aldrich, Cat A9418), 0.04-0.72 U/μL polymerase (various), and for the detection of RNA (RT-LAMP) reactions were supplemented with experiment specific concentrations of RTs (various) and 1 μL of target genetic material, or nuclease-free water (Sigma-Aldrich, Cat W4502) for negative control reactions.

Initial comparisons of different polymerases were carried out as per manufacturer's instructions with 5 μL LAMP reactions consisting of 1x buffer supplied with each enzyme, 6 mM MgSO₄ (NEB), 1.4 mM dNTPs (Thermo Fisher Scientific), 0.04-0.72 U/μL polymerase (various), 0.45 U/μL RTx (NEB, Cat M0380) and 0.5 μM SYTO 9 (Thermo Fisher Scientific). Amplification was achieved with primer sets specific for SARS-CoV-2 with 200 copies/μL SARS-CoV-2 RNA (Twist Bioscience) and the inclusion of 0.45 U/μL RTx (NEB). The polymerases used included 0.04-0.72 U/μL each. The buffers supplied with each DNA polymerase included: the 10x *Bsm* buffer used in combination with the *Bsm* DNA polymerase, large fragment (Thermo Fisher Scientific, Cat EP0691), consisting of, 200 mM Tris-HCl (pH 8.8 at 25°C), 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1 % (v/v) Tween 20. The 10x isothermal buffer used in conjunction with *Bst* 2.0 DNA polymerase (NEB, Cat M0537M), *Bst* 2.0 WarmStart (WS) DNA polymerase (NEB, Cat M0538M) and WS *Bst* 2.0 DNA polymerase glycerol-free (NEB, Cat M0402B-BM) consisting of 200 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1 % (v/v) Tween 20. The 10x ThermoPol reaction buffer was used in conjunction with *Bst* LF (NEB, Cat M0275M), consisting of 200 mM Tris-HCl (pH 8.8 at 25°C), 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1 % (v/v) Triton-X-100. The 10x isothermal amplification buffer II was used in conjunction with *Bst* 3.0 DNA polymerase (NEB, Cat M0374M), consisting of 200 mM Tris-HCl (pH 8.8 at 25°C), 1500 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1 % (v/v) Tween 20. The 10x isothermal buffer B was used in conjunction with the *Bst* DNA polymerase (Lucigen, Cat 30028-1) consisting of 200 mM Tris-HCl (pH 8.8 at 25°C), 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1 % (v/v) Triton-X-100.

4.3.3. One-step RT-LAMP

The evaluation of different RTs was performed using primer sets specific for SIV, BVS and SARS-CoV-2 with 10,000, 1,000 and 200 copies/μL of their specific RNA templates, with 0.5 U/μL WS *Bst* 2.0 DNA polymerase (NEB) and 0.325-0.825 U/μL of AMV-RT (Promega, Cat M5108) or 0.15-0.65 U/μL of WarmStart RTx (NEB). The comparison of RTs manufactured by Lucigen were performed in the SARS-CoV-2 assay only, with 200 copies/μL of SARS-CoV-2 RNA (Twist Bioscience) using 0.5 U/μL of WS *Bst* 2.0 DNA polymerase (NEB). Titrations of EpiScript RNase H- RT (Lucigen, Cat ERT12925K) were performed between 2.5-8.75 U/μL and RapiDxFire Thermostable RT (Lucigen, Cat 30250-1) was performed between 0.06-0.21 U/μL, using LAMP optimised reaction conditions.

The LavaLAMP RNA component kit without dye (Lucigen, Cat 30096-1) was assessed with primer sets specific to SARS-CoV-2, FluA, FluB, RSVab, rhinovirus and hgDNA (Promega) each at 2,000 copies/μL and as per the manufacturer's instructions. The 5 μL LavaLAMP reactions consisted of 1x LavaLAMP buffer, 5.21 mM MgSO₄ (NEB), 0.83 mM dNTPs (Thermo Fisher Scientific) and 0.21 μL of LavaLAMP RNA enzyme. Amplification was

measured in real-time with the inclusion of 0.5 μM of SYTO 9 dye (Thermo Fisher Scientific). Reactions were incubated for 30 minutes at 71.7°C, as this yielded the fastest amplification in a previous experiment in the CFX connect Real-Time PCR thermocycler (Bio-Rad, Cat 1845097), with fluorescence readings taken every 30 seconds. The optimised conditions using *WS Bst* 2.0 DNA polymerase (NEB) and RTx (NEB) were run in parallel using the same template dilutions and final copy numbers per μL reaction in a LightCycler 96 (Roche, Cat 05815916001) for 45 minutes at 63°C, with fluorescence readings taken every 30 seconds.

4.3.4. Two-step RT-LAMP

The transcription step of the two step RT-LAMP reactions consisted of 25 μL reactions consisting of 0.4 μM of F3 and B3 primers targeting SARS-CoV-2, 1x Thermostable RT reaction buffer (Lucigen, Cat F735111), 0.5 mM dNTPs (Thermo Fisher Scientific), 0.5 U/ μL of RI (Thermo Fisher Scientific, Cat EO0381) and 0.12 U/ μL of RapiDxFire RT (Lucigen, Cat F735110), with the addition of 5×10^3 , 5×10^2 , 5×10^1 or 0 copies/ μL of SARS-CoV-2 RNA (Twist Bioscience). RNA transcription took place for 5 minutes at 60°C, followed by heat inactivation of 95°C for 5 minutes and reactions subsequently placed on ice for 1 minute. The transcription with EpiScript first required the annealing of the SARS-CoV-2 RNA (Twist Bioscience) to the primers, with 0.8 μM of each F3 and B3 primers being heated in 10 μL reactions consisting of 1×10^4 , 1×10^3 , 1×10^2 or 0 copies/ μL of SARS-CoV-2 RNA for 2 minutes at 65°C. The production of cDNA using EpiScript RT (Lucigen, Cat ERT12910) took place in 20 μL reactions containing 1x RT reaction buffer, 10 mM DTT (Lucigen), 0.5 U/ μL RI (Thermo Fisher Scientific), 0.5 mM dNTPs (Thermo Fisher Scientific), 5 U/ μL of EpiScript RT (Lucigen) and 10 μL of the annealed template and primers (1:1 dilution), equating to starting RNA concentration of 5×10^3 , 5×10^2 , 5×10^1 and 0 copies/ μL . The transcription took place for 60 minutes at 37°C prior to heat inactivation for 5 minutes at 85°C. Subsequent LAMP and RT-LAMP reactions were performed in 5 μL volumes containing 1 μL of complementary DNA (cDNA) or RNA diluted to 5×10^3 , 5×10^2 , 5×10^1 and 0 copies/ μL for each reaction to have a final RNA or cDNA concentration of 1×10^3 , 1×10^2 , 1×10^1 or 0 copies/ μL . The RT-LAMP and LAMP reactions consisted of 1x isothermal buffer (NEB, Cat B0537), 6 mM MgSO_4 (NEB), 1.4 mM dNTPs (Thermo Fisher Scientific), 0.5 mg/mL BSA (NEB, Cat M0537L), 320 U/ μL *Bst* 2.0 DNA polymerase (NEB) and 0.5 μM SYTO 9 dye (Thermo Fisher Scientific). The addition of RNA in some reactions served as a control with the addition of 0.45 U/ μL RTx (NEB). Amplification occurred at 63°C with fluorescence readings taking place every 30 seconds for 45 minutes in a CFX connect Real-Time PCR thermocycler (Bio-Rad).

4.3.5. RNase inhibitors (RI) in LAMP

The performance of two RIs, Riboguard (Lucigen, Cat RG90910K) and RiboLock (Thermo Fisher Scientific, Cat EO0382) were titrated into 10 μL RT-LAMP reactions with concentrations ranging between 0.5-1.75 U/ μL . Aliquots of both Riboguard and RiboLock were heat-inactivated by incubation at 95°C for 5 minutes prior to being added to the LAMP reaction. RT-LAMP reactions were run in the presence of the SARS-CoV-2 specific primer set and 200 copies/ μL of SARS-CoV-2 RNA (Twist Bioscience). The RT-LAMP reaction consisted of reagents at concentrations consistent with the optimised LAMP protocol (**Section 4.3.7**).

4.3.6. pH-LAMP conditions

The isothermal buffer without Tris was produced at a 10x stock concentration consisting of 500 mM KCl (Sigma-Aldrich, Cat 60135-250mL), 20 mM MgSO₄ (Sigma-Aldrich, Cat M3409), 100 mM (NH₄)₂SO₄ (Sigma-Aldrich, Cat 60135-250mL) and 1% Tween-20 (PanReac AppliChem, Cat A4974,0250), adjusted to a pH of 8.5-9.0 with the addition of 5 M NaOH (Honeywell, Cat 011-022-00-6). Lucigen buffer B without Tris was made 10x concentrated, consisting of 100 mM KCl (Sigma-Aldrich), 20 mM MgSO₄ (Sigma-Aldrich), 100 mM (NH₄)₂SO₄ (Sigma-Aldrich) and 1 % Triton-X-100 (Sigma-Aldrich, Cat X100), adjusted to a pH of 8.5-9.0 with the addition of 5 M NaOH (Honeywell). The pH of the solution was measured with a Mettler Toledo™ Seven2Go™ pH/mV Meter (Thermo Fisher Scientific, Cat 15315073).

4.3.7. Optimised LAMP protocol

Subsequent LAMP and RT-LAMP reactions were run in 'optimal' conditions at 1x isothermal buffer without Tris, 5 mM MgSO₄ (NEB), 1.4 mM dNTPs (Thermo Fisher Scientific), 1.2 mg/mL of glycerol-free BSA (Sigma-Aldrich, Cat A9418), 5 mM NaOH (Honeywell) and 0.5 U/μL of *W**Bst* 2.0 DNA polymerase (NEB). RT-LAMP reactions included 0.45 U/μL of RTx (NEB). The addition of 0.5 μM SYTO 9 (Thermo Fisher Scientific) was used to acquire real-time data with incubation at 63°C for 45 minutes in a CFX connect Real-Time PCR thermocycler (Bio-Rad) or a LightCycler 96 (Roche).

4.3.8. LAMP additives

A selection of 14 chemicals previously found to be beneficial to either the SPE or production of amplicon polymerase performance was titrated into LAMP reactions. The performance of the LAMP reactions was determined using two LAMP assays, APP and MH, as well as the SARS-CoV-2 RT-LAMP assay. Reactions were performed in 5 μL volumes with the addition of 1,000 gDNA copies/μL of APP and MH, and 200 copies/μL of SARS-CoV-2 RNA. The addition of additives into the LAMP reactions included final concentrations of 0-10% PEG8 (Sigma-Aldrich, Cat 1546605), 0-10% PEG35 (Sigma-Aldrich, Cat 81310-1KG), 0-10% glycerol (Sigma-Aldrich, Cat E7889-100mL), 0-2 M 2-pyrrolidinone (Sigma-Aldrich, Cat 240338), 0.001-4% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat D8418-50ML), 0.1-5% formamide (Sigma-Aldrich, Cat 47671-250ML), 0.1-2% Triton X-100 (Sigma-Aldrich), 0.1-1.8% Tween-20 (PanReac AppliChem), 0.001-4% Brij-35 (Millipore, Cat 1.01894.0100), 0.1-1.5% Tergitol (NP-40, Sigma-Aldrich, Cat NP040S), 0.1-5 mM spermidine (Sigma-Aldrich, Cat S2626-1G), 0.1-5 mM spermine (Sigma-Aldrich, Cat S4264-1G), 10-100 mM guanidine hydrochloride solution (GuHCl, Sigma-Aldrich, Cat 50937-100ML) or 10-100% guanidinium thiocyanate (GTC, Sigma-Aldrich, Cat 50981-100G). Reactions were run at 63°C for 45 minutes, with fluorescence acquisition taken every 30 seconds in a CFX connect Real-Time PCR thermocycler (Bio-Rad) or a LightCycler 96 (Roche).

4.3.9. dUTP titration in LAMP

The substitution of dTTP to dUTP was performed with nucleotides obtained from NEB, with individual dNTPs (NEB, Cat N0446S) and dUTP (NEB, Cat N0459S) purchased separately. The dUTP and dTTP were mixed at

dUTP:dTTP ratios of 0:1 (0%), 1:8 (12.5%), 1:4 (25%), 3:8 (37.5%), 1:2 (50%), 5:8 (62.5%), 3:4 (75%) and 1:1 (100%) such that the final concentration remained at 25 μ M. The mixed volumes were then combined with the other dNTPs such that the final concentration of total dNTPs was 100 μ M. The performance of the inclusion of dUTP at different amounts compared to dTTP was assessed in SIV, BVS and SARS-CoV-2 assays with the relevant RNA added at 1,000 copies/ μ L for SIV, 100 copies/ μ L for BVS, and 200 copies/ μ L for SARS-CoV-2. RT-LAMP reactions were performed in a total volume of 5 μ L with a final total dNTP concentration of 5.6 mM (the same as typically used at 1.4 mM each dNTP). RT-LAMP reaction components contained concentration as set out in the optimised LAMP reactions.

4.3.10. UDG in LAMP reactions

To optimise UDG inclusion in LAMP reactions, the effect of the concentration of UDG and incubation time were investigated. RT-LAMP reactions were all carried out with the final reagent concentrations as detailed in the optimised RT-LAMP protocol, but with the substitution of dTTP with a mixture containing 50% dUTP and 50% dTTP. Initially, the inclusion of UDG in RT-LAMP experiments was in the presence or absence of 1.45 U/ μ L Antarctic thermolabile UDG (NEB, Cat M0372L), with the effect of RT-LAMP assays assessed in SARS-CoV-2, SIV, and BVS RT-LAMP assays containing 200, 1,000 and 100 copies/ μ L of SARS-CoV-2, SIV and BVS RNA, respectively. The results were analysed in GraphPad Prism v9.0, with significant differences being determined using the Mann-Whitney test. Subsequent titrations of UDG ranged from 0.01-0.2 U/ μ L in 5 μ L RT-LAMP reactions using SIV and BVS-specific primer sets at the same copy number concentration as above, with the addition of 2×10^5 and 2×10^4 copies/ μ L of the relevant amplicon. Pre-incubation of the reactions was for 5 minutes at 37°C before the main LAMP reaction was performed at 63.5°C for 45 minutes. The pre-incubation time for UDG activity was altered from 1-30 minutes at 37°C in the presence of 0.08 U/ μ L UDG with the inclusion of 2×10^5 and 2×10^4 copies/ μ L of the relevant amplicon. The ability of UDG to prevent carry-over contamination was performed by the addition of 0.08 U/ μ L of UDG with pre-incubation occurring for 5 minutes at 37°C, followed by LAMP reactions as per the optimised RT-LAMP reaction instructions.

The estimation of carryover contamination was generated from LAMP reactions performed as detailed in the optimised RT-LAMP protocol but using 50% of the usual dTTP concentration and with 50% of dUTP. Amplicons produced were quantified with a Qubit 4 fluorometer (Thermo Fisher Scientific, Cat Q33238) as per manufacturer instructions, in combination with a Qubit dsDNA Quantification, high sensitivity kit (Thermo Fisher Scientific, Cat Q32851). Estimations of copy numbers were based on the amplicon length of the furthest primers F3 and B3 diluted according to the Thermo Fisher Scientific copy number calculator (<https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html>). The diluted product was spiked into subsequent reactions containing the UDG enzyme as described previously.

4.3.11. LAMP compatibility with excipients

A panel of six excipients supplied by Biofortuna Ltd. were titrated into LAMP reactions at various concentrations. The disaccharide-based excipients, sucrose, and trehalose were titrated between 1-10% W/V. The polyalcohol sugar-based excipients, sorbitol, and mannitol were titrated between 1-4% W/V. The polysaccharide-based excipients, dextran and Ficoll-400 were titrated between 0.5-2% W/V. The six excipients were each titrated into three LAMP assays. The SARS-CoV-2 RT-LAMP reaction consisted of 200 copies/ μ L of SARS-CoV-2 RNA. The APP and MH LAMP assays each consisted of 1,000 copies/ μ L of APP and MH gDNA.

4.4. LAMP lyophilisation

4.4.1. Freeze-dried microscopy

Freeze-dried microscopy (FDM) was performed externally by Biofortuna Ltd. FDM data was produced by Dr Thanos Papas (Biofortuna Ltd.) using the lyophilisation compatible LAMP reactions mixture. The reaction mixture was frozen at -50°C , and after 2 minutes, a vacuum was pulled. Upon the formation of the freeze-drying front, the temperature increased at a rate of $2^{\circ}\text{C}/\text{min}$.

4.4.2. Lyophilisation of LAMP reactions

Lyophilised LAMP reactions were produced externally, by Biofortuna Ltd, in 2x concentrated mixes in order to account for the 10 μ L maximum dispense volume constraint specified by Biofortuna Ltd (personal correspondence). Therefore, 2x LAMP mixes consisted of 4 μ M each FIP and BIP, 2 μ M each LF and LB, 0.5 μ M each F3 and B3, 2x isothermal buffer without Tris, 10 mM MgSO_4 , 2.8 mM dNTPs (each), 2.4 mg/mL glycerol-free BSA, 4% mannitol, 2% ficoll, 1 U/ μ L WS *Bst* 2.0 DNA polymerase glycerol-free. For RNA targets, 0.9 U/ μ L of WS RTx was included. The 10 μ L LAMP reactions were dispensed directly into liquid nitrogen, and the frozen spheres underwent lyophilisation using the conditions set out in **Table 4.2**. Lyophilised reactions were sealed with either a foil or a plastic cap in a low humidity (<2%) environment and packaged in a sealed foil pouch along with desiccant silica gel. The lyophilised reactions were unsealed immediately prior to use and reconstituted with 20 μ L of water. For real-time detection, 0.1 μ M SYTO 9 (Thermo Fisher Scientific) was included in the water, and real-time data was obtained in a LightCycler 96 (Roche).

Table 4.2. Freeze drying conditions. RT-LAMP reactions with 2x final concentrations were lyophilised in reaction volumes of 10 μ L.

	Temperature ($^{\circ}\text{C}$)	Step (Ramp/Hold)	Time (minutes)	Vacuum (mTorr)
Pre-cooling	-45	R	30	N/A
	-45	H	30	N/A
Primary drying phase	-40	R	60	60
	-40	H	1100	60
	-40	H	1000	60
Secondary drying phase	+20	R	120	60
	+20	H	360	60

4.5. pH of LAMP reactions

pH-LAMP reactions were setup using the optimised RT-LAMP conditions (**Section 4.3.7**), with the exclusion of SYTO 9 dye. The SARS-CoV-2 assay was utilised to compare liquid (fresh) LAMP reactions against rehydrated lyophilised reactions using various input concentrations of SARS-CoV-2 RNA. The pH of LAMP reaction was measured with a SI400 pH meter using an MicroFET pH electrode (Sentron, Cat 9270-010).

Results

4.6. Optimisation of LAMP reactions

The majority of experiments performed using the SARS-CoV-2 assay for the purpose of optimisation of LAMP conditions were conceptualised, performed and analysed in conjunction with Dr Kenny Malpartida-Cardenas.

4.6.1. Alternative polymerases

Since the first description of LAMP amplification using *Bst* DNA polymerase and *BcaBEST* DNA polymerase (Notomi et al., 2000), several other polymerases displaying strong strand displacement activity have been commercialised for use in LAMP. We evaluated six different commercially available polymerases in a SARS-CoV-2 LAMP assay. Although the performance of the different polymerases was varied, they each displayed a decrease in TTP at higher polymerase concentrations (**Figure 4.1A**). Compared to the other polymerases, *Bst* LF supplied by NEB exhibited the most rapid amplification at any given concentration. I found that Lucigen's *Bst* polymerase plateaued at a lower concentration than the other polymerases (i.e., resulted in rapid amplification with lower activity units). Thus, less of Lucigen's *Bst* polymerase would be required to achieve the same performance as the other polymerases. The polymerase *Bst* 2.0 performed well and has been used historically in our laboratory for pH detection. Additionally, the WS *Bst* 2.0 is available in a glycerol-free format beneficial for lyophilisation. Therefore, we sought to improve the reaction conditions using the WS *Bst* 2.0 supplied by NEB.

4.6.2. Alternative reverse-transcriptases

For LAMP detection of RNA, an RT compatible with the LAMP incubation temperature (63°C) is required. Two different RTs were compared, one marketed by NEB, RTx, and one by Promega, AMV-RT. Although AMV-RT has been historically utilised in our laboratory, AMV-RT displays optimal activity across a temperature range of 37-58°C. A comparison of these two RTs across three RNA LAMP assays indicated that AMV-RT and RTx achieved similar activity (**Figure 4.1B**). However, RTx resulted in a more consistent amplification. Additionally, RTx can be supplied in the absence of glycerol and therefore is more compatible with the lyophilisation procedure discussed later in this Chapter. I also performed titrations with additional RT enzymes supplied by Lucigen, with EpiScript RT and RapiDxFire RT; both failed to produce detectable amplification in conjunction with LAMP (data not shown). However, this was rectified by a two-step protocol, requiring the RT to be incubated prior to addition into the LAMP reaction (**Supplementary Figure 8**). The EpiScript RT requires a 60-minute transcription step at 37°C, whilst RapiDxFire RT appeared more amenable to a PoC LAMP reaction, only requiring 5 minutes at 60°C. However, the performance of the two-step RapiDxFire RT resulted in slower amplification than the one-step RT-LAMP using RTx, even over a wide range of incubation temperatures and RapiDxFire RT enzyme concentrations (**Supplementary Figure 9A and Supplementary Figure 9B**). Lucigen's LavaLAMP kit uses a single polymerase which exhibits strong RT activity, therefore negating the need for a separate RT enzyme. However, on testing, I found that across seven assays, four displayed amplification in the absence of template (data not shown). Of the

three assays which displayed no FP amplification, the TTP was greatly increased over the use of WS *Bst* 2.0 in combination with RTx (**Supplementary Figure 10**). Therefore, RTx was taken forward as the most appropriate RT enzyme to optimise the conditions of RT-LAMP reactions.

RNA is notoriously prone to degradation. Often, a RI is included in a diagnostic pathway to limit the degradation of RNA by environmental RNases. However, I found that no glycerol-free RI was commercially available at the time, and when two RIs were evaluated, their inclusion had no effect on the performance of the LAMP assay (**Supplementary Figure 11**).

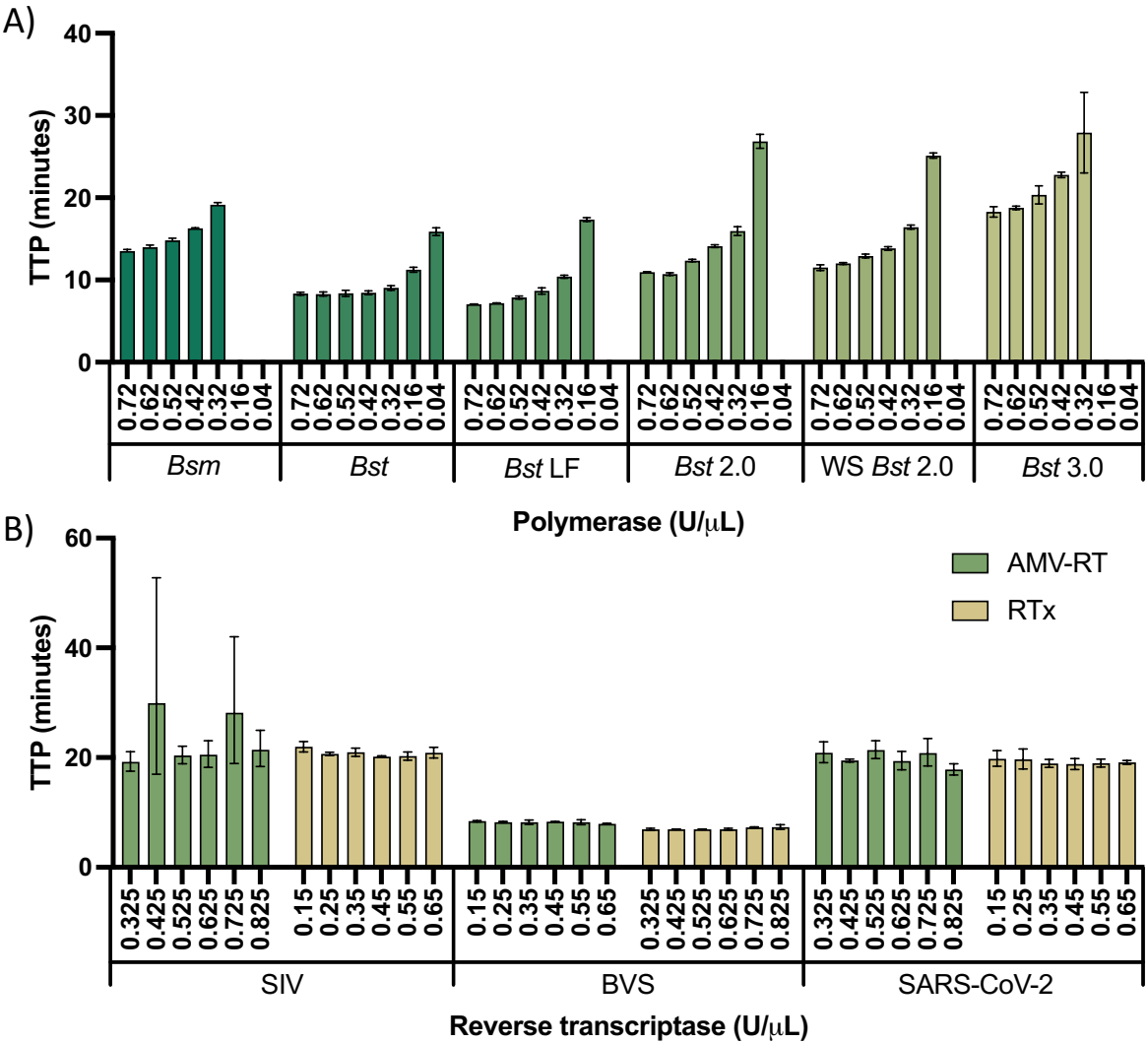


Figure 4.1. Polymerase and reverse transcriptase titration in RNA LAMP assays. Titration of six different commercially available polymerases in the SARS-CoV-2 LAMP assay (**A**). Titration of two different RTs into three different RNA LAMP assays, targeting SIV, BVS and SARS-CoV-2 (**B**). Each column shows the geometric mean achieved across three replicates and error bars depict the S.D.

4.6.3. Optimisation of LAMP components

Thus, we sought to optimise the performance of the LAMP reactions using 0.52U/μL of WS *Bst* 2.0 polymerase and 0.45 U/μL of the RTx RT. As both the polymerase and RT utilise Mg^{2+} as a co-factor and dNTPs for

transcription/polymerisation, these two components were titrated into three different RT-LAMP assays. The optimal condition for MgSO_4 was 5 mM (**Figure 4.2A**) and 1.5 mM for the dNTPs (**Figure 4.2B**). The presence of hygroscopic components such as glycerol and betaine negatively affects the lyophilisation process, as water is more difficult to remove, and the dried material displays decreased stability (Pack and Deng, 2007). Therefore, using two DNA LAMP assays, targeting APP and MH, and the SARS-CoV-2 RNA LAMP assay, betaine was titrated out of the reactions, with negligible impact on amplification (**Figure 4.2C**). The BSA is supplied in 50% glycerol. Therefore, a glycerol-free BSA was produced and titrated into the LAMP reactions. The inclusion of BSA had a negligible impact on the TTP of the reactions, with no clear trend across the three LAMP assays tested, thus 1.2 mg/mL of glycerol-free BSA was chosen as it showed a slightly lower TTP in two out of the three LAMP assays (**Figure 4.2D**). Commercially available enzymes are typically supplied in a storage buffer containing 50% glycerol, a glycerol-free version of the enzyme was available for the WS *Bst* 2.0 and the RTx. A comparison between *Bst* 2.0 and the glycerol-free WS *Bst* 2.0 found both polymerases produced comparable amplification at identical concentrations (**Figure 4.2E**); the same was found when RTx was replaced with its glycerol-free variant (**Figure 4.2F**).

4.6.4. Additives in LAMP reactions

Several compounds have been reported to improve the efficiency of molecular amplification techniques. Such compounds include; crowding agents, which mimic the conditions of the enzyme's biological environment (Tong et al., 2011), detergents, surfactants and solvents, which reduce non-specific interactions of PCR primers. Additionally, the inclusion of zwitterionic detergents and non-detergent surfactants has been reported to increase the stability of thermostable polymerases (Shultz and Huang, 1999). A literature search identified 14 compounds for evaluation as to their ability to increase LAMP reaction efficiency. A titration of three crowding agents, PEG8, PEG35 and glycerol, resulted in a TTP reduction by 22.63-29.16% when using 3-6% PEG8 in the APP assay, and 7.23-9.16% when PEG8 was added at 3-4% in the SARS-CoV-2 assay (**Supplementary Figure 12A**). The TTP also decreased by 14.85-22.72% with the addition of 2-6% PEG35 in the APP assay (**Supplementary Figure 12B**). PEG8 and PEG35 appeared to be slightly inhibitory to the MH LAMP assay, with the TTP increasing as the concentration of PEG (8 and 35) increased. A slight decrease in the TTP was observed in the DNA assays in the presence of 2-6% glycerol, with the TTP decreasing by 17.83-23.97% in the APP assay, and 15.32-21.26% in the MH assay. Conversely, there was little alteration to the TTP across glycerol titrations 0-6% in the SARS-CoV-2 RNA assay (**Supplementary Figure 12C**). The titration of three solvents, 2-pyrrolidinone, DMSO and formamide showed no decrease in TTP, with 2-pyrrolidinone being well tolerated in LAMP (**Supplementary Figure 12D**), and DMSO and formamide being inhibitory at the higher concentrations tested (**Supplementary Figure 12E** and **Supplementary Figure 12F**). None of the three surfactants, Triton X-100, Tween-20 or NP-40, resulted in any improvements to the LAMP assay TTP (**Supplementary Figure 12G**, **Supplementary Figure 12H** and **Supplementary Figure 12I**, respectively). Nor did the addition of the solvent, DMSO (**Supplementary Figure 12J**). The titration of two polyamines, spermidine and spermine, were inhibitory to the LAMP reactions, with up to 1% spermidine tolerated in LAMP reactions (**Supplementary Figure 12K**), and up to 0.1% spermine tolerated in the RNA reaction, but both DNA reactions tolerated up to 2.5% spermine (**Supplementary Figure 12L**). Two

chaotropic salts were tested, with GuHCl displaying a maximum TTP decrease of 20.6% in the presence of 20% GuHCl in the APP assay, and 20.06% in the presence of 30% GuHCl in the MH assay. Although no decrease was seen in the TTP of the RNA-based LAMP assay, detecting SARS-CoV-2 (**Supplementary Figure 12M**). The other chaotropic salt, GTC was slightly inhibitory to all three LAMP assays, with an increased TTP and complete inhibition of the reactions at 60 mM for the DNA assays, and 40 mM for the RNA assay (**Supplementary Figure 12N**). Therefore, none of the additives tested improved the TTP of all LAMP assays and, therefore, none were used in subsequent LAMP assay protocols.

4.6.5. Optimised LAMP protocol

To ensure the changes in the protocol did not negatively impact the SEN of LAMP reactions, we compared the standard versus optimised pH-LAMP protocols in three LAMP assays detecting RNA targets. The optimised protocol decreased the TTP on average by 48.60% in the SIV LAMP assay, 51.85% in the BVS LAMP assay and 33.42% in the SARS-CoV-2 assay (**Figure 4.3**). Increased SEN was also seen in the SIV and BVS assays, both amplifying 20 copies/ μ L when the optimised LAMP formulation was used, whilst the standard LAMP formulation did not detect SIV or BVS RNA at this copy number. Furthermore, we verified that the optimised pH-LAMP protocol resulted in more rapid detection of LAMP amplification across all NEB *Bst* DNA polymerases when compared to their recommended conditions (**Supplementary Figure 13**). Amplification was most rapid with *Bst* 2.0 at higher copy numbers of SARS-CoV-2 RNA, taking only 6.41 minutes at 400,000 copies/ μ L. At the lowest copy numbers, the WS *Bst* 2.0 showed the most rapid amplification, with a TTP of 10.81 minutes at 40 copies/ μ L, whilst the glycerol-free WS *Bst* 2.0 was the second most rapid amplification at this DNA concentration, taking 13.54 minutes. The *Bst* 3.0 displayed FP amplification in both the recommended and optimised protocols. However, the optimised protocol decreased the TTP (44.8%) in the presence of the template and created a higher degree of separation between target- and self-amplification events. In the manufacturer's protocol, the *Bst* 3.0 negative control was amplified within 0.16 minutes of the positive control, which increased by 5.95 minutes in the optimised protocol.

An alternative *Bst* enzyme which exhibited low TTP in the initial polymerase screen (**Figure 4.1A**) was Lucigens *Bst*, the optimised protocol was also compared to the manufacturer's protocol in the SIV, BVS and SARS-CoV-2 RT-LAMP assays, with comparable results to NEB (**Supplementary Figure 14**). Thus, using our optimised protocol, both NEB *Bst* 2.0 variants and Lucigens *Bst* display equivalent amplification. Therefore, the LAMP reactions were optimised to decrease the TTP for both the detection of both RNA and DNA.

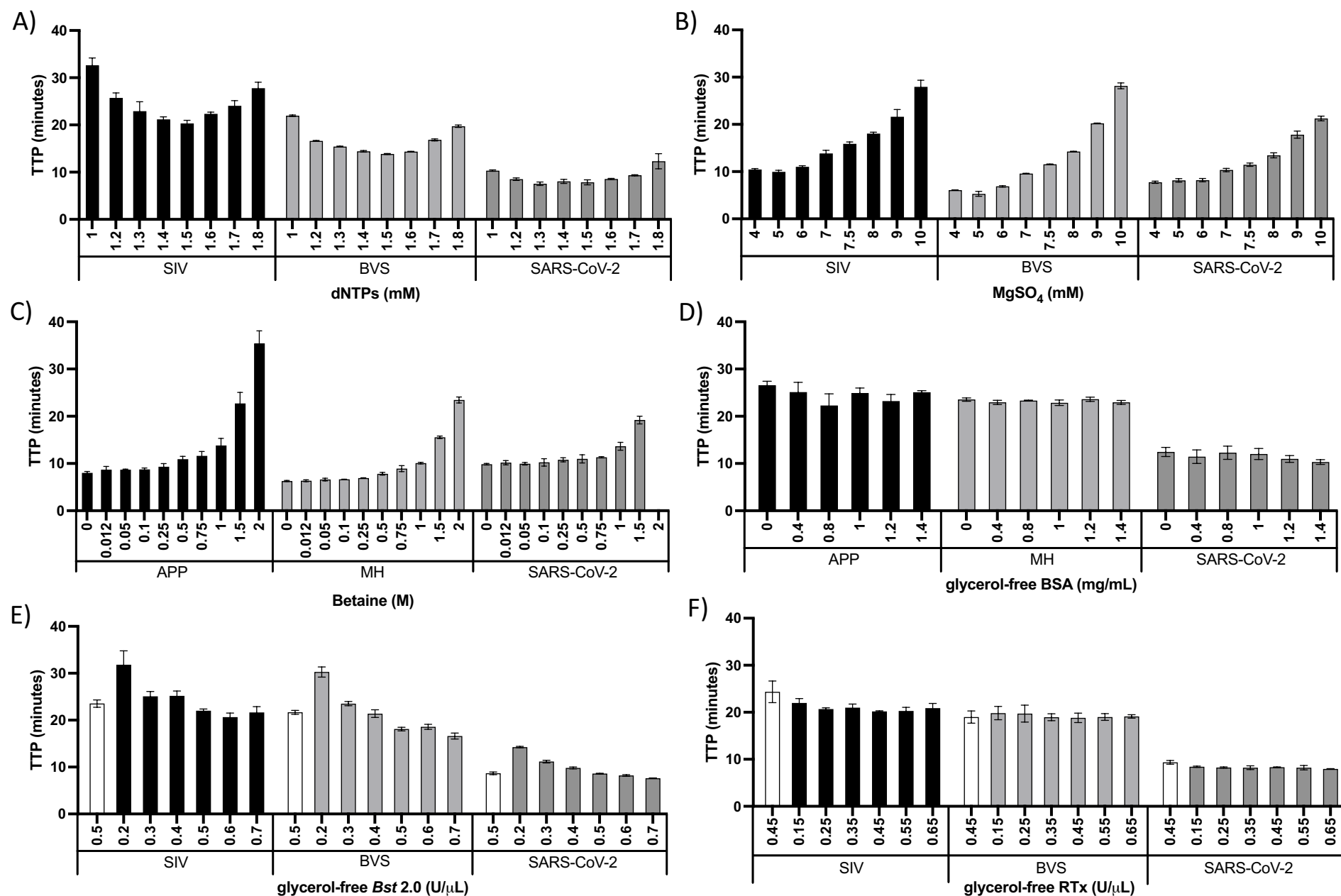


Figure 4.2. Optimisation of LAMP and RT-LAMP reactions. Titration of dNTPs (A) MgSO₄ (B) was performed in RT-LAMP reactions detecting SIV, BVS and SARS-CoV-2. Titration of betaine (C) and glycerol-free BSA (D) was performed in LAMP and RT-LAMP reactions detecting APP, MH and SARS-CoV-2. Titration of glycerol-free *Bst* 2.0 (E) and RTx (F) was performed in RT-LAMP reactions detecting SIV, BVS and SARS-CoV-2. Columns represent the geometric mean achieved from three replicates, and error bars depict the S.D.

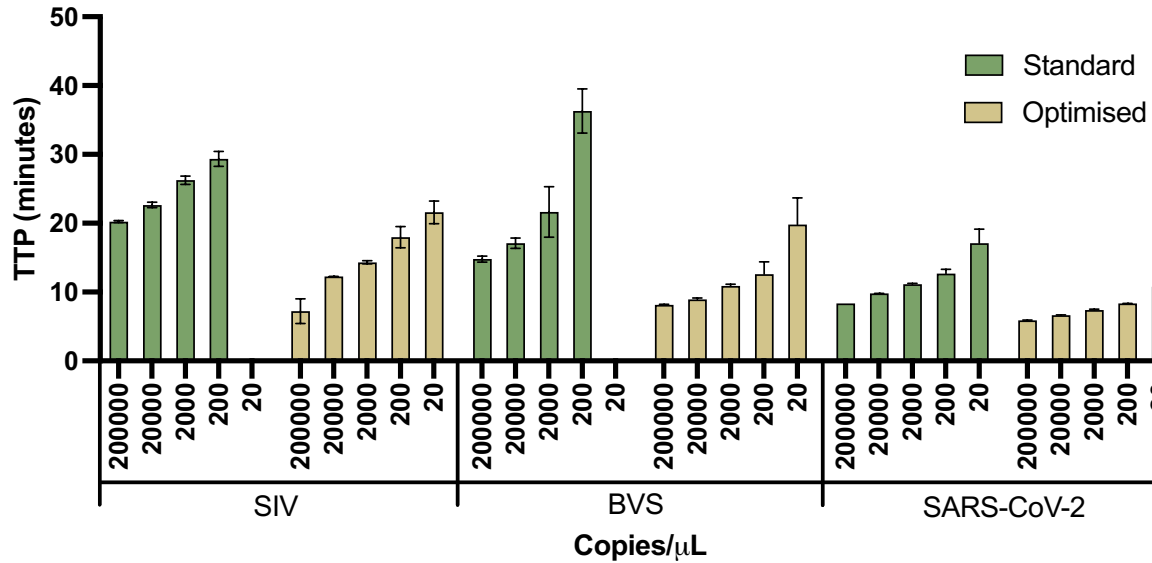


Figure 4.3. LoD achieved using standard and optimised pH-LAMP protocol. Amplification of 10-fold serial dilutions of SIV, BVS and SARS-CoV-2 performed in RT-LAMP reactions using the standard or optimised pH LAMP reactions. Columns represent the geometric mean across three replicates, with error bars representing the S.D.

4.7. Contamination control

As with any molecular diagnostic technique, there is a high risk of carry-over contamination from previously completed LAMP amplicons, especially when an IVD device is being reused in combination with a disposable cartridge. From a device design standpoint, design decisions can be made to minimise the risk that a used cartridge may contaminate the device. In molecular diagnostics, the use of DNA-repair enzymes in combination with dUTP in place of dATP can prevent the carry-over contamination of amplified products into pre-amplified cartridges. In this Section, I sought to validate the use of the UDG in LAMP reactions. Firstly, I determined that the addition of the UDG enzyme into the LAMP reaction did not impact the TTP, by evaluating the TTP achieved when LAMP was performed in the presence or absence of UDG in five LAMP assays (three RNA assays and two DNA assays). No significant difference was found between the TTP medians with or without the addition of UNG, with a Mann-Whitney test displaying P values of 0.67-1.00 (**Supplementary Figure 15**).

It has been reported that the polymerase commonly used in LAMP, *Bst* 2.0, successfully incorporates dUTP into DNA strands (Hsieh et al., 2014). Therefore, I sought to determine how the addition of dUTP in place of dTTP impacted the amplification across the five LAMP assays. For all LAMP assays, the TTP increased with the increasing addition of dUTP. In the LAMP assay detecting SIV, the TTP was not significantly changed until 75% of the dATP in the reaction was replaced with dUTP (Figure 4.4A), compared to >62.5% dUTP in the BVS assay (Figure 4.4B) and 12.5% in the APP assay (Figure 4.4C). The higher the proportion of dUTP is incorporated into the amplicon, the more sites UDG will have to nick the amplicon and, therefore the less likely the amplicon will be able to contaminate future reactions. Thus, although resulting in a slower TTP in some LAMP reactions, 50% dUTP is often used in published UDG contamination protocols and so was chosen for future experiments.

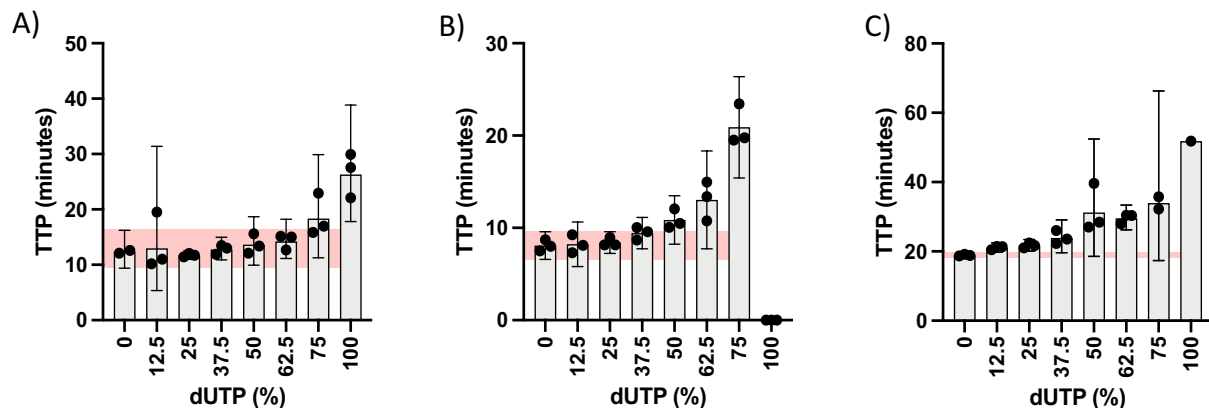


Figure 4.4. Titration of dUTP into LAMP reactions. The effect the addition of increasing concentrations, given as a % of dUTP compared to dATP has on the TTP achieved from LAMP reactions containing SIV primers and 10,000 copies/ μ L SIV RNA (A), BVS primers and 1,000 copies/ μ L of BVS RNA (B) and APP primers with 500 copies/ μ L of APP serovar 8 gDNA (C). Columns represent the geometric mean, with error bars representing 95% CI, and red bands signifying the 95% CI error for the control (0% dUTP).

The substrate of UDG is uracil bases within dsDNA. To assess the SPE of UDG against ssRNA targets containing uracil, three RNA LAMP assays were used. Completed LAMP reactions containing 50% of their dATP as dUTP were quantified and 10-fold serial dilutions were made from approximately 1 million copies of each amplicon. Subsequent LAMP reactions were spiked with the different concentrations of contamination amplicon in the presence (positive) or absence (negative) of RNA template. LAMP reactions were incubated in the presence of 1.45U/ μ L of UDG for 5 minutes prior to amplification. UDG was confirmed to prevent cross-contamination when LAMP reactions were spiked with 10,000 copies of SARS-CoV-2 amplicon (Figure 4.5A), 1,000 copies of SIV amplicon Figure 4.5B) or 100,000 copies of BVS amplicon Figure 4.5C).

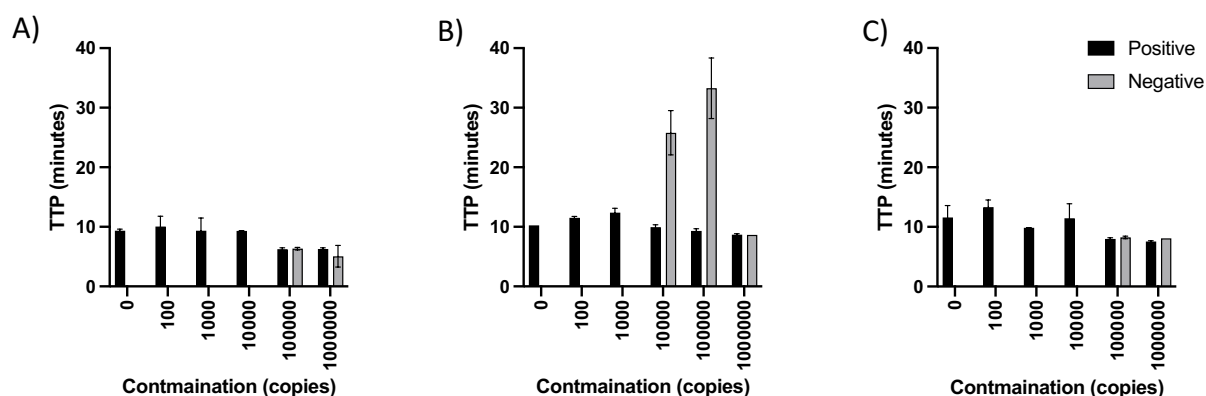


Figure 4.5. UDG elimination of carry-over contamination. The ability of UDG to eliminate amplicon produced in previous reactions during the 5-minute preincubation step prior to amplification with LAMP occurring. 10-fold serial dilutions were made of SARS-CoV-2 (A), SIV (B), and BVS (C) amplicons and spiked into reactions in the presence (positive) or absence (negative) of target RNA. Bars depict the geometric average of amplification time performed in triplicate, and error bars show the S.D.

LAMP amplicons are difficult to quantify due to amplification being a concatenation of the primers rather than a defined amplicon sequence, as seen in PCR; therefore, the copy numbers used are an approximation. However, when the estimated copy numbers were back-calculated, they were equivalent to a hundredth of a microliter of

the finished LAMP product. Therefore, UDG can successfully prevent the detection of minor cross-contamination events that may occur in the device and cause FP events in subsequent LAMP reactions.

4.8. Lyophilisation of LAMP reactions

The process of lyophilisation can cause structural stress to biological material and therefore cause a reduction in the functional activity of proteins. The addition of chemicals, known as excipients, that aid in retaining the structural characteristics during the freeze-drying process is critical to the success of the lyophilisation process. Therefore, we titrated a range of commonly used excipients into our LAMP reactions to assess if the addition of excipients results in an inhibitory effect on the TTP. Six excipients, consisting of two disaccharides, two sugar alcohols and two polysaccharides, were added at concentrations deemed relevant by Biofortuna Ltd (personal correspondence) into three LAMP assays (**Figure 4.6**). Across all three LAMP assays, an RNA assay (SARS-CoV-2), and two DNA assays (APP and MH), the two polysaccharide compounds, dextran and Ficoll, were the least inhibitory to the LAMP reactions. The disaccharide excipients, sucrose and trehalose, were more inhibitory in the DNA compared to the RNA assays (**Figure 4.6B** and **Figure 4.6C**), and the sugar alcohol compounds, sorbitol and mannitol, were slightly inhibitory but did not delay the TTP of the reactions in a concentration-dependent manner, at the concentrations tested.

Dextran and Ficoll were the least inhibitory excipients to the LAMP reaction, shown by the minimal impact they exhibited on the TPP compared to the control of no excipient. However, after consultation with our lyophilisation partner, Biofortuna Ltd., a combination of a sugar alcohol (sorbitol or mannitol) and a polysaccharide (dextran or Ficoll) would result in the highest stabilisation of the enzymes in the LAMP reaction. Therefore, we cross-titrated the least inhibitory of each of these groups of compounds (mannitol and dextran) into the SARS-CoV-2 LAMP assay (**Figure 4.7A**). The results suggest that at higher concentrations, mannitol is slightly inhibitory. In contrast, when Ficoll is also present, the inhibitory effect of mannitol is reduced. Therefore, FDM was performed on the LAMP reactions containing excipient concentrations of 2% Ficoll and 4% mannitol. The FDM determines the freeze-drying parameters, by performing lyophilisation on a small sample under a microscope. The sample is placed under a vacuum at a low temperature, the temperature is slowly raised allowing sublimation to occur from the edges of the material, forming a sublimation front. When too much heat is applied to the dried material, the structure becomes weak and collapses, known as the onset of collapse. The temperature at which the material completely collapses and there is no longer any definition between the sublimation front and the frozen material is known as total collapse and is the critical temperature, or T_c .

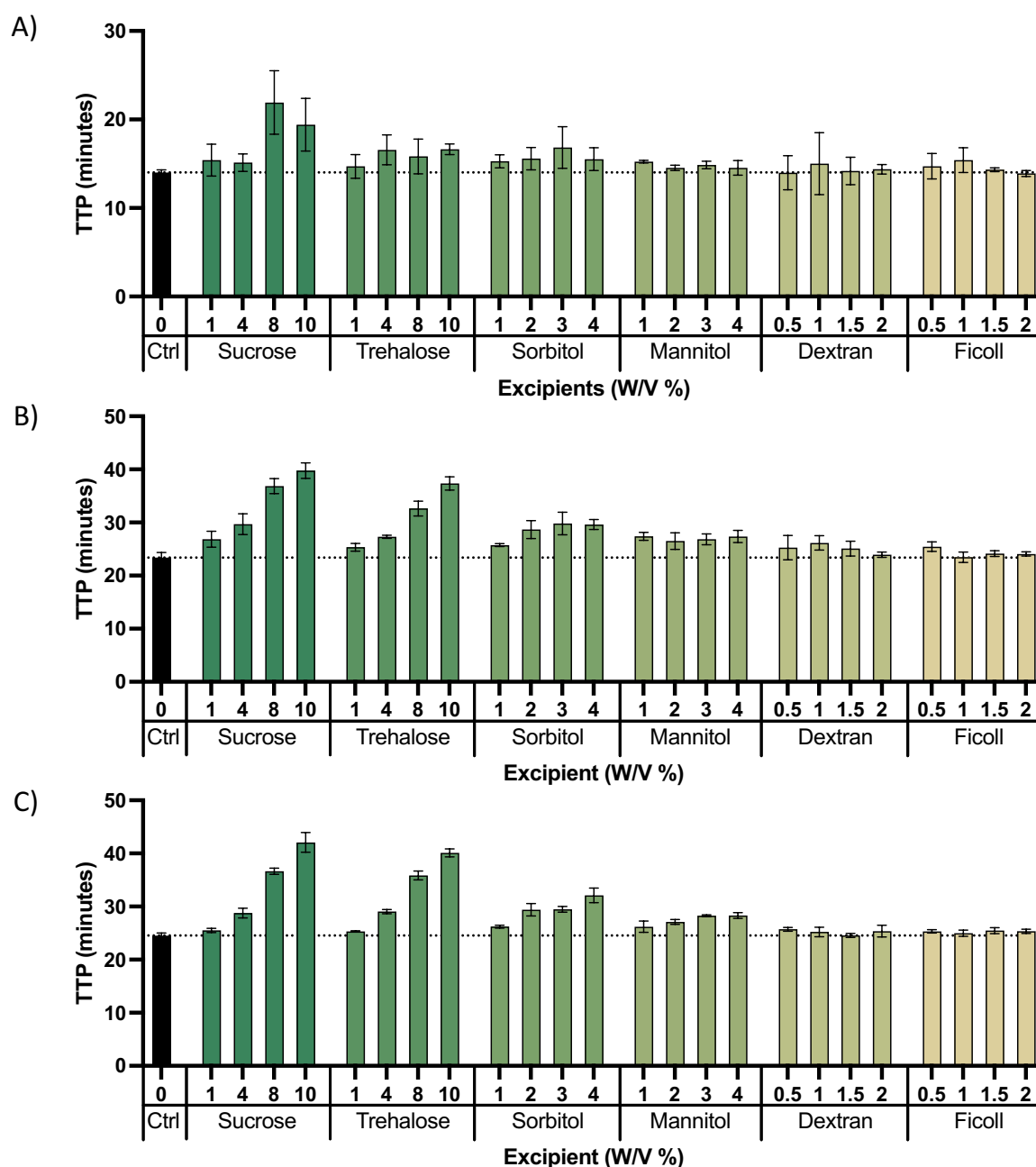


Figure 4.6. Excipient titration into LAMP reactions. The effect of a range of excipients at biologically relevant concentrations in SARS-CoV-2 assay (A), the APP LAMP assay (B) and the MH LAMP assay (C). Columns show geometric mean of triplicate reactions, and error bars depicts the S.D.

The FDM data performed by Dr Thanos Papas showed that the onset of collapse occurs at -38.6°C (Figure 4.7B). The total collapse of the material occurred at -37.9°C under 5.4 Pa of pressure. The onset of collapse temperature is indicative of the temperature at which the primary drying phase should be performed. Therefore, the primary drying phase occurred at -40°C , as indicated in Table 4.2.

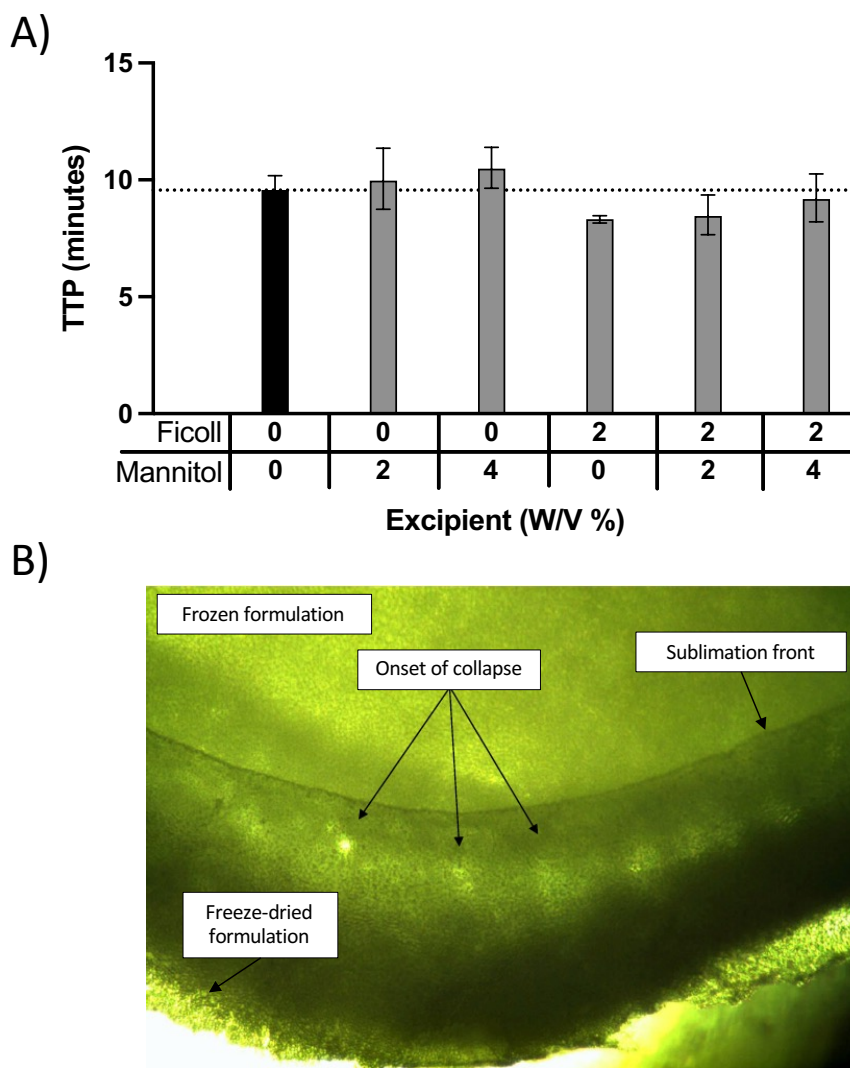


Figure 4.7. Determining lyophilisation parameters. Cross-titration of varying concentrations of Ficoll and mannitol excipients in SARS-CoV-2 reactions performed in triplicate (A). Freeze drying microscopy of the SARS-CoV-2 LAMP reactions, image taken at -38.6°C (B). Columns depict the geometrical mean and error bars S.D. Freeze drying microscopy data was obtained by Dr Thanos Papas and published with permission from Biofortuna Ltd.

Subsequent freeze-dried reactions contained primers for the SARS-CoV-2 LAMP assay (Figure 4.8A). An analytical SEN experiment showed the lyophilised LAMP reactions produced a slight delay in TTP to the LAMP reaction mixture produced fresh (liquid) (Figure 4.8B). However, the lyophilised reactions were slightly more sensitive, with all three replicates of the lowest copy number, 10 copies/ μL , compared to the liquid LAMP reaction, which produced detectable amplification from 200 copies/ μL (Figure 4.8B).

To confirm that the lyophilisation process did not impact the ability of the CMOS-ISFET sensor to detect amplification, the analytical SEN experiment was repeated, and the pH of the reactions was measured pre- and post-incubation. The results indicated that the SARS-CoV-2 lyophilised reactions produced a much stronger pH change during amplification than the liquid (fresh) reactions (Figure 4.8C). Therefore, we had successfully

produced a minimally buffered lyophilised LAMP reaction that is suitable for storage in the absence of a cold chain. Furthermore, the lyophilisation of the LAMP reactions does not compromise the rapidity or SEN of the LAMP reaction and produces a robust pH change for electrochemical detection of amplification events.

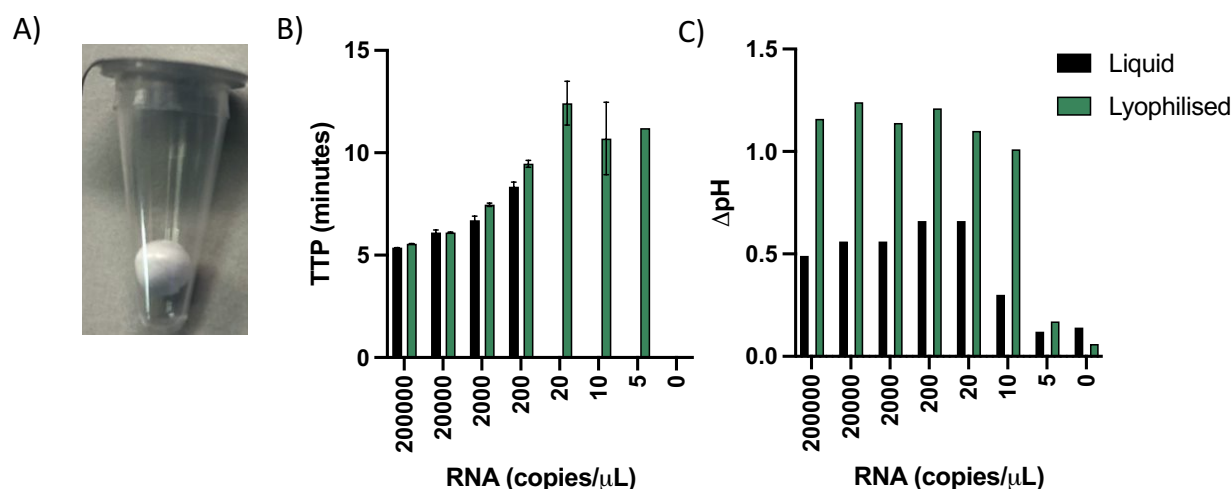


Figure 4.8. Lyophilisation of RT-LAMP reactions. An image captured of the SARS-CoV-2 RT lyophilised reaction in a 0.2 mL tube that has been heat-sealed with a foil cap (A). The performance of the liquid (fresh) and lyophilised (rehydrated) SARS-CoV-2 RT-LAMP reactions performed using a serial dilution of SARS-CoV-2 RNA, with the time taken for amplification to occur, shown by TTP (B) and the pH change achieved before and after amplification, shown by the delta pH (C). Columns depict the geometric mean obtained across three replicates and error bars depict S.D.(B). Columns depict the results of a single replicate (C).

4.9. Results summary

In this Chapter Dr Kenny Malpartida-Cardenas and I have optimised LAMP and RT-LAMP conditions to produce more rapid and sensitive amplification. With the optimal conditions being: 1x custom buffer, 1.4 mM dNTPs (each), 5 mM MgSO₄, 1.2 mg/mL BSA, 5 mM NaOH, 0.52 U/μL *Wt Bst* 2.0 DNA polymerase supplemented with 0.45 U/μL RTx for RNA detection. Using the optimised protocol in conjunction with 2% Ficoll and 4% mannitol the RT-LAMP reactions containing SARS-CoV-2 primers were successfully lyophilised. The lyophilised reactions were determined to have a slightly delayed TTP compared to when the LAMP reagents were prepared fresh, however an a 40x increase in SEN was observed with the lyophilised mixtures. Additionally, a much greater pH change was observed to occur in the lyophilised LAMP reaction compared to the fresh mixture.

Discussion

In this Chapter, the performance of several polymerases displaying strand-displacement activity was investigated, with LAMP and RT-LAMP reactions being optimised using DNA and RNA assays, respectively. A substantial decrease in TTP for three RNA assays in comparison to the recommended protocols of the manufacturers. Additionally, we have successfully lyophilised the RT-LAMP reactions and validated their compatibility with electrochemical sensing, therefore, enabling cold-chain free storage of the LAMP reactions - a key milestone in the development of a IVD PoC device.

Strand displacing DNA polymerases are necessary for efficient primer infiltration during LAMP amplification (Notomi et al., 2000). Several commercially available polymerases were evaluated in this Chapter for their performance using their manufacturer's protocol. I found that initially that *Bst* LF and Lucigens *Bst* produced the most rapid amplification, with *Bst* 3.0 resulting in FP amplification in the DNA assay, which was not observed with other polymerases using the same primer set. Subsequent optimisation of the LAMP reactions using the most appropriate polymerase compatible with lyophilisation, WS *Bst* 2.0. The optimised protocol not only increased the speed of the amplification for WS *Bst* 2.0 to have equivalent performance to *Bst* LF, but also exhibited cross-translational improvements to all polymerases, apart from *Bst* LF, whose performance remained the same. The false amplification observed with *Bst* 3.0 is in line with a recent study using digital LAMP (dLAMP) to evaluate the performance of different polymerases in LAMP (Rolando et al., 2020). Rolando et al. reported that *Bst* 3.0 displayed a high rate of non-specific amplification, even in the presence of the target, evident by the increase in the T_m of the product polymerases. It is of note that *Bst* 3.0 has been reported to produce ultra-rapid (<6 minutes) and specific amplification when the primers undergo extensive screening and optimisation using *Bst* 3.0 (Schoepp et al., 2017). Therefore, *Bst* 3.0 may have been placed at a disadvantage in our testing, as only one assay was used in the comparison (SARS-CoV-2), with the primers selected based on their performance with another iteration of *Bst*. Nonetheless, the optimised protocol developed in this Chapter increased the differential time between specific and non-specific amplification using *Bst* 3.0 compared to use of the manufacturer's recommended protocol. Thus, the optimised protocol not only decreased the time taken for amplification to initiate but also decreased the rate of non-specific amplification events, a key accomplishment in reducing the likelihood of FP results in our diagnostic platform. An additional polymerase that exhibits efficient RT activity is PyroPhage 3173, the homologue of which is supplied with the LavaLAMP component kit (Schoenfeld et al., 2006). The activity profile for the LavaLAMP enzyme is 68-74°C. The use of this enzyme was associated with non-specific amplification with three of the six primer sets tested. Whilst there are no guidelines given by Lucigen for the design of LAMP assays specifically for LavaLAMP, the assays I utilised to evaluate the performance of LavaLAMP were optimised using *Bst* 2.0. This, similarly, to *Bst* 3.0, may have accounted for the increased false-positive amplification seen using LavaLAMP. Whilst it would be beneficial to the reagent costs of the device to have a single enzyme capable of both the RT step and DNA amplification, neither of the polymerases evaluated in this Chapter performed as well on RNA targets as using a dedicated RT.

This Thesis has focused on the detection of the main five bacterial pathogens involved in the PRDC. However, it was important also to consider the compatibility of RT-LAMP because SIV and BVS are RNA viruses. Thus, I also optimised several parameters related to enzyme efficiency, namely the dNTP and Mg^{2+} concentration in relation to SIV and BVS RT-LAMP reactions. An RT-LAMP assay for the detection of the human respiratory pathogen SARS-CoV-2 was also evaluated. A intention was to produce a cross-translational protocol between both LAMP and RT-LAMP assays, facilitating the implementation of LAMP assays with RNA targets in the future. Whilst all polymerases discussed above have been shown to display some degree of RT activity (Shi et al., 2015b), only *Bst* 3.0 is advertised as possessing RT activity. *Bst* 3.0 is an in silico-designed homologue of *Bst* LF which exhibits RT activity (Ong et al., 2015). However, as *Bst* 3.0 it displays an increased TTP and non-specific amplification, it would not be appropriate for use in our LAMP assays. Five commercially available RTs were evaluated, with two, EpiScript and RapiDxFire RT, not displaying detectable amplification in our LAMP assays. Amplification was only achieved when these RTs were used in a two-step protocol where the RNA was transcribed separately to the amplification taking place. For EpiScript, this was unsurprising, as this RT has an activity range up to 55°C, 7°C lower than the temperature LAMP amplification (LGC Biosearch). Thus, it is likely that in the one-step RT-LAMP reaction, EpiScript is undergoing heat denaturation. Meanwhile, the RapiDxFire RT is reported as displaying an activity temperature range of between 50-80°C (LGC Biosearch), within the target range of LAMP amplification. Therefore, the lack of activity of RapiDxFire in the LAMP reaction is likely due to buffer incompatibility. However, as the RapiDxFire buffer is undisclosed, this hypothesis was not tested in this Chapter. Additionally, as both EpiScript and RapiDxFire RT displayed delayed TPP in two-step LAMP compared to RTx in one-step LAMP, neither of the RTs were investigated further.

A selection of additives, based on previous reports that indicated some improvement in enzyme activity and amplification, were evaluated for their ability to increase the speed of LAMP reactions. Whilst the APP-LAMP assay supported previous reports that 6% PEG8 increases the rate of the reaction (Nose et al., 2013), the SARS-CoV-2 RT-LAMP assay showed no observable improvement at this concentration, with a slight decrease in TTP at 3-4% PEG8. In contrast, the MH LAMP assay showed no improvement at any of the concentrations of PEG8 tested. Similarly, PEG35 has previously been reported to reduce nonspecific amplification in RT-LAMP reactions at 5% (Gaiteri et al., 2019). However, only APP-LAMP reactions showed a decreased TTP, across PEG35 concentrations of 3-6%. Glycerol has also been found to slightly decrease the TTP in LAMP reactions when added at 5% (Gao et al., 2019). This is not supported by my findings, in which glycerol was tolerated well in LAMP reactions up to 8%, with some benefit seen in LAMP reactions when added at 2-6%, and a negligible effect on the TTP in the RT-LAMP reactions. The only other additive tested in this Chapter which displayed a decreased TTP on addition to the LAMP reactions was GuHCl. The addition of 30% GuHCl displayed a beneficial effect on the TTP of the two LAMP reactions tested, however, no benefit was seen at any concentration in the RT-LAMP reaction. This is contrary to previous reports that 40-60% GuHCl greatly increases the reaction rate of the RT-LAMP reactions (Zhang et al., 2020). A major limitation of the results in this Chapter, overlooked at the time, was that the addition of GuHCl would have decreased the pH of the reaction. Conversely, Zhang et al (2020) accounted for this by adjusting pH of the GuHCl to pH 8.0, prior to addition into the RT-LAMP reaction. Therefore,

we cannot discount that the altered pH may have altered my RT-LAMP conditions and may account for the differences seen between the achieved and reported effect GuHCl on the RT-LAMP assay. The beneficial effect GuHCl had on the LAMP reactions compared to the RT-LAMP reactions may suggest that the RT is more pH sensitive than the polymerase. Therefore, none of the additives tested resulted in significant improvements at concentrations that were translational across all assays tested, thus, none were implemented into our optimised protocol.

We have optimised the LAMP conditions to not only improve the rate of the reaction, indicated by the decreased TTP, but also, these conditions proved favourable across a range of different polymerases tested. The decreased TTP may be a result of a decrease in primer interaction or off-target events, as when the optimised protocol was used in conjunction with *Bst* 3.0, there was a greater differentiation between amplification and false-positive amplification events. Previously, the difference between TP and FP amplification time was associated with increased SPE (Ku et al., 2022). Although WS *Bst* 2.0 was utilised in the optimised protocol, improvement across all polymerases tested was found. Additionally, Lucigens *Bst* was shown to have comparable performance when used with the standard buffer as well as Lucigens buffer B. A subsequent analysis between NEB WS *Bst* 2.0 glycerol-free and Lucigens *Bst* suggest that at low-production scales, Lucigens *Bst* would be most cost-effective, reducing the GBP £ per unit cost of the polymerase by 45.22%, equating to a reduction of 7.14% to the total reagent costs (personal correspondence with each supplier).

Contamination is a concern in molecular techniques with high SEN, especially in LAMP, where a small quantity of amplicon can contaminate the reaction (Tomita et al., 2008). Therefore, to minimise the risk of contamination events, laboratories often have dedicated workspaces for manipulation of reactions pre- and post-amplification. Additionally, it is considered good laboratory practice to include negative controls for each target being run to ensure the validity of results. In a PoC scenario, the use of negative controls for each assay would double material costs. Therefore, manufacturers frequently mitigate the risk by having a single-use cassette, considered a closed system and therefore unlikely to introduce amplicon into subsequent cassettes (QuantuMDx, 2021, Abbott laboratories, 2021, Cepheid, 2020). However, cartridge leaking has been found to occur in an analysis study of the Cepheid GeneXpert system (McHugh et al., 2017). Strategies to minimise the detection of contamination rather than prevent the occurrence include the use of the base-excision enzyme UDG (Slupphaug et al., 1995), previously implemented in molecular amplification chemistries, including LAMP (Kil et al., 2015). In this Chapter, I have also validated the use of UDG with our optimised protocol, determining that a 5-minute incubation at room temperature was sufficient to prevent at least 1,000 copies, equating to 3×10^{-10} μL of amplicon being detected in subsequent LAMP reactions. Although, in two assays, contamination of 10,000 copies was prevented, equating to 5×10^{-9} μL of the amplification reaction, the volume of a single 5 μm aerosol. In the literature, UDG prevented the detection of down to 10^{-8} ng (Hsieh et al., 2014) and 10^{-7} ng (Tang et al., 2016). The results achieved in this Chapter are at 100x higher, with 2×10^{-6} - 2×10^{-5} ng of the carry-over amplicon successfully eliminated from the RT-LAMP reaction. However, a more recent report found UDG to be capable of

eliminating 2.3×10^{-3} ng of contaminating amplicon, x100 higher than our study (Quyen et al., 2022). The large discrepancies between the amount of amplicon being removed by UDG may be due to the SEN of specific assays, with those that are highly sensitive requiring less contaminating DNA to be present to produce FP results. Additionally, the GC content of the target sequence is an important factor, with the more AT-rich the amplicon, the increased frequency of UTP bases for UDG to excise. Nonetheless, I have validated that the use of UDG as a contamination control has the potential to eliminate potential amplicon aerosolization events which may occur to a PoC cartridge. However, in its current form, the CMOS-ISFET platform is heated to 63°C prior to the addition of the LAMP reaction. Therefore, the UDG-LAMP implementation would require a re-design of the platform, and as such, the use of UDG-LAMP was not taken further in this Thesis.

Although the electrochemical sensing of LAMP amplification has previously been validated on the CMOS-ISFET chip with a range of assays (Malpartida-Cardenas et al., 2019, Rodriguez-Manzano et al., 2021, Rodriguez-Manzano et al., 2020), the accessibility of the CMOS-ISFET detection platform is limited by the use of LAMP reagents requiring a cold chain. Although ready-lyophilised LAMP reactions are commercially available (e.g., OptiGene ISO-DR001, MAST IOPLEX DNA Lyo), these solutions contain Tris for buffering, rendering them incompatible with the CMOS-ISFET chip. Therefore, Dr Kenny Malpartida-Cardenas and I optimised LAMP and RT-LAMP reactions for compatibility with the lyophilisation procedure, minimising the reaction glycerol contents, removing the betaine and implementing compatible excipients. We found that 2% Ficoll and 4% mannitol had a limited impact on the assay SEN, and lyophilised material exhibited equivalent results to the freshly prepared LAMP reactions. This is the first instance, to my knowledge, of minimally buffered LAMP reactions undergoing lyophilisation for the purpose of electrochemical detection. Previously, 10% trehalose was found to be compatible with the lyophilisation of RT-LAMP (Carter et al., 2017). Whilst 10% trehalose only inhibited our RT-LAMP reaction by 2.62 minutes, in the LAMP reactions detecting APP and MH, 10% trehalose inhibited the TTP by 13.98 and 15.58 minutes, respectively.

The production of lyophilised LAMP reactions has previously been achieved using the metal indicator dye, hydroxy naphthol blue (HNB), added on rehydration of the mixture, in a two-step approach (Chen et al., 2016b). The same group subsequently included the DNA intercalating dye, SYBR green, in combination with sucrose for the lyophilisation and one-step detection of LAMP amplification with a UV light (Chen and Ching, 2017). Neither of Chen's papers describe the concentrations of the sucrose sugar used in their lyophilisation protocols, in our testing, the addition of 10% sucrose was more inhibitory to the reactions than trehalose.

An alternative to freeze-drying is air-drying, where the reactions are heated, and the water is evaporated from the product over time. This is known to be compatible with the addition of 3 μ M trehalose and 21% glycerol. However, the buffer, magnesium and fluorescent dye were separated from the enzymes and dNTPs (Hayashida et al., 2015). Air-drying has been used in combination with metal-chelating detection of LAMP reactions (Hayashida et al., 2015, García-Bernalt Diego et al., 2019), however, it is yet to be confirmed as compatible with pH-sensing techniques.

In the liquid form, the enzymes used in LAMP will be held in their quaternary structure and be biologically active via the formation of intramolecular hydrogen bonds made with water. The removal of water from these structures results in the destruction of hydrophobic bonds and the disruption of these intramolecular bonds. As the free water is removed from the reaction, the protein is subject to physical denaturing, which is minimised by the addition of excipients to retain proteins structural integrity. In an unbuffered solution, the removal of water will also result in an increase in pH to the solution, known to decrease protein functionality. The performance of the lyophilised SARS-CoV-2 RT-LAMP reactions displayed higher SEN compared to the liquid formation, with a slight increase in the TTP of the lyophilised reactions. Indicative that the formulation of the lyophilisation mixture was sufficient to retain DNA polymerase activity. However, the lyophilisation was only performed in combination with either Ficoll and dextran or dextran and trehalose, investigating how different excipients may protect the protein activity during the lyophilisation process was beyond the scope of this work.

In this Chapter, I aimed to create an optimised LAMP chemistry that remains compatible with pH detection methods, such as the CMOS-ISFET platform. Ultimately, we were successful in creating a rapid, sensitive, and lyophilised LAMP reaction. However, limitations of this work for a PRDC diagnostic remain. Due to the SARS-CoV-2 pandemic and lock-down and the rules regarding the type of work permitted in laboratories, there was a shift in emphasis to that virus. However, in terms of optimisation of the biochemistry, the appropriate controls were included in all assays to ensure that the target pathogen nor the efficiency of the primer set would impact any observed trends. Furthermore, the majority of testing was performed using at least three different assays, combining RNA and DNA detection to confirm amplification trends were universal, and any inter-assay variability was minimised. Secondly, lyophilised RT-LAMP reactions were only produced for the detection of SARS-CoV-2, owing to the high costs associated with developing a proof-of-concept lyophilisation protocol. Lastly, the long-term stability of the lyophilised reactions was not assessed due to the limited number of lyophilised reactions available once initial performance testing was completed. It would have been beneficial to perform an accelerated ageing study, where the reactions are incubated at a higher temperature, and their activity is periodically tested at appropriate time points. The loss of activity is therefore accelerated and represents the length of time the reactions will retain their activity if stored at room temperature. Nevertheless, it is likely that the results obtained with SARS-CoV-2 are applicable to the PRDC pathogens.

4.10. Conclusions

In conclusion, we have optimised the LAMP and RT-LAMP protocol resulting in a reaction that is more rapid than the recommended manufacturer's protocol and compatible with the lyophilization process. The optimised LAMP conditions have enabled the production of a lyophilised reaction which is not only comparable with reactions that were produced fresh but resulted in increased SEN. Importantly, we have also verified the lyophilised reactions produce a very strong pH change and are, therefore compatible with the CMOS-ISFET detection platform. Thus, I have produced dried reactions that are suitable for deployment in a PoC device, negating the need for cold-chain storage. Enabling the deployment of it in previously inaccessible settings, such as on-farm.

Chapter 5: Development of DNA extraction methods from solid tissues

Introduction

The vast majority of diagnostic systems have a requirement for some degree of sample analyte purification prior to detection. The purpose of a sample purification procedure is to separate the target analyte from cellular components which inhibit downstream analysis, for example, blood components inhibiting Taq polymerase, commonly used in PCR. There is variation in the tolerance of different amplification methods to inhibition by cellular components. For example, LAMP amplification typically has a higher tolerance to whole blood inhibition than PCR (Francois et al., 2011). Therefore, the removal of potentially inhibitory macromolecules ensures target amplification is not impeded. In this Chapter, I will explore different sample purification methods with focus placed on solid tissue extraction, namely that originating from that lung.

5.1. Tissue homogenisation

The process of breaking down tissue structures to form a suspension of liquid is known as tissue homogenisation. The effective homogenisation of tissue will not only expose internal structures of the tissue to the sample preparation method but may also aid in the lysis of pathogens through mechanical force. All of the bacterial pathogens of the PRDC are considered primarily extracellular, although there are reports of internalisation of all of the bacterial species of interest, for instance, internalisation of APP by endothelial cells (Plasencia-Muñoz et al., 2020) and MH internalisation has been shown to be responsible for phagocytic evasion (Raymond et al., 2018b). SS has been found within epithelial cells with survival for at least 24 hours; however, there is no evidence that this occurs in vivo (Valentin-Weigand, 2004). Thus, the vast majority of PRDC bacteria are extracellular in the lung tissue during an active infection. Whereas the vast majority of viral particles will be intracellular as part of their replication process.

There are several tissue homogenisation methods, such as grinding, shearing, beating and shocking. Whilst numerous homogenisation instruments have been designed, such as the MP-Biomedicals FastPrep-24 Tissue instrument they are often large and expensive (>GBP £4,000), favouring high throughput over portability and affordability. Far fewer homogenisation devices have been designed for use at the PoC. The most primitive form of homogenisation would be the manual use of a pestle and mortar, with the PowerMasher II utilising a similar process in which the pestle is rotated by a battery-operated motor to grind the tissue (**Figure 5.1**). The benefit of this system is that the pestle is plastic and, therefore, single-use and can be obtained sterile (BioMasher V). The BioMasher has been previously used in the homogenisation of rodent brain tissue and plants (Yamamoto et al., 2012, Benga et al., 2004) and, in this Chapter, I describe the evaluation of the PowerMasher II in combination with the BioMasher V for the homogenisation of lung tissue samples.



Figure 5.1. Commercially available portable tissue homogenisation devices. The handheld battery-operated electric homogeniser, the PowerMasher II, including a rubberised pestle holder to the rapid change of homogenisation pestles (A). The BioMasher V is a sterile, single-use homogenisation system consisting of a plastic pestle with a textured tip encapsulated into an inner filter tube (80-145 µm pore size) which filters homogenised tissue into the supplied 15 mL centrifuge tube (B). Image used with the permission of the copyright holder, nippi-inc.

5.2. Pathogen lysis

The lysis of pathogens within a sample involves the breakdown of cellular components, which ultimately results in the disruption of the bacterial membrane and liberation of intracellular genomic material, a key process in the diagnostic pathway. The lysis of cells can occur through several techniques, such as thermal, mechanical, chemical, and electrical methods. In thermal lysis, the cells are subject to thermal stress, damaging the cell membrane and denaturing cellular proteins. Alternatively, several freeze-thaw cycles produce the same result. In mechanical lysis, the cells are placed under mechanical strain, and the cell membrane is ruptured by force. A common method is passing bacterial cells through a space smaller than the cell diameter, with jagged edges which puncture the cellular membranes of the cells (Di Carlo et al., 2003). In chemical lysis, the cells are subject to buffers which damage the cells through several mechanisms. Detergents, such as sodium dodecyl sulphate (SDS) and Triton X-100, form pores in the cell membrane through solubilising proteins and lipids embedded in the cell membrane (Linke, 2009). Ionic salts such as NaCl, KCl and $(\text{NH}_4)_2\text{SO}_4$ increase the osmotic stress on the cells, resulting in cellular rupture (Logisz and Hovis, 2005). Chaotropic salts, such as GTC, phenol and urea, disrupt the structure of macromolecules, including those within the cell membrane. Additionally, proteolytic enzymes such as proteinase K can be used to aid in the degradation of proteins. In electrical lysis, cells are placed under a high-intensity pulsed electric field, which creates nanopores in the cell membrane and, over time, causes the membrane to disintegrate (Chang et al., 1991).

The bacterial PRDC pathogens consist of four Gram-negative (APP, MH, PM and GPS) and one Gram-positive (SS) bacterium. Gram-negative bacteria contain increased peptidoglycan cross-linkage within their cell walls and

possess an outer membrane containing LPS. Gram-positive bacteria contain a thicker peptidoglycan layer than Gram-negative bacterium, and most Gram-positive bacteria contain anionic glycopolymers; membrane-bound lipoteichoic acid and cell wall-bound teichoic acid (**Figure 5.2**). Generally, it is more difficult to disrupt the rigid structure of Gram-positive bacterial membranes, and thus they are considered more difficult to lyse (Mahalanabis et al., 2009, Fujiwara et al., 2005, Cabeen and Jacobs-Wagner, 2005).

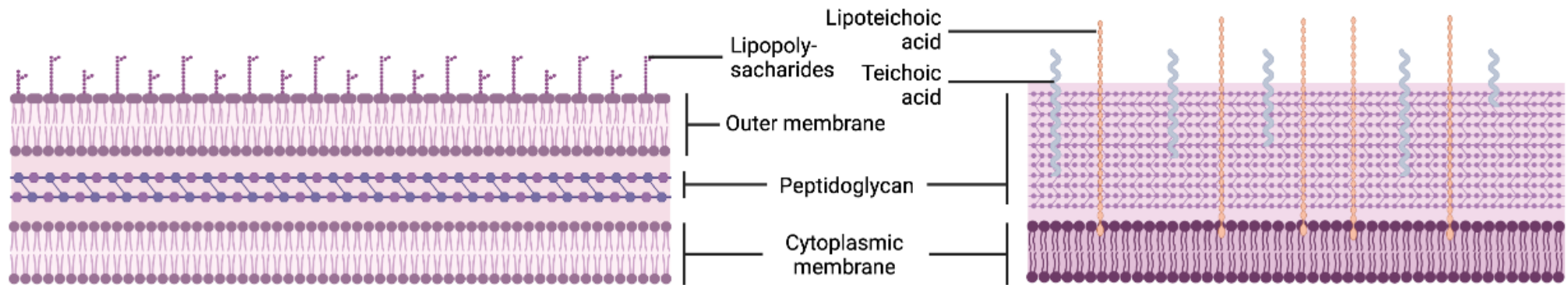
A key advancement in the isolation of nucleic acids from complex samples was the identification that strong chaotropic salts, such as GTC, possess two important properties, the ability to lyse cells and denature proteins (such as RNases and DNases) and its use releases genetic material from the cell whilst simultaneously preventing RNase and DNase-based degradation (Gordon, 1972, Chirgwin et al., 1979). The addition of phenol-chloroform fast-tracked the isolation of nucleic acids as proteins, lipids and genetic material separate based on their association with different aqueous and organic phases (Chomczynski and Sacchi, 1987). However, the isolation of nucleic acid using chaotropic salts coupled to phenol-chloroform remained lengthy, taking up to four hours and requiring chemicals which are unsuitable for use outside of a laboratory environment. It was not until the 1990s that it was discovered that chaotropic salts also enable nucleic acids to selectively bind to solid supports, namely silica (Boom et al., 1990), reducing nucleic acid purification time to less than one hour.

5.3. Binding of nucleic acids

The capture of DNA or RNA onto a silica-based solid support in the presence of chaotropic salt is known to be pH-dependent. Although weak interactions between DNA and silica have been observed under basic pH conditions, as the pH approaches the isoelectric point of silica, ~pH 3.0, these interactions become stronger (Fujiwara et al., 2005). Once the pH is increased to alkaline conditions, the interactions become weakened such that the nucleic acid is released into the solution, known as a simplistic pH-modulated charge switch. Further to silica, nucleic acids have been reported to charge switch with several other materials, including glass (Nanassy et al., 2007) and several polymers (Zhang et al., 2019d, Al Balawi et al., 2019, Pandit et al., 2015).

An alternative to materials which form interactions with nucleic acids is the use of paper membranes which entrap nucleic acids, such as Whatman filter paper, cotton fibres and polyethersulfone membranes (Zou et al., 2017, Ahmed et al., 2022, Linnes et al., 2016). The benefit of paper-based extraction methods is that they are very economical to produce, abundant and biocompatible. Previously, the Langford group described the use of FTA cards which are chemically coated Whatman paper, lysing bacteria and viruses on contact and compatible with multiplex PCR for the detection of APP (Stringer et al., 2021). However, the rapid entrapment of genetic material on paper-based material often requires lengthy incubation times to release the DNA back into the solution, with the most efficient solubilisation of DNA taking as long as 33 minutes (Tang et al., 2022).

Gram-negative



Gram-positive

Figure 5.2. Bacterial cell membrane components of Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess an outer membrane embedded with LPS. A thin layer of peptidoglycan is situated in the periplasmic space between the outer and inner membranes (cytoplasmic membrane). Gram-positive bacteria contain a peptidoglycan layer that is several times thicker than that of Gram-negative bacteria. Within the peptidoglycan layer, there are membrane-bound lipoteichoic acid glycopolymers and cell wall-bound teichoic acid polymers. Figure created with BioRender.

Therefore, a paper-based extraction solution might not be the most suitable for PoC testing involving multiplexing, such as that described in this Thesis. Thus, the most appropriate sample preparation protocol would ensure the rapid separation of DNA from other macromolecules present in the sample and the equally rapid release of the DNA back into the aqueous phase for amplification. The use of silica-based solid phase isolation remains the most common method of DNA purification today in a laboratory setting. The use of silica-based spin columns, in which centrifugal force is used to pass solutions through the silica filter is regarded as the gold-standard technique for sample preparation methods.

A major advancement in the throughput of silica-based sample purification methods was the development of magnetic particles which are encapsulated in silica (Bruce et al., 2004). Now widely commercially available, the silica particles can be rapidly collected based on their magnetic strength with lysis, washing and elution solutions applied to the sample in a single tube without the need for expensive laboratory equipment. Therefore, in this Chapter, I evaluated the use of silica-based magnetic beads compared to the gold-standard method of DNA extraction, using a silicon-based spin-column kit.

5.4. Contaminant removal

After the sample has been associated with a suitable solid-phase support, the material is often washed several times in a wash buffer. The wash buffer, typically salt or ethanol, removes contaminating proteins, LPS and smaller nucleic acids, increasing the purity of the nucleic acid. Historically, a high percentage of ethanol in combination with salt was used to reduce the free water in a solution, forcing the phosphate groups of DNA to be attracted to the salt cations in the solution and precipitate out of the solution, known as 'salting-out' (Green and Sambrook, 2016). This allows the genetic material to be pelleted by centrifugation. In solid-phase sample preparation, two-wash protocols frequently employ a similar strategy, with the first wash buffer containing ethanol and salt to precipitate the DNA within the solid phase, whilst contaminating macromolecules are solubilised into the solution. A secondary wash in a higher percentage of ethanol, reduces the salt concentration, which can inhibit amplification. Finally, the purified nucleic acid is eluted, with water or TE buffer which re-solubilise the nucleic acid off the surface of the substrate and back into the solution.

The purification of nucleic acids from lung tissues can be categorised into four key processes, discussed in detail below:

- 1) The homogenisation and lysing of the tissue to release the nucleic acids
- 2) The binding of the nucleic acids to the silica surface
- 3) The washing of the beads, typically in alcohol
- 4) The release of nucleic acids into a solution

5.5. Aims

In this Chapter, I evaluated two rapid homogenisation methods to determine if either are appropriate for use on a farm. Additionally, I optimised the extraction of gDNA from several bacterial pathogens of the PRDC, evaluating several commercially available magnetic separation products, with the view to implementing them into the rapid (taking <5 minutes) extraction process conceptualised, developed and validated previously by Matthew Cavuto, Dr Ivana Pennisi and Dr Jesus Rodriguez Manzano at Imperial College London, named SmartLid.

Methods

5.6. Lung tissue

Lung tissue was kindly supplied by Paul Penny or Eduardo Velasquez (Ceva, UK) and stored at -20°C prior to use. Lung tissue was thoroughly defrosted prior to use and excised with a sterile scalpel into approximately 100 mg pieces. Pathogen-negative lung samples had been previously characterised by a PCR designed by the Langford laboratory to detect the five PRDC pathogens by Dr Yanwen Li (unpublished data).

5.7. Homogenisation methods

Two pathogen-negative lungs were used to evaluate three homogenisation methods. Slices of lung tissue were weighed, and 100 mg of tissue was homogenised in 400 µL of PBS (Gibco, Cat 10010015) in duplicate for each method and each lung sample (a total of 12 lung slices). The gold-standard method was the use of MP Biomedicals lysing matrix A (MP Biomedicals, Cat 116910050-CF) in conjunction with FastPrep-24 5G (MP Biomedicals, Cat 116005500) set to 6.0 m/s for 60 seconds. The gold standard was compared to the use of the lysing matrix A (MP Biomedicals) and a vortex for 30 seconds, or the use of a PowerMasher II (2BScientific, Cat 891300) in combination with BioMasher V tubes (2BScientific, Cat 891393) for 30 seconds.

5.8. Lysis buffers

Three bacterial isolates from three different species, APP - MIDG 2331, PM - MIDG 2314 and SS - P1/7, were grown overnight on BHI (BD, Cat 2237500) containing 1.5% agar (Sigma-Aldrich, Cat W201201) and supplemented with 0.01% NAD (MP Biomedicals, Cat 100319) and 10% DHB (TCS biosciences, Cat HB035) at 37°C. 10 µL loops were used to resuspend the bacterial colonies to an OD₆₀₀ of 0.1 in BHI broth (BD) supplemented with NAD (MP Biomedicals) and 10% DHB (TCS biosciences), incubated on a shaker at 100 rpm at 37°C. Each hour an OD₆₀₀ measurement was taken for each of the cultures and plotted. Once the cultures had reached the early growth phase, equating to an OD₆₀₀ of 1.11 for APP, 0.85 for PM and 0.86 for SS. The cultures were split into 1 mL aliquots into 1.5 mL microcentrifuge tubes and pelleted by centrifugation at 5,724 x g for 5 minutes for APP and SS, whilst PM required to be centrifuged at 12,879 x g for 5 minutes to form a compact pellet. The supernatant was carefully removed and discarded, and the pellets were gently resuspended in 1 mL of each lysis buffer.

A panel of seven lysis buffers (including commercial and formulated) were used to evaluate their ability to lyse, APP, PM and SS. The panel included the lysis and binding buffer (LBB), which is included in the TurboBeads extraction kit (TurboBeads, Cat 5006), which contains an undisclosed mixture of chemicals. The eNAT buffer consists of 42.5-45% GTC (Copan, 2014) and is the lysis and stabilisation medium used in COPAN eNAT swabs (Copan, Cat 6U072S). Buffer ATL is supplied with the DNeasy blood and tissue kit (Qiagen, Cat 69556) and is most likely composed of 1-10% SDS, 30-50% GuHCl and 1-10% Triton X-100 (Pastorino et al., 2020). The buffer ATL was used with (ALT +) and without (ATL -) the addition of proteinase K at 60 mAU/mL (Qiagen, Cat 19157). The

MagMax lysis and binding solution (LBS) is supplied with the MagMax DNA multi-sample kit (Thermo Fisher Scientific, Cat 4413020), and contains 15-24% non-ionic surfactant, 30-50% GuHCl and <0.01% acrylamide (Thom et al., 2021). Two further buffers were produced in-house from a literature search. Custom buffer 1 (CB1) was adapted from a Human Lung tissue lysate buffer protocol, which is based on a radioimmunoprecipitation assay (RIPA) buffer (Cell Sciences). The adapted CB1 was devoid of chemicals that aid in protein extraction: 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL Aprotinin and 5 µg/mL Leupeptin. Therefore, CB1 consisted of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% w/v SDS, 1 mM EDTA and 1% Triton X-100. Custom buffer 2, (CB2) was described as a Tissue lysis buffer and contained 100 mM Tris-HCl pH 8.0-8.5, 200 mM NaCl, 0.2% w/v SDS, 5 mM EDTA with (CB2 +) or without (CB2 -) 100 µg/mL proteinase K (Sigma-Aldrich, Cat 3115887001) (Cold Spring Harbor, 2009).

5.9. Bacterial extractions

Whole gDNA was extracted from APP, SS and MH using isolates MIDG 2220, P1/7 and MH232, respectively. MH gDNA was supplied by bacterial gDNA from MH was supplied by Professor Andrew Rycroft (Royal Veterinary College). Isolates of APP and SS were cultured on BHI agar (BD) supplemented with NAD (MP Biomedicals) and 10% DHB (TCS biosciences) at 37°C overnight. 10 µL loops of bacterial colonies were resuspended in 200 µL of sterile PBS (Gibco, Cat 20012027). DNA extraction was performed using a FastDNA Spin Kit (MP Biomedicals, Cat 116540600-CF), as described in **Chapter 2; Section 2.8.1**.

5.10. Spin-column based protocol

5.10.1. Gold-standard (Qiagen) protocol

1 g of pathogen-negative lung tissue was homogenised in 1.5 mL of buffer ATL (Qiagen, Cat 939011) using lysing matrix A (MP Biomedicals) and a FastPrep-24 5G (MP Biomedicals) set to 6.0 m/s for 60 seconds. 1 mL of the supernatant was subsequently diluted in 19 mL of buffer ATL (Qiagen) and aliquoted into 320.4 µL aliquots prior to being combined with 39.6 µL of bacterial preparation at different OD₆₀₀ dilutions made in buffer ATL (Qiagen). 360 µL of the bacterial preparations were then used as the sample input into the DNeasy Blood and Tissue Kit (Qiagen). 20 µL of proteinase K (Qiagen) was added, and the tubes were thoroughly vortexed and incubated for 1 hour at 56°C in a heat block. Subsequently, 200 µL of buffer AL (Qiagen, Cat 19075) was added, and the mixture was vortexed for 15 seconds prior to being incubated for 10 minutes at 70°C to inactivate the proteinase K. Subsequently, 200 µL of ethanol (Sigma-Aldrich, Cat 51976-500mL) was added, and the solution thoroughly pipette-mixed prior to application to a spin column by centrifugation for 1 minute at 6,000 x g. The flow-through was discarded, and the sample was washed with the addition of 500 µL of buffer AW1 (Qiagen, Cat 19081), which was centrifuged for 1 minute at 6,000 x g. The flow through was discarded, and the column washed with the addition of 500 µL of buffer AW2 (Qiagen, Cat 19072), and centrifuged for 3 minutes at 20,000 x g. The flow-through was discarded, and the sample was centrifuged for 1 minute at full speed 20,000 x g for one minute to ensure no ethanol carry-over into the sample. The filter section was subsequently transferred to a new collection tube, and 100 µL of buffer AE (Qiagen, Cat 19077) was applied to the filter. The sample was left to sit for 5

minutes at room temperature prior to being centrifuged for 1 minute at 6,000 x g. The filter was discarded, and the sample eluent was kept on ice.

5.11. Magnetic beads protocols

A comparison was made of the magnetic bead protocols to determine which combination of bead type and protocol from each manufacturer produced the highest yield of DNA. The Magnetic bead kits compared were: MagMax DNA multi-sample kit (Thermo Fisher Scientific), Sera-Xtracta Virus/Pathogen kit (Cytiva, Cat 29506009) and TurboBeads for viral RNA extraction kit (TurboBeads, Cat 5006). Extraction protocols were performed as per the manufacturer's protocols, with the following alteration, input volumes were adjusted to be consistent between protocols, with 400 μL of a suspension of 10^5 , 10^4 and 10^3 copies/ μL of APP serovar 8 gDNA, and all with 10^5 copies/ μL of the IC synthetic DNA. Additionally, all elution volumes were altered to be in a final volume of 400 μL to match the sample input.

5.11.1. MagMax protocol

The MagMax protocol consisted of 10^5 , 10^4 and 10^3 copies/ μL of APP serovar 8 gDNA and IC synthetic DNA made in the lysis buffers supplied with each magnetic bead kit. 400 μL of each preparation was added to a 2 mL microcentrifuge tube and mixed by vortexing for 3 minutes with 300 μL of 100% isopropanol (IPA, Sigma-Aldrich, Cat 59304-1L-F). The equivalent of 16 μL of MagMax/Sera-Xtracta or TurboBeads were then added with 24 μL of nuclease-free water prior to being vortexed for 3 minutes to bind the DNA to the beads. The mixture was then separated using a magnetic stand (DynaMag-2 Magnet, Thermo Fisher Scientific, Cat 12321D) for 5 minutes, the supernatant was discarded, and the beads were washed with 300 μL of Wash Solution 1. The preparations were removed from the magnetic stand and vortexed for 30 seconds prior to being placed back into the magnetic stand and left for 1 minute to separate the magnetic beads from the solution. This process was then repeated with 300 μL of Wash Solution 2 and left to air dry without the lids being closed for 3 minutes on the magnetic stand to evaporate off any residual ethanol. 100 μL of freshly prepared RNase A mix containing 5 μL of RNase A and 95 μL of nuclease-free water was added to each tube and mixed by inversion for 2 minutes. 100 μL of Multi-Sample DNA Lysis Buffer and 120 μL of isopropanol were added to the sample and shaken for a further 3 minutes. The preparation was then separated by being placed in the magnetic stand for 1 minute, and the supernatant was removed. The beads were washed twice, as described above, in 300 μL of Wash Solution 2 and air-dried without the lid being closed for 3 minutes. Once the residual ethanol had evaporated, 200 μL of DNA Elution Buffer 1 was added to each tube and vortexed prior to incubation for 5 minutes at 70°C in a heat block. The tubes were then vortexed for a further 5 minutes prior to 200 μL of DNA Elution Buffer 2 being added. The tubes were gently vortexed for 30 seconds and placed on the magnetic stand for 5 minutes. Finally, the supernatant was removed and kept on ice in a clean microcentrifuge tube.

5.11.2. Sera-Xtracta protocol

The Sera-Xtracta protocol was as recommended by the manufacturers of the Sera-Xtracta Virus/Pathogen kit (Cytiva, Cat 29506009). Briefly, 10 μ L of proteinase K was added to 400 μ L of the DNA preparations diluted in Tris-EDTA buffer (Sigma-Aldrich, Cat 93283-500ML). The beads were subsequently added to the tube as a 20 μ L mix, consisting of either a 1:1 volumetric ratio of SeraSil-Mag 400 and SeraSil-Mag 700, or 20 μ L of MagMax or TurboBeads. 570 μ L of binding/lysis reagent (Cytiva) was then added, and the preparations were mixed via a vortex for 1 minute prior to being incubated on a heat block for 10 minutes at 60°C. The magnetic beads were then separated via being placed on a magnetic stand for 1 minute. The supernatant was discarded and 950 μ L of Wash Buffer 1 (Cytiva) was added and mixed by gentle pipetting 10-times. The solution was then mixed for 1 minute with a vortex and separated by being placed on the magnetic stand for 1 minute and the supernatant removed. The beads were washed with addition of 950 μ L of freshly prepared 80% ethanol (Sigma-Aldrich, Cat 51976-500mL), which was mixed by pipetting. The magnetic beads were separated from the solution by being placed on the magnetic stand for 1 minute and the supernatant was removed. The tubes were subsequently removed from the magnetic stand, and the beads were allowed to return to the bottom of the microcentrifuge tube prior to being returned to the magnetic stand and any residual supernatant was removed with a 10 μ L pipette. The tubes were again removed for the magnetic stand and left to air dry without the tubes being closed for 2 minutes. The samples were eluted by the addition of 400 μ L of preheated (70°C) nuclease-free water (Thermo Fisher Scientific, Cat AM9939) and mixed via pipetting 20-times. Finally, the tubes were returned to the magnetic stand and left for 1 minute prior to the supernatant being transferred to a clean microcentrifuge tube and placed on ice.

5.11.3. TurboBeads protocol

The TurboBeads protocol involved addition of 400 μ L of the sample with 300 μ L of IPA (Sigma-Aldrich) and 300 μ L of LBB (TurboBeads) prior to mixing by inversion for 30 seconds in the presence of 20 μ L of TurboBeads (silica), MagMax or SeraSil-Mag beads. The microcentrifuge tubes were then placed on the magnetic stand for 1 minute, and the supernatant removed and discarded. The beads are then removed from the magnetic stand, and 400 μ L of 80% ethanol (Sigma-Aldrich) was added and mixed by inversion for 30 seconds. The microcentrifuge tubes were returned to the magnetic stand and left for 1 minute prior to the supernatant being removed. The tubes were removed from the magnetic stand and left to stand, open, at room temperature for 10 minutes to evaporate any residual ethanol. For elution, 200 μ L of nuclease-free water was added (Thermo Fisher Scientific), and the solution mixed by inversion for 30 seconds. Finally, the microcentrifuge tubes were returned to the magnetic stand for 1 minute, and the supernatant transferred to a clean microcentrifuge tube and placed on ice.

5.11.4. SmartLid protocol

For extraction with SmartLid, I implemented the pre-designed patented design (GB2102573.9) for the purification of nucleic acids, optimised by Dr Ivana Pennisi and Matthew Cavuto. The SmartLid system uses a pre-moulded magnetic adapter that fits microcentrifuge tubes, used in conjunction with a detachable magnet

for the rapid collection and transfer of magnetic beads (**Figure 5.3**). This arrangement allows for the binding, washing and elution steps to be in three pre-aliquoted microcentrifuge tubes. The tubes were prepared as follows: Tube 1 contained 400 μL of eNAT buffer containing patient sample (Copan, Cat 6U073S01) and 700 μL of a proprietary solution consisting of TurboBeads silica (TurboBeads) and a lysis and binding buffer (LBB) containing isopropanol (IPA) ($\leq 61\%$) and GTC ($\leq 25\%$). Tube 2 contained 400 μL of 80% ethanol (Sigma-Aldrich, Cat 51976-500mL). Tube 3 contained 200 μL of nuclease-free water (Thermo Fisher Scientific). The instruction guide on the SmartLid process is shown in **Supplementary Figure 16**.

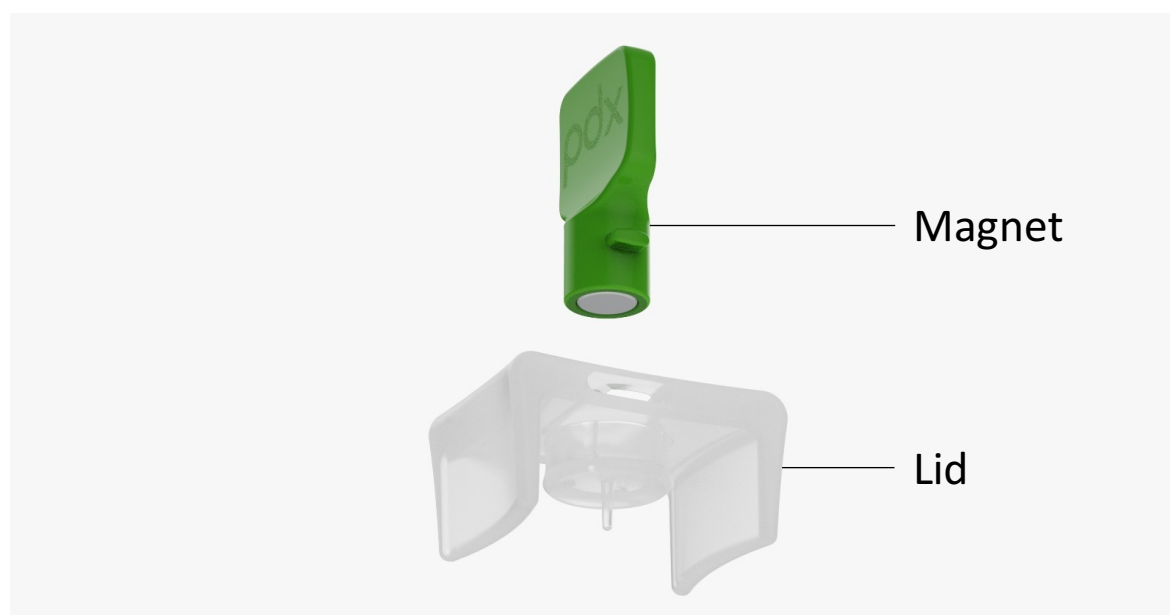


Figure 5.3. Render of SmartLid sample extraction tool. Reproduced with permission from the Copyright holder, ProtonDx Ltd.

5.12. Magnetic-based extraction optimisation

5.12.1. Optimisation of SmartLid protocol

The optimisation of the SmartLid protocol, was originally validated for the use of eNAT buffer originating from human nose and throat swabs for the detection of SARS-CoV-2 (unpublished data). For adaptation to pig lung samples, the sample input was modified with 0.1 g of tissue per mL of lysis buffer, and homogenisation with lysing matrix tube A (MP Biomedicals, Cat 116910050-CF), which was vortexed for 30 seconds on full speed, being used. The supernatant was spiked with purified gDNA from APP serovar 8 isolate, MIDG 2220 at 10^5 , 10^4 and 10^3 copies/ μL . In the initial optimisation, a number SmartLid protocol parameters were altered. These included the number of washes ranging from no wash to three washes in 400 μL of 80% ethanol (Sigma-Aldrich, Cat 51976-500mL). The volume of one wash in 80% ethanol ranged between 100-800 μL , with the sample input remaining at 700 μL and the elution at 200 μL of nuclease-free water. Next, the ratio of IPA to sample in SmartLid Tube 1 was varied from 10-80% of the sample, with the total sample volume input remaining consistent with the elution volume, using a single 400 μL wash in 80% ethanol (Sigma-Aldrich).

The volume of TurboBeads was altered to be between 10-40 μL of 50 mg/mL silica coated TurboBeads using both 400 and 700 μL spiked lung homogenate input, with 300 μL IPA, beads were washed once in 80% ethanol (Sigma-Aldrich) and eluted into 400 and 700 μL of nuclease-free water (Thermo Fisher Scientific), respectively. The length of time the SmartLid underwent vigorous inversion, without the magnet inserted, to mix the solution components ranged from 10-120 seconds, with a 400 μL sample input at 10^5 copies/ μL of APP MIDG 2220, 400 μL of 80% ethanol (Sigma-Aldrich), 80% 2-mercaptoethanol (Sigma-Aldrich, Cat M6250-100mL) or 80% tripropylene glycol (Sigma-Aldrich, Cat 187593-1L) in the wash tube and 200 μL sample elution in nuclease-free water (Thermo Fisher Scientific).

5.12.2. Wash buffer optimisation

A panel of six wash buffers were trialled in the SmartLid protocol using the optimised parameters of 0.5 g of the pathogen-negative lung being homogenized in 2 mL of tissue lysing buffer, in lysing matrix A for 30 seconds on a vortex. Pooled homogenates were then aliquoted into appropriate working volumes and spiked with gDNA extracted from APP serovar 8 isolate, MIDG 2220 to 10^5 copies/ μL . 700 μL of the sample preparation was washed once, with no drying step apart for when ethanol was used. The extracted DNA was eluted into a constant volume of 200 μL . The panel of wash buffers consisted of 2-methyl-1,3-propanediol (Sigma-Aldrich, Cat 375721-1L), methanol (Sigma-Aldrich, Cat 322415-100mL), 1,2-butanediol (Sigma-Aldrich, Cat 177652-250G), tripropylene glycol, 2-mercaptoethanol (Sigma-Aldrich) and ethanol (Sigma-Aldrich) were diluted in nuclease-free water (Thermo Fisher Scientific) to 70% V/V. Subsequently, only four wash buffers were compared at 400 μL : 2-methyl-1,3-propanediol, 1,2-butanediol, tripropylene glycol and ethanol, at concentrations ranging from 50-80% V/V. After which the range was increased between 50-100% V/V and the elution volume varied between 100-400 μL .

5.12.3. Optimised SmartLid protocol

The optimised SmartLid protocol consisted of three tubes. The first tube contained 700 μL of homogenised lung tissue being combined with 320 μL of a proprietary solution containing TurboBeads and $\leq 61\%$ IPA. The second tube contained 400 μL of 80% tripropylene glycol (Sigma-Aldrich), and the third tube contained 200 μL of nuclease-free water (Thermo Fisher Scientific). Once the magnetic beads had been released into the solution, by the removal of the magnet from the SmartLid, the tube is mixed by inversion for 30 seconds prior to the magnet being added and the magnetic TurboBeads being collected by 10x inversions of the tube. The SmartLid is then transferred to tube 2 and the process is repeated i.e., release of beads, mixing, and collection prior to the SmartLid being transferred to tube 3 and the process is repeated - resulting in purified nucleic acids in tube 3.

5.13. qPCR amplification

The qPCR primers used in this Chapter are shown in **Table 5.1**. The assessment of the extraction efficiency occurred with qPCR. The qPCR reactions consisted of 1x GoTaq probe qPCR MM (Promega, Cat A6102), 1 μL of

sample eluent, 0.5 μM of F and R primer and 0.3 μM of TaqMan probe and made up to a final volume of 5 μL with nuclease-free water. All primer concentrations for the qPCR assays remained constant, apart from the APP qPCR primers, which were used at 0.25 μM F and R. The qPCR amplification took place with 1 cycle of 2 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C after which the fluorescence reading for each well was measured. Amplification took place in a CFX Connect Real-Time PCR thermocycler (Bio-Rad, Cat 1855201) and data was analysed with the CFX Maestro software.

Table 5.1. qPCR primers used in Chapter 5.

Species detected	Primer ID	Sequence (5' – 3')	Reference
APP	APP_F	GGGGACGTAACCTCGGTGATT	(Tobias et al., 2012)
	APP_R	GCTCACCAACGTTTGCTCAT	
	APP_PrB	FAM-CGGTGCGGACACCTATATCT-BHQ1	
PM	PM_F	CAGAGTTTGGTGTGTGA	(Settyipalli et al., 2016)
	PM_R	CAGACTGACAAGGAAATATAAAC	
	PM_PrB	FAM-AATC+TGC+TTCCTT+GAC-BHQ1	
SS	SS_F	TCCRATRCTGCTCTGCCATT	(Srinivasan et al., 2016)
	SS_R	TGATAGTAGAAGTCCAGCAACT	
	SS_PrB	FAM-AATAGCCC"T"GAAAAMCAGCCACWYTTTGARA-BHQ1	
MH	MH_F	CCAGAACCAAATTCCTTCGCTG	(Strait et al., 2008)
	MH_R	ACTGGCTGAACCTTCATCTGGGCTA	
	MH_PrB	FAM-AGCAGATCTTAGTCAAAGTGCCCGTG-BHQ1	
<i>S. scrufa</i>	IC_F	TCGGAACAGACCTCGTAGAA	This Thesis
	IC_R	AGGGCGGTAATGATGAATGG	
	IC_PrB	FAM-AACCCTCAC-ZEN-ACGATTCTTCGCCTT-Iowa Black FQ	

+ signifies LNA modification to the following base

"T" indicated abasic site

ZEN is an IDT proprietary quencher

5.14. LAMP amplification

The compatibility of the extraction protocol, or the comparison between extraction protocols were verified in LAMP reactions. The LAMP reactions used in this study are shown in **Table 5.2**. LAMP reactions were performed in a total reaction volume of 5 μL , consisting of 1 x custom isothermal buffer – Tris, 5 mM MgSO_4 (NEB, Cat B1003S), 1.4 mM dNTPs (Thermo Fisher Scientific, Cat 10297018), 1.2 mg/mL glycerol-free BSA (Sigma-Aldrich, Cat A9418), 5 mM NaOH (Honeywell, Cat 011-022-00-6), 0.5 U/ μL *Wst* 2.0 (NEB, Cat M0538M), 0.5 μM SYTO 9 (Thermo Fisher Scientific, Cat S34854), 0.25 μM of F3 and B3, 1.0 μM of LF and LB and 2 μM of FIP and BIP primers. LAMP amplification took place at 63°C for 45 minutes, with a fluorescence reading taking place every 30 seconds using a CFX Connect Real-Time PCR thermocycler (Bio-Rad).

Table 5.2. LAMP primers used in Chapter 5.

Species detected	Primer ID	Sequence (5' – 3')	Reference
APP	APP_F3_A	CTGAAAGTTTCGTCAGCAC	(Ji et al., 2012)
	APP_B3_A	TCTGAAACTGAAAAGTATCCAC	
	APP_LF_A	TTTTTGTGGTAACGTAC	
	APP_LB_A	ACTATTGCAAAAAATCGC	This Thesis
	APP_FIP_A	GCTCGAAATCCGGGTTATATCCT-ATTTAATTGATAAACACGGTGTG	
	APP_BIP_A	TCGTTTAAAAGCGTTACAAGAGGA-AACTGAAGACAAGCGGTA	
PM	PM_F3_A	GGTGCCTATCTTGCTTCG	(Bhimani et al., 2015)
	PM_B3_A	TCGATCGCTAGCACCACA	
	PM_LF_A	CGACCATCGGTTGCATTTTC	
	PM_LB_A	TGGCATTGCATGGCTATCA	

	PM_FIP_A	TGCCGTAGCAGAACTGGACTTTTAGGTGAGCCATGTGAGCT	
	PM_BIP_A	CACACCGAAGCCAGGACTTTTTTCACCAAATGTACTGGTTGCTTC	
SS	SS_F3_A	TGTCGATGATGTTTGGACTA	(Arai et al., 2015)
	SS_B3_A	GCTTCTCCATATACAAGTCTTG	
	SS_LF_A	AGATAAATCATTGCCT	This Thesis
	SS_LB_A	GGTCAGCTCAGCCAATCACGC	
	SS_FIP_A	TTGCGAGTCCGTAGAGAACGCCAT-CTGACCTTGATGCC	(Arai et al., 2015)
	SS_BIP_A	CGCTGGTGGTGTAGCTGTAT-TTCACGAGTCCATGACAAG	
MH	MH_F3_B	TGTTGAGATCGCATCAAGAA	
	MH_B3_B	CCTACCAAACAAAACAAGGTAGT	
	MH_LF_B	CCAAACACTAAATTATGCC	
	MH_LB_B	GGAGGCTTTTAAAAGGG	(Li et al., 2013)
	MH_FIP_B	GGCGCTCATGAATATGGTCATCTTTTCTGGATCGGAAATATCCTG	
	MH_BIP_B	TTCCCGTAGTTTTAAAAAATGGCGTTTTCCAATCATCCCGAATTCCTT	
<i>S. scrofa</i>	IC_F3_A	ATTCAATTGACCTCCCAGC	
	IC_B3_A	GTTTGCATGTAGATAGCGAAT	
	IC_LF_A	CAAGATTAGGCAGATGCCAAGAG	
	IC_LB_A	ACAACAGCTTTCTCATCAGTTACAC	
	IC_FIP	AAGAACAGGCCTGTTAGGATTTG-ATCATGATGAACTTCGGTTC	
	IC_BIP	AGCAATACATTACACATCAGACACA-ATCCGTAATTTACGTCTCGA	This Thesis

5.15. Qubit quantification

Quantification of free gDNA was made using a Qubit 4 fluorometer in conjunction with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Cat Q32854), as per the manufacturer's instructions. To limit error, sterile lysis buffers were used to blank the solutions and readings were taken in duplicate.

Results

5.16. Chemical lysis of bacterial pathogens

Several lysis buffers were evaluated for their ability to lyse PRDC bacterial pathogens to increase the availability of nucleic acid for detection in LAMP assays, two Gram-negative (APP and PM) and one Gram-positive (SS) bacteria were used. A total of eight different lysis buffers were evaluated, with the inclusion of PBS as a control. Bacterial isolates MIDG 2331 (APP), MISG2214 (PM) and P1/7 (SS) were grown to early-growth phase (**Supplementary Figure 17**), pelleted, and resuspended in the nine different buffers. The addition of the custom buffer, CB1 and CB2, resulted in increased DNA in the preparations, as determined by Qubit quantification (**Figure 5.4**). The custom buffers were especially efficient at liberating free gDNA from the SS isolate, with both CB1 and CB2 producing the highest quantification, a result which was mirrored in the qPCR amplification of the supernatant of the buffer (**Supplementary Figure 18**). SS is a Gram-positive bacterium which contains thicker peptidoglycan cell wall than that found in Gram-negative bacteria (Cabeen and Jacobs-Wagner, 2005). Therefore, CB1, CB2 with proteinase K (+) and the CB2 without proteinase K (-) were evaluated in further experiments.

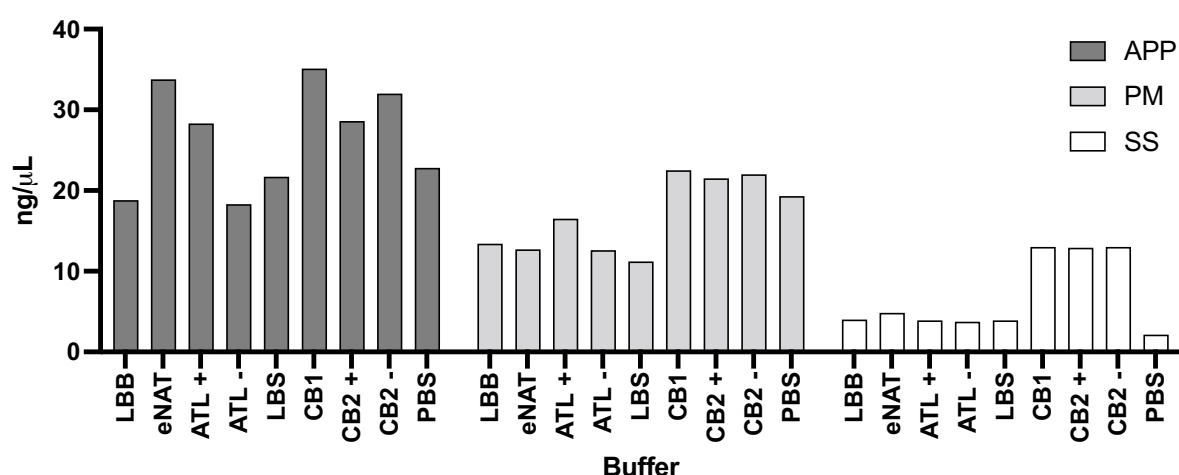


Figure 5.4. Quantity of free DNA present within each lysis suspension. The quantification by Qubit of each bacterial isolate, APP, PM and SS, which had been subject to chemical lysis via the addition of a range of different lysis buffers. Including those supplied by TurboBeads (LBB), Copan (eNAT), Qiagen (ATL), MagMax (LBS) and those produced in house (CB1 and CB2). Buffers suffixed by '+' indicate the inclusion of proteinase K, whilst their paired buffers suffixed by '-' are devoid of proteinase K. Columns depict the value obtained from each Qubit reading.

5.17. Mechanical homogenisation of lung tissue

To ensure adequate tissue mastication, chemical lysis can be used in conjunction with a mechanical homogenisation method. For the gold standard Qiagen method, lung tissue was homogenised using an MP Biomedicals FastPrep-24 bead-beater in combination with the MP Biomedicals lysing matrix A. The MP Biomedical FastPrep-24 bead beater is a large and expensive (list price: GBP £9,180) benchtop instrument. Therefore, two homogenising alternatives were explored; the use of a PowerMasher II, a hand-held battery-powered homogenisation device with a BioMasher V, a disposable homogenisation tube designed for the

homogenisation of soft tissue, and the use of MP Biomedicals lysing matrix A, containing a 1/4" ceramic ball a garnet matrix in combination with a simple benchtop vortex. A comparison between these three homogenisation methods was performed using two pig lung samples diluted in 400 mL to 0.1 g of tissue in PBS, so as to minimise the chemical lysis process. The assessment was performed using qPCR against the swine housekeeping gene, *cytB*. The results showed that the FastPrep-24 resulted in the greatest tissue homogenisation, with an average C_t value of 19.11 for lung sample A and 19.44 for lung sample B. The alternative homogenisation methods, using the vortex or BioMasher V, displayed similar results to one another, with less than a single cycle between their results (Figure 5.5).

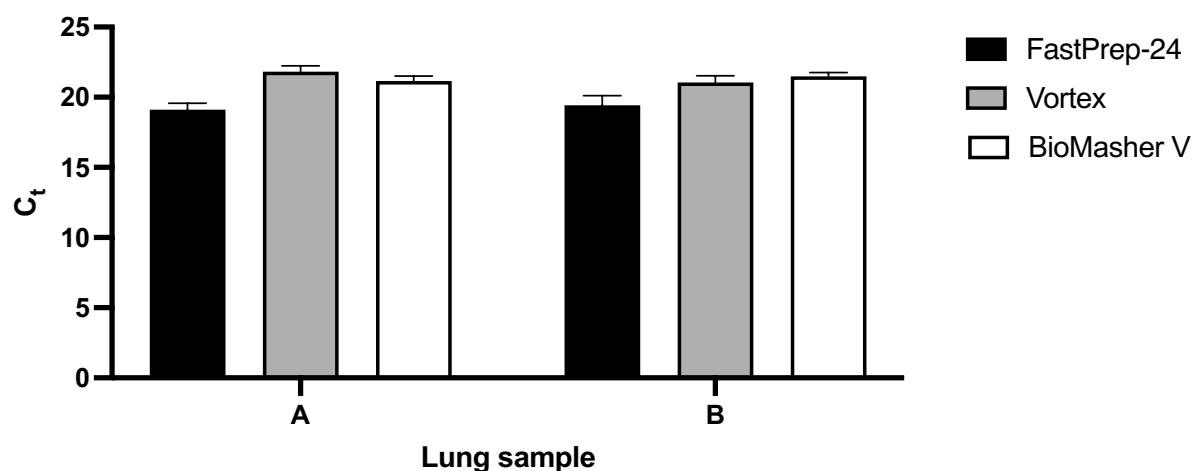


Figure 5.5. Mechanical homogenisation of lung tissue. Two lung tissues, A and B, were homogenised with lysing matrix A tube in either the FastPrep-24 (Gold-standard) or a vortex compared to the use of the BioMasher V tube used in conjunction with the PowerMasher II. Columns depict triplicate C_t results obtained from the *cytB* qPCR assay, with one replicate performed from each homogenisation method. Error-bars depict the S.D.

5.18. Magnetic capture of nucleic acids

Several magnetic beads have been developed for the capture of nucleic acids; I next assessed three magnetic beads produced by three different manufacturers in their recommended protocols, as well as the protocol described by the other manufacturers. The MagMax protocol resulted in the highest recovery of bacterial gDNA across all three bacterial copy input numbers (Figure 5.6A). The SeraSil-Mag protocol displayed the next highest recovery for 100,000 and 10,000 copies/ μ L of gDNA (Figure 5.6B). The TurboBeads protocol produced higher C_t values indicative of a lower recovery than the other protocols (Figure 5.6C). Using the TurboBeads protocol, two bead types, SeraSil-Mag and TurboBeads, captured sufficient gDNA from the lowest 1,000 copies/ μ L DNA concentration and resulted in amplification in qPCR. Whilst extracting gDNA following the SeraSil-Mag protocol, only the SeraSil-Mag magnetic beads captured enough gDNA for amplification in qPCR. Across the bead types, the SeraSil-Mag magnetic beads displayed the lowest C_t , indicative of the highest recovery across both TurboBeads and SeraSil-Mag protocols. Additionally, both the TurboBeads and SeraSil-Mag beads produced amplification in both these protocols in the initial preparation of 1,000 copies/ μ L. The general trends observed across different concentrations of APP gDNA were mirrored in the qPCR results for the IC synthetic construct (data not shown). However, whilst the MagMax protocol displayed the highest recovery, the protocol also

required the most steps and, therefore, hands-on time, with the incubation times alone requiring almost 45 minutes to complete. The SeraSil protocol requires almost 20 minutes to complete, requiring a heating step in the initial binding of the sample to the beads. The TurboBeads protocol requires just over 5 minutes. Therefore, due to the rapidity of the TurboBeads protocol, it was taken forward for potential implementation in the PoC device. The TurboBeads protocol has been previously optimised and adapted into a system known as SmartLid, for the development of equipment free and rapid (<5 minutes from sample to extraction) protocol (**Supplementary Figure 16**). Furthermore, for RNA extraction, the SmartLid protocol had a high correlation compared with the use of the Qiagen RNA extraction kit comparator (Pennisi et al., unpublished data). Therefore, I used the SmartLid protocol, performing optimisation to adapt the SmartLid system for solid tissue input.

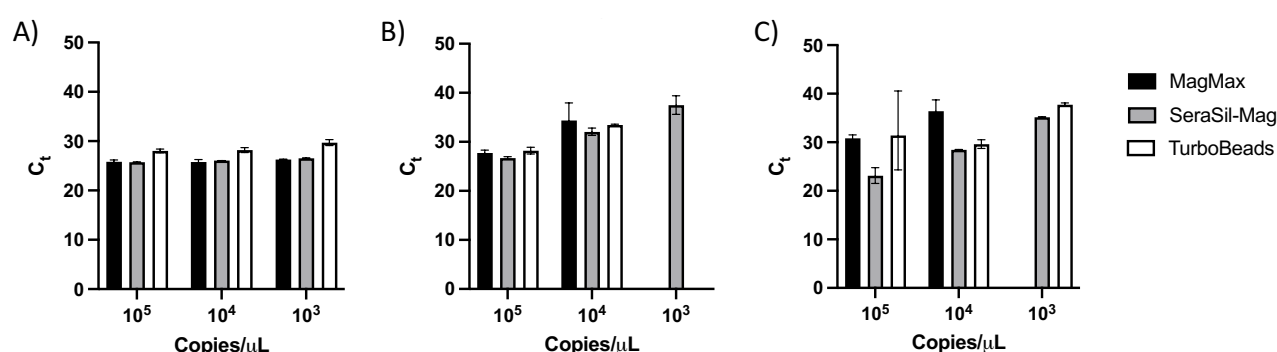


Figure 5.6. Extraction protocols across different magnetic bead types. The comparison of the MagMax (A), SeraSil-Mag (B) and TurboBeads (C) extraction protocols across different commercially available magnetic beads, represented by the coloured columns. A 10-fold serial dilution of APP serovar 8 gDNA input into each extraction protocol and a qPCR of eluent from each. Columns represent the geometric mean across three replicates originating from the same eluent, and error bars the S.D.

Next, I sought to optimise the TurboBeads protocol, using the three magnetic beads evaluated previously, to determine if any conditions increased the extraction efficacy with one bead type over another. First, I evaluated the compatibility of our in-house bacterial lysis buffers, CB1 and CB2, with and without proteinase K, with the SmartLid extraction process. Dilutions of APP serovar 8 gDNA were run at concentrations of 10⁵, 10⁴ and 10³ copies/μL. The CB1-APP preparations resulted in the highest C_t at low concentrations of bacterial DNA (**Figure 5.7A**), with an average C_t of 38.36 using MagMax beads, 34.99 using SeraSil-Mag beads and 37.10 using TurboBeads. The CB2-APP preparations with proteinase K (**Figure 5.7B**) produced the lowest C_t values at 10³ copies/μL, achieving an average C_t with the MagMax beads of 37.15, 31.11 with the SeraSil-Mag beads and 32.17 with the TurboBeads. The CB2-APP preparations without proteinase K (**Figure 5.7C**) resulted in a slightly higher C_t than when proteinase K was included, with the average C_t 10³ copies/μL 38.02, 32.71 and 35.20, for the MagMax beads, SeraSil-Mag beads and TurboBeads, respectively.

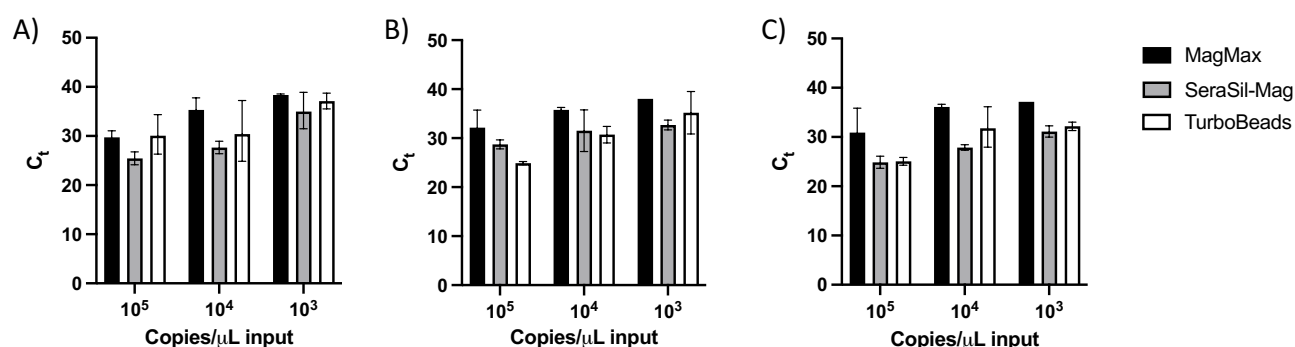


Figure 5.7. Compatibility of lysis buffers in the SmartLid protocol. The use of three in-house lysis buffers, CB1 (A), CB2 with (B) and without (C) proteinase K. Lysis buffers were spiked with gDNA of APP serovar 8 at 10⁵, 10⁴ and 10³ copies/μL, as sample input. Sample eluent, after being extracted with the SmartLid, was run with the APP qPCR in triplicate. Columns depict the geometric mean of the achieved C_t values and error bars the S.D.

The MagMax bead consistently displayed the highest C_t across all conditions tested, with the extraction performed with the SeraSil-Mag beads achieving a 1-3 C_t decrease compared to TurboBeads. Next, I evaluated the ability of the three bead types to move in and be attracted to a magnet in fluid. I found that the TurboBeads displayed the fastest collection speed, with all beads suspended in a 12 mL Falcon tube attracted to the magnet within 90 seconds, compared to the SeraSil-Mag beads, which took 5 minutes. The MagMax beads displayed the slowest collection time, taking over 10 minutes. On a smaller scale, each bead type was suspended in the 400 μL, with TurboBeads being instantly collected by the magnet, SeraSil-Mag beads took 7 seconds to collect and MagMax bead, just over a minute. Taken together, the TurboBeads were the most appropriate bead type to evaluate in future experiments.

5.19. Optimisation of SmartLid protocol

The standard SmartLid protocol calls for 400 μL of sample input, historically eNAT buffer with 300 μL of LBB and 400 μL of IPA. I evaluated how the replacement of the proprietary LBB with the sample impacts the ability of the beads to bind DNA. The replacement of LBB with either CB1 or CB2 lysis buffers produced equivalent results when using bacterial gDNA spiked into the buffers (**Supplementary Figure 19A**). The experiment was subsequently repeated with bacterial pathogen-negative lung tissue that was homogenised in the presence of each of the buffers with the lysing matrix A and vortexed for 30 seconds. The CB2 produced results equivalent to the LBB, whilst the CB1 and CB2 with proteinase K appeared to result in a loss of SEN (**Supplementary Figure 19B**). Therefore, the CB2 without proteinase K was implemented in all future experiments. Other parameters of the protocol were optimised, such as the number of washes which indicated that one wash in 80% ethanol was sufficient to remove inhibitors to amplification which may be present in the sample (**Figure 5.8A**). No decrease in C_t of the qPCR assay was observed when the volume of the ethanol single was increased from the usual 200 μL (**Figure 5.8B**), nor when the time of the wash increased (**Figure 5.8C**).

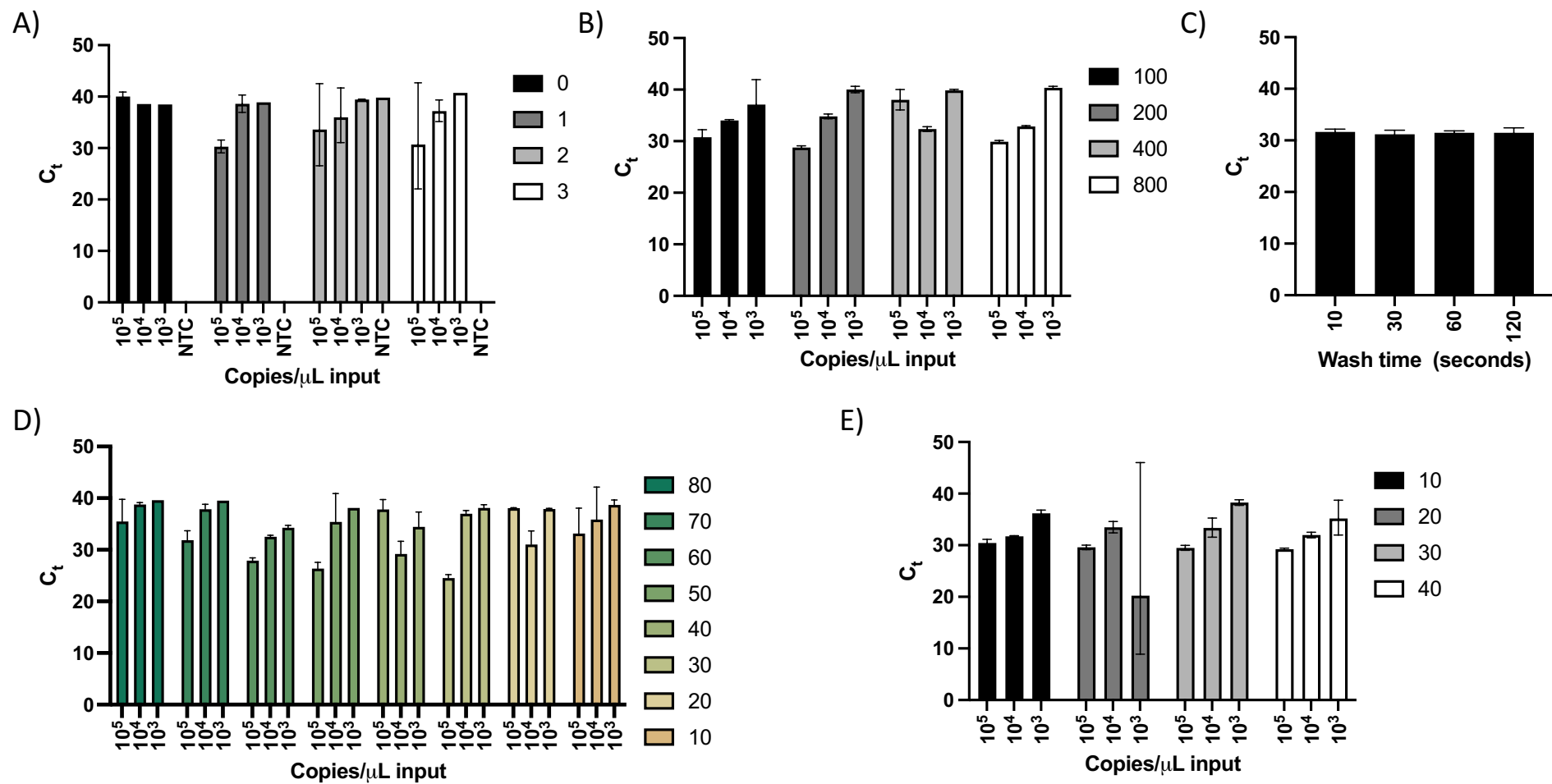


Figure 5.8. Optimisation of SmartLid protocol. The SmartLid protocol altered by the number of washes (A), the wash volume (B) and wash time (C), as well as the IPA:sample % (D) and volume of TurboBeads (μ L) used to capture nucleic acids (E). Using pathogen-negative lung sample spiked with gDNA from APP. Figure C used 10^5 copies/ μ L of sample input and was performed in triplicate. All other experiments were performed in duplicates across a range of DNA concentrations. Columns represent geometric mean and error bars S.D.

Additional parameters that were evaluated included the ratio between IPA and the sample input and the amount of magnetic capture beads added to the sample, neither of which displayed any decreases to the C_t , and therefore were not considered to result in higher yield of DNA, as determined by qPCR (**Figure 5.8D** and **Figure 5.8E**, respectively). Therefore, neither of these parameters were changed from the original SmartLid protocol, as optimised by Dr Ivana Pennisi.

The original SmartLid protocol, like numerous other protocols, calls for high percentages of ethanol as a wash buffer, however, ethanol is difficult to transport due to being highly flammable. Additionally, ethanol evaporates over time which is problematic to shelf-life estimates. Therefore, to combat the issues with the storage and shipment of ethanol, I trialled several other alcohol-based substances for use. The inspiration for utilising different alcohols was based on findings that polyols allow the nucleic acid to remain in a solution bound to a solid support (Ellis et al., 2010). An initial titration of the panel of alcohols showed that LAMP was more susceptible to inhibition than qPCR, if any of the wash buffers were to be carried over into the elution. The two LAMP assays were completely inhibited by the addition of 10% 2-methyl-1,3-propanediol, 1,2-butanediol, tripropylene glycol, and 3.2% 2-mercaptoethanol significantly delayed or prevented LAMP amplification (**Supplementary Figure 19A**). The qPCR data showed that only 10% 1,2-butanediol and 3.2-10% mercapto-glycol ethanol inhibited amplification completely (**Supplementary Figure 19B**). Therefore, subsequent testing of the wash buffers was performed in both qPCR and LAMP to ensure the conditions chosen were not detrimental to LAMP amplification. The full panel of wash buffers at 70% V/V resulted in inconsistent results in both qPCR and LAMP (**Supplementary Figure 21**). The panel was reduced to just buffers which have no globally harmonized system (GHS) criteria on their label and are therefore deemed non-dangerous for supply and transportation globally. Three of the polyols within the original panel met our GHS criteria, 2-methyl-1,2-propanediol, 1,2-butanediol and tripropylene glycol, and subsequent washes of lung samples with different V/V% of these compounds produced promising results in both qPCR and LAMP. At 80% V/V, both 2-methyl-1,2-propanediol and tripropylene glycol performed equivalently to 80% ethanol (currently used) across the two LAMP assays tested (**Figure 5.9A** and **Figure 5.9B**). Additionally, neither of the two wash buffers inhibited the LAMP reaction through carry-over into the elution, with no increase in the TTP by increasing the V/V% of the wash buffer. It is of note that when this experiment was repeated with lung spiked with APP gDNA, the trend was unclear, with amplification poor when low V/V% buffer was used, and equally inconsistent at high elution volumes, indicative of poor extraction efficiency, rather than inhibition in LAMP (data not shown). Therefore, the percentage of wash buffer was selected to be 80%, and the elution volume 200 μ L, as the qPCR data from the same experiment showed that elution in 100 μ L may result in decreased recovery of gDNA (**Figure 5.9C** and **Figure 5.9D**). Additionally, the tripropylene glycol produced consistent recovery across 70-80% V/V wash buffer, and therefore, the use of 80% tripropylene glycol wash buffer was implemented in the final protocol.

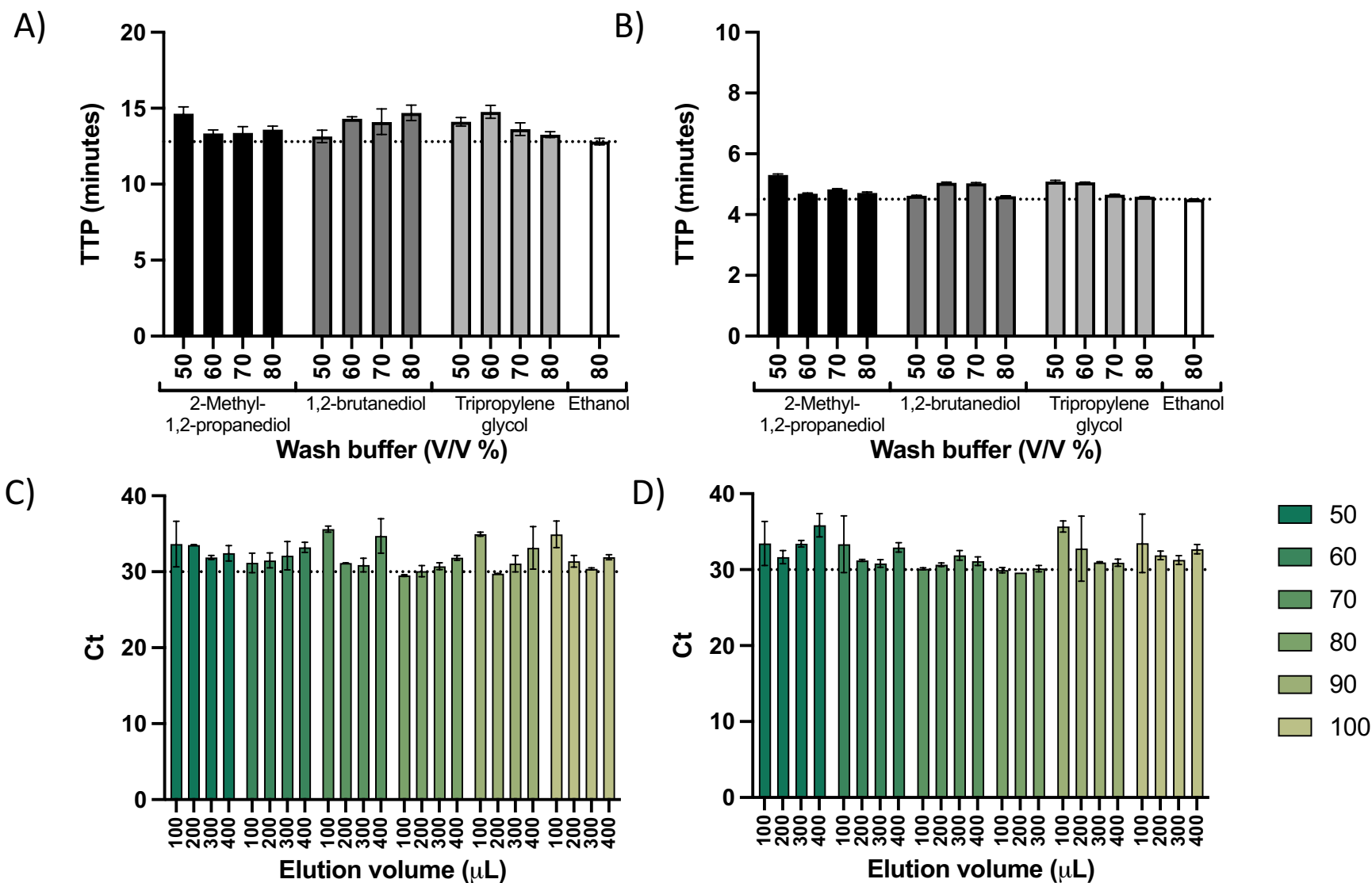


Figure 5.9. Evaluation of polyol wash buffers. The use of three polyol wash buffers in LAMP reactions detecting APP (A) and *cytB* (B). The use of two polyols, 2-methyl-1,2-propanediol (C) and tripropylene glycol (D), in qPCR across different V/V percentages as depicted by column colours, using a range of elution volumes. LAMP and qPCR experiments were performed in triplicate, with columns showing the geometric mean and error bars the S.D.

The optimised SmartLid protocol, therefore, consisted of a lung homogenate produced by vortexing lung tissue with CB2 lysis buffer for 30 seconds in lysing matrix A. 700 μ L of the lung homogenate is combined with 320 μ L of a proprietary solution containing TurboBeads and $\leq 61\%$ IPA, mixed by vigorous inversion for 30 seconds. Next, the magnet is inserted into the SmartLid, and the magnetic beads are captured by slow inversion. The SmartLid, including the magnet, is then transferred to the wash buffer tube consisting of 80% tripropylene glycol and the magnet is removed to release the magnetic beads and the mixture is inverted vigorously for 30 seconds. The beads are again captured by insertion of the magnet and gentle inversion. Finally, the SmartLid is transferred to the elution tube containing 200 μ L of nuclease-free water and with the magnet removed, the water and beads are mixed for 30 seconds by vigorous inversion prior to the beads being collected again by the magnet and disposed of. The tube now contains purified nucleic acid from the sample.

As such, the optimised protocol produced more consistent amplification in LAMP across three assays (**Figure 5.10A**) and qPCR (**Figure 5.10B**). The optimised protocol detected all nine data points (three replicates of the three extractions) of lung tissue spiked with gDNA from APP, compared to only four replicates detected using the original protocol. Similar results were found when MH gDNA was spiked into the Lung sample (**Figure 5.10A**). No detectable amplification was observed in SS-LAMP using lungs spiked with SS gDNA (**Figure 5.10A**), however in SS-qPCR, one replicate was observed in the original protocol, and seven of the nine replicates in the optimised SmartLid protocol (**Figure 5.10B**).

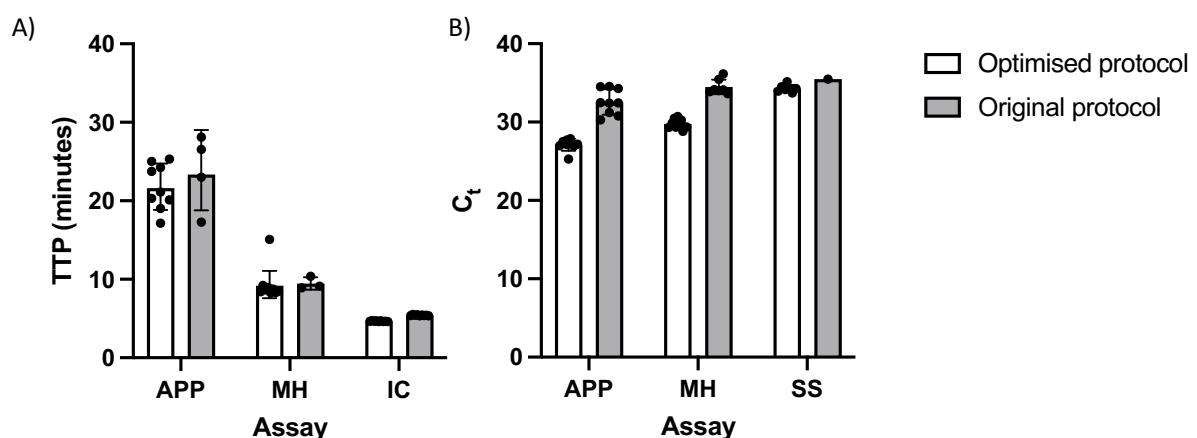


Figure 5.10. Performance of the optimised and original SmartLid protocols. Pathogen-negative lung samples spiked with APP, MH or SS gDNA. The lung samples were both homogenised with a vortex in lysing matrix A for 30 seconds and then passed through the optimised (clear columns) or original (grey columns) SmartLid protocol in triplicate for each condition. Sample eluent was run in triplicate in LAMP (**A**) and qPCR (**B**) reactions containing the appropriate assay primers. Data points represent a single replicate out of nine possible replicates (triplicate amplification of extractions performed in triplicate). Columns represent the geometrical mean of the replicates which amplified, and error bars the S.D.

5.20. Validation of SmartLid

A comparator was made between the gold-standard and optimised SmartLid (henceforth referred to as SmartLid) extraction methods, using a pathogen-negative lung sample that was spiked with serial dilutions of bacterial preparations made with APP, SS and PM. The results indicate that at least 100-fold SEN is lost between

Qiagen and SmartLid extractions with APP and 50-fold in SS (**Table 5.3**). It was unclear how much SEN is lost with PM, as all dilutions amplified in qPCR for Qiagen and SmartLid. However, given the issues with contamination previously encountered in **Chapter 3** in this assay, it is not unexpected. No difference was seen in the SEN of qPCR and LAMP using Qiagen or SmartLid extractions with APP, nor with Qiagen extractions in PM or SS. At least a 1,000-fold decrease in SEN is seen between qPCR and LAMP in SmartLid extractions for the detection of PM. However, this may be due to the contamination in the qPCR PM assay. Only a 50x decrease in SEN is seen between qPCR and LAMP in the SmartLid extraction of SS (**Table 5.3**). Across the qPCR data, the average ΔC_t between the Qiagen and SmartLid protocols ranged between 3-14 cycles in favour of the Qiagen extraction protocol.

Table 5.3. Detection of bacterial isolates in spiked lung tissue. Pathogen negative lung samples homogenised in lysing matrix A, with either the FastPrep-24 (Qiagen) or vortex (SmartLid) in the presence of ATL (Qiagen) or CB2 (SmartLid) and spiked with dilutions of bacterial isolates from APP, PM or SS. The preparations then underwent genomic extraction with either DNeasy blood and tissue kit (Qiagen), or the SmartLid optimised for lung tissue (SmartLid). Sample eluents were run in duplicate with appropriate qPCR and LAMP assays for the detection of target DNA.

Bacteria Extraction	APP				PM				SS			
	Qiagen		SmartLid		Qiagen		SmartLid		Qiagen		SmartLid	
OD ₆₀₀	qPCR	LAMP	qPCR	LAMP	qPCR	LAMP	qPCR	LAMP	qPCR	LAMP	qPCR	LAMP
1	++	++	++	++	++	++	++	++	++	++	++	++
0.1	++	++	++	++	++	++	++	++	++	++	++	++
0.01	++	++	++	++	++	++	++	++	++	++	++	++
0.001	++	++	--	+-	++	++	++	--	++	++	++	+-
0.0005	++	++	--	+-	++	++	++	--	++	++	++	+-
0.0001	++	++	--	++	++	++	++	+-	++	++	--	--
0.00005	++	++	--	+-	++	++	++	--	++	++	--	--
0.00001	++	++	--	--	++	++	++	+-	++	++	--	--

++ indicates detectable amplification of both replicates

+- or -+ indicate only one replicate amplified

-- indicates neither replicate produced detectable amplification

The performance of the Qiagen extraction protocol was compared to the SmartLid extraction protocol using 10 clinical lung samples. The lung samples were extracted using the two methods in parallel and run in qPCR assays for the detection of the APP, MH, PM, SS and GPS. For APP, five samples extracted with Qiagen were positive, with an average C_t of 19.67 SD 2.46. All five were also positive by qPCR when extracted with the SmartLid protocol, with an average C_t of 25.65 and a SD of 0.71. In the detection of MH, eight of the 10 samples were positive when extracted with Qiagen, but only five were positive when extracted with SmartLid. The samples that were missed with SmartLid, displayed an average C_t of 29.27, 28.62 and 35.48 in the Qiagen extractions. The five paired samples that were positive in both extraction protocols, their C_t increased by an average of 3.01 with a SD 0.89 when extracted with SmartLid. For the detection of PM, three samples had a C_t below 30 cycles when extracted in Qiagen, and one sample amplified below 32 cycles. The three low C_t (below 30 cycles) samples also amplified with a C_t below 30 cycles when extracted with the SmartLid, with an average increase in their C_t of 5.75 and a SD of 1.85.

All six samples that were positive in SS-qPCR using the Qiagen extractions were also positive in the SmartLid extraction, with an increased C_t , on average, of 3.82 with an SD of 1.31. All three samples that were positive for GPS in the Qiagen extraction were positive in the SmartLid extraction, with the C_t increasing by an average of 2.87 and the S.D. 2.12.

In order to validate the end-to-end systems of gold standard Qiagen extraction and qPCR to SmartLid extraction and LAMP amplification, the extracted lung samples were also amplified in LAMP reactions. The LAMP amplification detected all five APP-positive samples, only three of the eight positive MH samples, all three positive PM samples, five of the six positive SS samples, and all three of the positive GPS samples. Of the samples that the SmartLid combined with LAMP missed, the majority displayed a high C_t in qPCR (**Figure 5.11**). Three of the five MH-positive samples missed in LAMP were also missed in the qPCR for MH using SmartLid elution. All samples that were positive for PM by qPCR-SmartLid were also positive in LAMP. One sample was negative for SS in LAMP but positive in qPCR using eluents from both SmartLid and Qiagen extractions. However, the SmartLid extraction had a C_t of 35.1, and the Qiagen extraction of this sample had the highest C_t of the SS-positive samples, of 32.55. All three GPS samples that were positive by Qiagen-qPCR were also positive by SmartLid-LAMP.

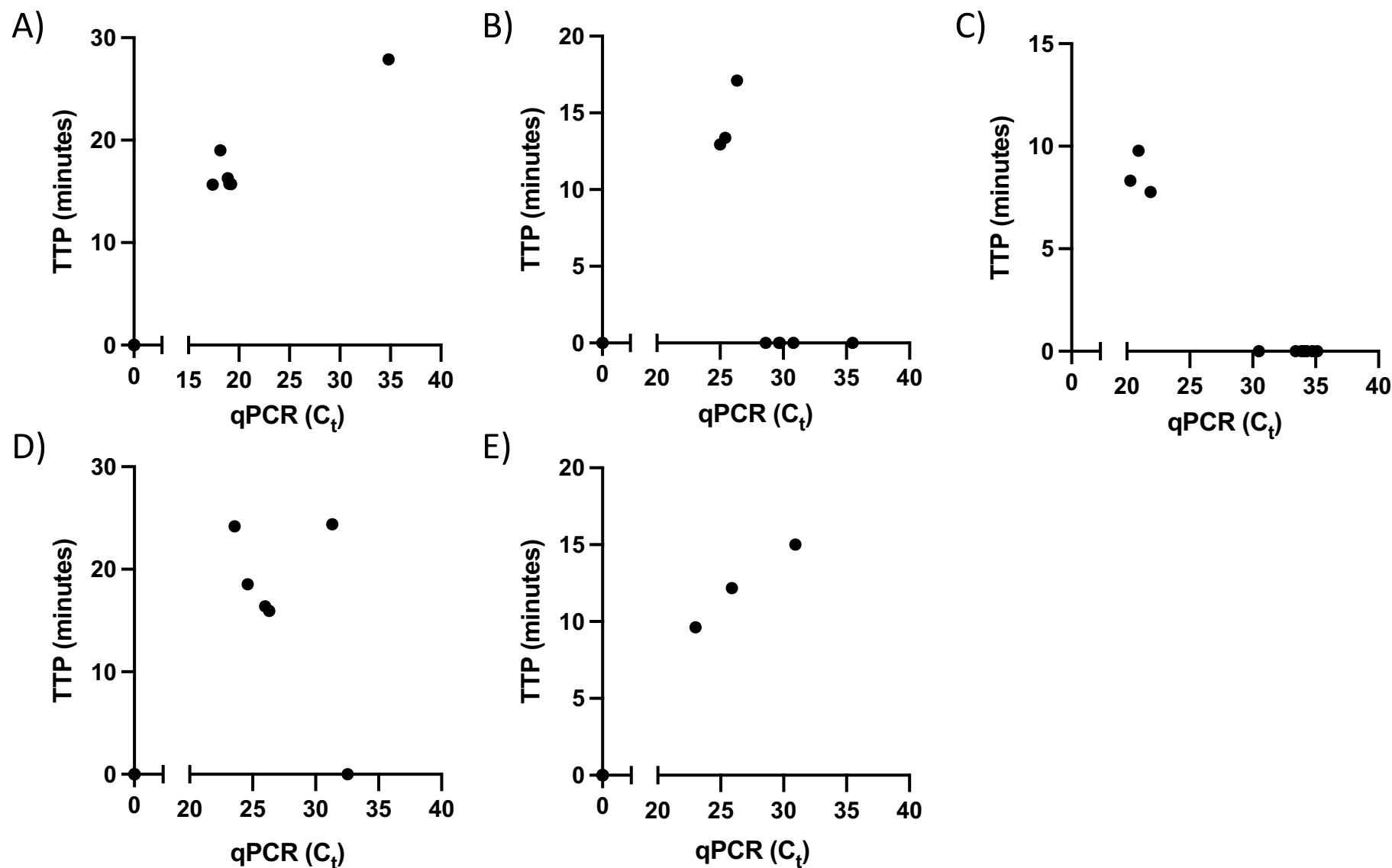


Figure 5.11. 10 clinical samples extracted and amplified by Qiagen-qPCR and SmartLid-LAMP. 10 clinical lung samples were extracted with the gold-standard extraction technique, including homogenisation and purification with a commercial kit combined with qPCR detection. Compared to the rapid homogenisation, vortexing for 30 seconds in lysing matrix A, purification with the optimised SmartLid protocol and detection with LAMP assays. Each lung sample was run with primers specific for APP (A), MH (B), PM (C), SS (D) and GPS (E). The TTP depicts the time taken to reach the threshold of each LAMP assay, and the qPCR Ct, the number of cycles taken to reach the threshold value for each qPCR assay. Reactions were run in duplicate for each assay, with the mean of each duplicate shown by each datapoint. Datapoints with a zero value did not amplify.

5.21. Results summary

In this Chapter, the previously validated SmartLid protocol was adapted for use on pig lung samples, including the re-optimisation of the SmartLid reagents to ensure maximum sample input and sensitive and specific results. A direct comparison was performed between the optimised SmartLid protocol developed in this Chapter and the gold standard DNeasy blood and tissue (Qiagen) extraction of three bacterial species (APP, PM and SS). The comparison indicated that SmartLid resulted in less extracted gDNA from the bacterial isolates compared to Qiagen, with both a decrease in SEN and increase in qPCR C_t observed when using the SmartLid extraction. On average the SmartLid resulted in an increase of 3-14 cycles before qPCR detection, and a 75-fold decrease in SEN.

In a clinical validation using 10 clinical lung samples, both the gold-standard and SmartLid extraction resulted in the detection of five APP-positive lung samples by qPCR and LAMP. MH was detected in seven clinical lung samples using gold-standard methods (Qiagen extraction and qPCR detection). However, SmartLid extraction failed to detect three of these samples, and when the SmartLid eluent was run in LAMP, a further two samples were not detected. In the detection of SS, SmartLid failed to detect one SS-positive sample, as determined by Qiagen and qPCR. However, identical results were seen for positive samples of PM and GPS assays. The result summary of this small-scale clinical validation is listed in **Table 5.4**.

Table 5.4. Clinical validation of end-to-end system. The amplification by qPCR and LAMP of extractions performed on 10 clinical lung samples either by the FastPrep-24 homogenisation and extraction with the DNeasy blood and tissue kit (Qiagen) or the vortex homogenisation and optimised for lung SmartLid extraction (SmartLid). Reactions were run in the amplification chemistry in duplicate, with ++ indicating detectable amplification of both replicates, +- or -+, if only one replicate and -- if neither replicate amplified. The detection of both replicates for each assay are considered positive for that pathogen, highlighted in grey.

Assay	Amplification	Extraction	Lung sample									
			1	2	3	4	5	6	7	8	9	10
APP	qPCR	Qiagen	++	++	++	++	--	--	--	--	++	--
		SmartLid	++	++	++	++	--	--	--	--	++	--
	LAMP	SmartLid	++	++	++	++	--	--	--	+-	++	--
MH	qPCR	Qiagen	++	++	--	++	++	++	++	++	++	--
		SmartLid	--	--	--	++	++	++	++	--	++	--
	LAMP	SmartLid	--	--	--	--	++	+-	++	--	++	--
PM	qPCR	Qiagen	--	--	--	++	++	--	++	--	--	--
		SmartLid	--	--	--	++	++	--	++	--	--	--
	LAMP	SmartLid	--	--	--	++	++	--	++	--	--	--
SS	qPCR	Qiagen	--	--	--	++	++	++	--	++	++	++
		SmartLid	--	--	--	++	++	++	--	++	+-	++
	LAMP	SmartLid	--	--	--	++	++	++	--	++	+-	++
GPS	qPCR	Qiagen	--	+-	+-	--	++	++	+-	--	+-	++
		SmartLid	--	--	--	--	++	++	--	--	--	++
	LAMP	SmartLid	--	--	--	--	++	++	--	--	--	++

The optimised SmartLid protocol used in combination with a simple benchtop vortex takes significantly less time to produce purified nucleic acids (<5 minutes) compared to the gold-standard technique (>70

minutes), requiring numerous hands-on steps (Figure 5.12), complex and expensive machinery to perform. Additionally, the sample must be transported from the abattoir or farm to centralised diagnostic laboratories. The transportation of animal tissue is not only costly, requiring specialist couriers, but also poses a biosecurity risk as lung samples harbouring potentially infectious material are transported throughout the country and often between countries. Thus, strategies have been developed to aid in the inactivation of samples taken for diagnostic purposes (Stringer et al., 2021). Therefore, this is the first instance, to our knowledge of a rapid extraction protocol that can be performed on whole tissue pen-side on farm.

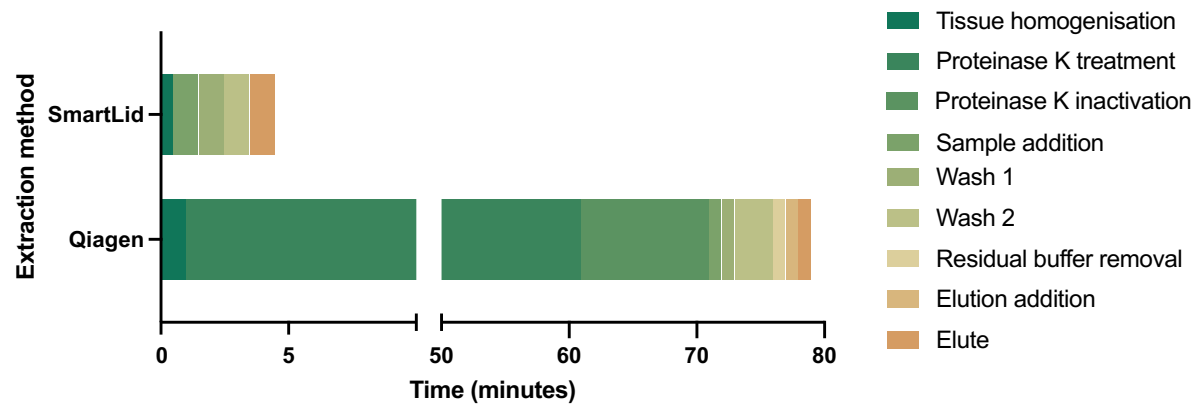


Figure 5.12. Comparison of Qiagen and SmartLid extraction protocols. The extraction of SmartLid optimised for lung samples, including the homogenisation of the sample for 30 seconds with lysing matrix A and a vortex for 30 seconds, followed by sample addition, wash and elution. Each SmartLid step consists of 30 seconds of mixing by inversion and 30 seconds of collection of the magnetic beads, for a total of 1 minute per step. The Qiagen protocol includes homogenisation of the sample with lysing matrix A with a FastPrep-24 homogeniser at 6 m/s for 60 seconds, followed by the Qiagen DNeasy blood and tissue kit.

Discussion

In this Chapter, I have developed a rapid and simple sample extraction protocol to purify nucleic acids from pig lung tissue in resource-limited settings, such as on farms. My developed protocol requires limited hands-on time, taking less than 5-minutes from lung to purified nucleic acid. Furthermore, the developed protocol exhibited higher extraction efficiency than the original protocol, across three bacterial species, including the Gram-positive bacterium, *SS*.

The implementation of an efficient sample-preparation method is the cornerstone of producing reproducible molecular diagnostic results. Lung tissue, in particular, is considered a troublesome sample type due to the presence of elastic connective tissue, dominated by collagen and elastin required to maintain the structural integrity and functionality of the lungs (Suki et al., 2005). Furthermore, I encountered several samples which contained sections of fibrous cartilage and bone, displaying the often-imprecise dissection of tissue on farms. Therefore, I sought to produce a sample preparation technique that would adequately homogenise the lung samples, including lysing of both animal and pathogenic bacteria.

A major obstacle I encountered was the limited availability of commercialised homogenisation solutions that are suitable for use on farms. Ideally, the solution would be single use to limit the risk of contamination, as well as being battery operated for portability. I evaluated the use of two homogenisation methods, using a lysing matrix A tube in combination with a bench-top vortex, and a commercially available handheld homogeniser distributed by Funakoshi, the PowerMasher II. The analysis showed that both methods produced equivalent tissue homogenisation, measured by direct amplification of the supernatant by qPCR, and both increased the detection by 1-2 cycle lengths compared to using the gold-standard benchtop homogeniser. The increase in qPCR cycles would equate to the rapid homogenisation methods producing around a quarter of the free gDNA compared to the benchtop method, assuming 100% efficiency of the qPCR assay. The use of a vortex has been reported to have a homogenisation rate of around 50% of that achieved with bead-beaters (Burden, 2008), a similar result to what was achieved with the AudioLyse. The AudioLyse is a similar system to what was evaluated in this Chapter, but instead of a benchtop vortex, an electromagnetic provides a magnetic field of 30 Hz to a tube containing a magnetic bead, moving the bead in the tube, and homogenise the tissue (Buser et al., 2015).

Both the rapid homogenisation methods evaluated in this Chapter performed equivalently and although the PowerMasher II met our criteria of having single-use homogenisation tips and being battery operated, it was cumbersome to use. Lung tissue being ground against the associated filter of the BioMasher V tubes frequently resulted in the filter becoming blocked. Additionally, for use on the farm, the end-user would be required to perform multiple steps with the tissue being added prior to a sachet of lysis buffer, followed by a plastic pestle for grinding the tissue. In the time taken to insert the pestle, I encountered the lysis buffer flowing through the filter into the collection tube, potentially limiting the chemical lysis contact with the tissue during homogenisation. Therefore, the use of the lysing matrix A tube in combination with a miniature-benchtop centrifuge was deemed the most appropriate for use, as the lysis buffer can be provided pre-aliquoted in the

tubes ready for sample input. Furthermore, this design decision was also more economical, with a single sample costing GBP £1.94, whilst the single-use BioMasher V tubes cost GBP £11.85 per sample. Alternative mechanisms that were not evaluated include the use of high-powered rotor-stators, which contain a stationary outer sheath and an inner turning shaft however they require a 240 V power source and are expensive. The Omni TH handheld homogeniser has a list price of GBP £922.09, with an additional GBP £15.60 per disposable metal or plastic disposable tip. The use of a battery-operated miniature homogeniser, the OmniLyse has been shown to be comparable to the bead-beating device produced by BioSpec, even for difficult-to-lyse bacteria such as *Mycobacterium tuberculosis* (Vandeventer et al., 2011). The OmniLyse has a list price of USD \$540.00 with 48 disposable lysing tubes, therefore, costing USD \$11.25, around GBP £10.17 per sample in today's economy (exchange rate 0.90 30 Sep 2022). However, to our knowledge, the OmniLyse has yet to be used with solid tissue. Alternatives that are yet to be commercialised are the AudioLyse which, again, uses a liquid sample input. More promising alternatives on the horizon include the BLENDER, a PoC device that homogenises large tissue samples, validated on small (3 mm) skin biopsies, producing similar DNA concentrations to the Qiagen extraction kit (McCloskey and Erickson, 2022). Thus, our results demonstrate that our cheap (GBP £1.94 per sample) system of lung tissue homogenisation by vortex in the presence of a ¼-inch ceramic ball and garnet, the constituents of the lysing matrix A, is sufficient to cause tissue destruction whilst keeping costs to a minimum, a factor which would significantly hamper the maximum market penetration of this PoC device.

The CB1 and CB2 contained nearly identical chemicals, with the CB2 containing higher concentrations of 50 mM more Tris-HCl and NaCl, 0.8% less SDS and 4 mM more EDTA, and the increased concentrations of these components may have led to increased lysing potential. Increased NaCl concentrations have been associated with the disruption of hydrogen bonds formed between the oxygen groups of water and silica, thus increasing interactions between silica, and negatively charged nucleic acids. EDTA has been reported to contribute to osmotic stress by sequestering divalent cations of the LPS and resulting in 40-50% loss of the LPS from the outer membrane of Gram-negative bacteria (Amro et al., 2000). The ability to be able to formulate lysis buffers in-house is advantageous, with the events of SARS-CoV-2 resulting in an industry-wide shortage of viral lysis buffers and led to the validation of in-house lysing buffers (Scallan et al., 2020). Additionally, the buffer chosen (CB2) conforms with REACH regulations. The European chemicals agency (ECHA) REACH committee prohibited the use of Triton X-100 in vitro diagnostics chemicals (European Chemicals Agency, 2021). However, it would be advantageous to have a more comprehensive evaluation of the concentrations of each of the reagents in the tissue lysis buffer, to ensure the appropriate concentrations are being used, although time constraints prevented this. Additionally, the comparison between using the tissue lysis buffer and chaotropic salts in relation to DNA-silica binding was not investigated in depth. For example, with the popularity of chaotropic salts in lysis buffers supplied with solid phase extraction kits, strategies such as utilising positively charged amino acid buffers employed to negate the necessity of chaotropic salt solutions have potential use (Vandeventer et al., 2013). I saw no difference between the inclusion or exclusion of proteinase K, which is contrary to other reports (Shahriar et al., 2011). A justification for this discrepancy between results might have arisen from our protocol incubating the proteinase K at room temperature, and although proteinase K displays activity across temperatures of 20-

60°C, the optimal temperature is 37 °C (Bajorath et al., 1988). Additionally, there is the possibility that proteinase K was carried over into our elution, which may have had an inhibitory effect on the amplification efficiency.

A wide range of magnetic particles have been commercialised with differing physiochemical properties such as size, magnetic potential, surface charge, shell thickness and coating. The coating of the bead and the bead size is of particular importance, as their interaction with nucleic acids dictates their binding potential, with the charge differential between the bead surface and the solution (known as the zeta potential) known to correlate with binding efficiencies, as determined by qPCR (Haddad et al., 2016). For this purpose, I evaluated three magnetic particles, MagMax, SeraSil and TurboBeads, each with differing properties altering their ability to bind nucleic acids. MagMax beads are the least defined in the literature, with undisclosed size, magnetic strength and coating, but it has been suggested they contain a magnetic core combined with more than one polymer (Latham et al., 2004). The Cytiva SeraSil beads are 40 nm in diameter with a nitrocellulose coating (Axen et al., 2006). The TurboBeads consist of a 20-100 nm diameter displaying a surface area of 15 m²/g (Grass and Stark, 2006). Although I found that the SeraSil beads consistently resulted in slightly increased extraction efficiency compared to the TurboBeads, the decreased size of the TurboBeads contributes to their ability to move through liquid phases and thus result in a rapid collection speed, which is more amenable to an on-farm PoC setting.

The TurboBeads used in this Chapter are coated with silica, which, as described previously has long been implemented in DNA purification and recovery methods. The extraction efficiency of the magnetic beads could potentially be increased. For example, a more specific approach involving conjugating custom antibodies to the magnetic beads and selectively targeting the DNA of interest has been reported to enrich the target template up to 11.5-fold (Feehery et al., 2013). However, the use of antibodies would result in an additional layer of complexity and cost to the assays, especially when it is unclear to what degree the enrichment process would benefit multiplex assays, such as required for PRDC pathogens.

An integrated solution which incorporates homogenisation, lysis, sample extraction and amplification within the CMOS-ISFET device would be the optimal solution. However, an integrated system cannot be achieved without the use of complex microfluidic systems requiring significant expertise and time to implement. The integration of several sample preparation methods with lab-on-chip devices has already been achieved for pork meat for the detection of *Salmonella* species. However, this study failed to detail the homogenisation steps for the tissue prior to the sample-preparation step, and no comparison was performed against standard laboratory extraction methodologies (Sun et al., 2015b). The more integrated use of magnetic beads in an on-chip design was designed by Li et al. using a magnet that is adjusted through a solenoid (Li et al., 2020b). A comparison showed that the integrated sample preparation produced comparable extraction efficiency to a lab-based magnetic bead extraction method. Although several laboratories have implemented the use of magnetic separation techniques, a recent study has found that column-based extraction typically results in consistently higher yields of nucleic acid (Sahu et al., 2021). This mirrors the results found in this study, where the extraction efficiency dropped by 100-fold, with an average C_t decrease of 10 between the optimised SmartLid extraction and running the same

spiked preparation through the Qiagen protocol. This is also a 10-fold decrease from our previous sample-preparation protocol using FTA cards and mPCR (Stringer et al., 2021). A potential source of this difference is that during this evaluation, bacteria were spiked into a set volume of already-homogenised tissue. Therefore, the bacteria were not re-homogenised in the presence of lung tissue, and thus the mechanical lysis section of this protocol was not compared. A more realistic comparison would be to repeat the homogenisation step after the introduction of the different quantities of bacteria.

Our previous qPCR results from clinical tissue were banded into high, medium, and low copy numbers of target based on the C_t value (**Chapter 3**). The expected reduction of 10 cycles between Qiagen and SmartLid would result in the majority of samples that were extracted by Qiagen being placed in the tier below i.e., medium bacterial load samples become low bacterial load, which will obviously have a significant impact on the clinical SEN of our test. Although 10 clinical samples were evaluated in this Chapter, the results suggest that SEN is not only lost in the rapid sample extraction protocol but also in the LAMP amplification step, a result also found in **Chapter 3**. However, the distribution of bacterial loads within active infected lung tissue is unknown and, therefore, cannot be properly assessed until the sample-to-result workflow is implemented. It is unclear if the lack of efficiency of the developed SmartLid protocol is due to the tissue homogenisation step, the cell lysis buffer or the SmartLid extraction protocol, with more work required to pin-point areas where efficiency is not comparable between the SmartLid and Qiagen methods.

In this Chapter, I employed the use of alternative wash buffers. A major contributing factor to this was that ethanol evaporates at a rate of 0.150 pg/minute (Budi et al., 2021), and over time the ethanol will evaporate faster than water – decreasing the ethanol content (Liu et al., 2008). Additionally, the shipment of ethanol can be problematic, with 80% ethanol considered as a dangerous good and subject to shipping conditions (Economic Commission for Europe Committee on Inland Transport, 2017). A panel of alternative wash buffers were selected due to their inclusion in a patent by LifeTech (Ellis et al., 2010), which describes alternative wash buffers to ethanol, mostly polyols, which do not require the evaporation step required to prevent carryover of ethanol into the eluent. My results suggest that the majority of the chemicals tested could be alternatives to the ethanol wash. However, some were still considered hazardous and were thus excluded from further use. The optimum wash buffer identified was tripropylene glycol, which displayed very similar performance to ethanol and is considered non-hazardous and non-flammable under the criteria of the Federal OSHA Hazard Communication Standard 29CFR 1910.1200, GHS, and EU Regulations EC 1272/2008. However, the selection of alternative wash buffers comes with the disadvantage that their implementation can be complex for the commercialisation of the device. Since the experiments were completed, TurboBeads introduced their own NoWait wash buffer, containing a non-disclosed proprietary solution, which could be an alternative to using tripropylene glycol once sufficient experimentation has been performed.

LAMP, as a biochemistry, is considered generally tolerant to inhibitors, for example, compared to standard PCR (Wang, 2020), and to this, LAMP kits have been commercialised specifically designed for direct amplification

with inhibitory sample types such as urine and whole blood (Meridian). Although not evaluated in this study, there may be some benefit in using alternative polymerases which display increased tolerance to common biological inhabitants, such as blood.

In this Chapter, only the extraction efficiency of DNA targets was evaluated due to time constraints and the availability of viral samples. Therefore, it is still unknown if the tissue lysis buffer is sufficient to lyse the viral envelopes. It has been reported that several commercial lysis buffers containing GTC are not sufficient to completely inactivate several viruses (Ngo et al., 2017), and often combining lysis buffers with heat are required (Avelin et al., 2022). Thus, whether the use of the optimised lysis buffer also works with virally infected pig lung samples remains to be determined. However, the CB2 buffer, which performed optimally in our testing, contains components commonly used on tissue samples, such as the commercially available RIPA buffer. The RIPA buffer contains half the amount of buffering salt (Tris-HCl), three-quarters the amount of ionic salt (NaCl), half the ionic detergent (SDS), and only 40% of the metal chelator (EDTA) compared to CB2. However, the RIPA buffer contains the extra detergents, 1% Triton X-100 and 1% sodium deoxycholate. Additionally, several deviations from the RIPA buffer have been cited in the literature, including one devoid of SDS and EDTA, with NP-40 substituted for Triton X-100, which was used on cells infected with BVS-1 (Mötz et al., 2022). Similarly, a RIPA buffer with the addition of 0.5% sodium deoxycholate was used to lyse cells infected with ASF (Eaton et al., 2017). Indicative that our CB2 buffer will certainly be suitable for the lysis of intracellular viral pathogens.

The exclusion of chaotropic salts, such as GuHCl, in the lysis buffer, is advantageous as GuHCl is classified by the GHS criteria as hazardous at the concentrations used for the purification of nucleic acids. However, the exclusion of chaotropic salts may impact the stability of the nucleic acids, rendering them susceptible to DNase and RNase degradation, especially as RNase A has been found particularly difficult to render inactive, being found to reassemble into its active structure after being denatured (Arnold, 2014). The assessment of the carryover of active RNaseA would be evident if RNA samples were used. Furthermore, it has been reported that ssDNA binds more strongly than dsDNA to silica due to the increased number of hydrophobic and phosphate-silanol binding interactions between unpaired ssDNA and the silica surface (Kang et al., 2001, Li et al., 2005, Isailovic et al., 2007, Shi et al., 2015a). This was not evaluated but perhaps should have been - especially as one of our viral targets, PCV2, is a ssDNA virus. However, RNaseA has also been found to bind to DNA and limit the extraction efficiency of phase separation protocols (Dona and Houseley, 2014).

5.22. Conclusion

In this Chapter, I have produced a simple and quick homogenisation of lung tissue that is compatible with the already-developed SmartLid extraction system, resulting in a purification time of gDNA from tissue in less than 5 minutes. Additionally, I have optimised the SmartLid protocol, resulting in higher SEN and greater reproducibility at lower DNA concentrations, for use with pig lung material. My optimised protocol was verified with three bacterial species of the PRDC, APP, PM and SS, demonstrating that the developed method is suitable

for both Gram-positive and Gram-negative bacteria. Therefore, I have successfully implemented an extraction protocol that is compatible with LAMP amplification, reducing the time taken to extract DNA from lung samples from several hours to minutes, ensuring rapid turnaround time with limited hands-on manipulation, and being ready for PoC implementation.

Chapter 6: Assessment of loop-mediated isothermal amplification detection methods

Introduction

In the early 2000s, the WHO produced criteria they consider necessary standards for PoC diagnostic devices, detailing the requirement for tests to be affordable, sensitive, specific, user-friendly, rapid and robust equipment free and deliverable to end users (ASSURED) (Kettler et al., 2004). In recent years, technological advancements have led to the addition of two more parameters: real-time connectivity and ease of specimen collection (REASSURED) (Land et al., 2019). Therefore, an ideal PoC device requires not only the amplification chemistry to be rapid but also the detection method used. The original publication describing LAMP amplification detailed two methods for analysing the amplicon, gel electrophoresis and qPCR with SYBR green I DNA dye (Notomi et al., 2000). The inclusion of fluorescent dyes does meet the REASSURED criteria of producing rapid real-time results and can produce semi-quantitative results. However, their use has limited multiplexing capacity as fluorescent DNA dyes integrate non-specifically into dsDNA and ssDNA and thus cannot differentiate between multiplex assays amplifying in a single tube. Fluorometers to measure real-time amplification are commercially available, including some with a small footprint (<20 cm²), which can simultaneously detect fluorescence readings from 8 or 16 reactions, such the Axxin T18 or T6 and OptiGene Genie II or Genie III devices. However, these fluorometers are expensive, with a single device costing >GBP £8,000. Several alternative approaches are described in the literature, including the multiplexing of fluorescent-based platforms into miniaturised microarrays, producing equivalent results to bench-top fluorometers at an affordable price (Zhou et al., 2014, Ahmad et al., 2011). However, no such products are on the market. Therefore, currently, fluorescent methods that detect LAMP amplification are impractical for PoC implementation, with the device cost being a major contributor to their limited utilisation, which has yet to transcend from specialist diagnostic laboratories into field use.

6.1. Lateral flow test

The most widely implemented PoC diagnostic platforms are based on LFT detection. LFT strips were first commercialised in the 1960s for the detection of glucose levels in the urine (Free et al., 1957), aiding in the diagnosis of diabetes. During the SARS-CoV-2 pandemic, the use of LFT strips was central to worldwide diagnostic strategies, enabling end-users to detect the SARS-CoV-2 virus at home. The popularity of LFT strips can be attributed to their ability to be mass-produced at low cost – with the materials estimated to cost as little as USD \$0.38 per test (Bangs Laboratories Inc, 2013), with more recent costings not-published most likely due to the high sale prices of SARS-CoV-2 antigen LFT strips. An additional benefit to LFTs is that they can be easily read by the naked eye, requiring no further processing to distinguish a positive from a negative result.

In its most simple form, a LFT strip consists of a porous membrane, such as nitrocellulose, that allows the analyte to flow from the sample pad to the wicking pad through capillary action (**Figure 6.1**). The analyte is captured and visualised on the strip via two types of Abs. One set of Abs are deposited on the nitrocellulose membrane, whilst the other set of Abs are conjugated to a visible tag (often gold particles), known as the conjugate-Abs. The sample is applied to the sample pad and flows over the conjugation pad, with the analyte of interest binding to

the conjugate-Abs. The conjugate-analyte continues to flow up the strip to the test line, where the analyte is captured by the test-line Abs. Typically, an additional control line is located near the top of the strip and contains another set of Abs, which will bind to the conjugate-Ab, and therefore signifies that the conjugate-Ab has flowed sufficiently up the strip.

The classical LFT strip has been subsequently adapted for the detection of products of amplification. Rather than the analyte being an antigen, the amplification product is labelled with two detectable moieties, typically carboxyfluorescein (FAM), fluorescein (FITC), digoxigenin (DIG) or biotin. To produce a dual-labelled amplicon, opposing primers contain different 5' label (FAM, FITC, DIG or biotin). During target amplification, the opposing primers are incorporated into the amplicon along with the 5' labels, creating a dual-labelled product. The completed reaction is then applied to the LFT strip, with one label being specific to the conjugate-Abs and the other label for the line-Abs, with sufficient amplification events resulting in a visible line. Different LFT strips have different target Abs, the layout of the HybriDetect2T LFT strips, compatible for use with several amplification chemistries, including LAMP, and used in this Chapter, is shown in **Figure 6.1**. LFT strips have been previously implemented in LAMP assays, resulting in rapid and sensitive detection of LAMP amplicon, including the detection of swine pathogens PCV2 (Jang et al., 2021) and SIV (Ummi Asih et al., 2021).

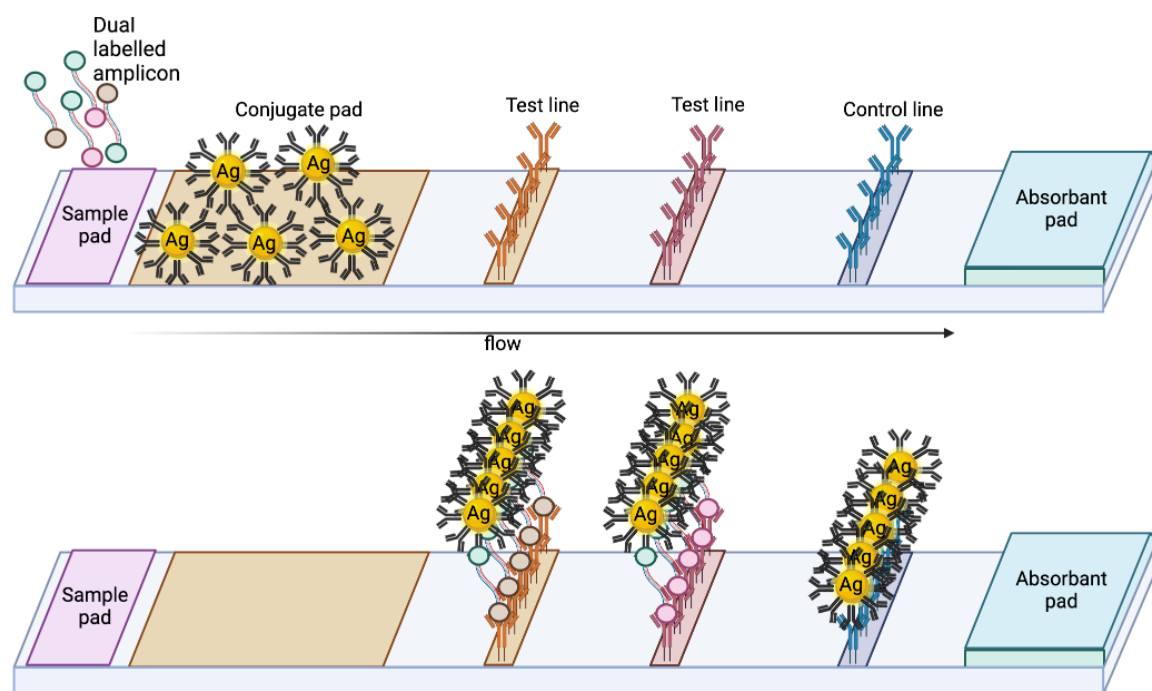


Figure 6.1. Lateral flow test detection of a molecular assay via a dual-labelled amplicon. The dual-labelled amplicon containing either FAM/FITC-Biotin or FAM/FITC-DIG is applied to the sample pad of the LFT strip, and the FAM/FITC label is captured by an anti-FAM/FITC Abs conjugated to a gold particle (conjugate-Abs) as it flows up the strip by capillary action. The HybriDetect2T contains two capture lines, one containing a biotin-ligand and the other anti-DIG Abs. Biotin and DIG of the primers and amplicon are immobilised by the relevant capture line on the test strip. If the amplicon also contains a FAM/FITC moiety, the amplicon will be sandwiched between the test line Ab and the conjugate-Ab allowing a visible line to form. Lastly, any conjugate-Abs that flows to the top of the strip are immobilised by anti-rabbit Abs on the control line. Figure adapted from Milenia Biotec GmbH and created with BioRender.

6.2. Colourimetric

It has long been reported that LAMP amplification results in the accumulation of magnesium pyrophosphate, formed by the pyrophosphate ions, released during DNA synthesis, combining with free-magnesium ions in the LAMP reaction. The increase in magnesium pyrophosphate results in a visible white precipitate in the reactions and a measurable increase in the turbidity (Mori et al., 2001). The drawbacks of magnesium pyrophosphate detection are that sufficient accumulation of product can take up to an hour, and the visible detection can be ambiguous - even under favourable lighting conditions (Mori et al., 2004, Zhang et al., 2009, Tomita et al., 2008). Alternative methods to detect LAMP amplification include the addition of pH- and metal-indicating dyes. Dye-based LAMP detection has grown in popularity as a low-cost method of distinguishing end-point amplification, being applied to several sample types and targets (Papadakis et al., 2022). Two major dye groups exist, those that are pH-sensitive and those that are metal ion-sensitive.

pH-sensitive dyes utilise the release of protons during LAMP amplification, which decrease the pH of the solution as the reaction proceeds. The increase in protons released during DNA amplification interacts with the pH-sensitive dye, and results in a conformational change in the dye structure and a visible colour change of the reaction occurs. Several pH-sensitive dyes have been previously utilised in LAMP, such as phenol red (PR) which changes from pink to yellow, and neutral red (NR) which changes from yellow to pink (Tanner et al., 2015). PR is widely used, implemented in NEBs commercialised colourimetric LAMP kit, previously implemented in the rapid detection of ASF (Yu et al., 2021). Bromothymol blue (BB), which changes from yellow to green, has also recently been described for the pen-side detection of foot and mouth (Zhang et al., 2022a). However, the difficulty with pH-sensitive dyes is that each dye has a specific pH range over which colour changes occur, and, therefore, the starting pH of the LAMP reaction is a key factor in determining the most appropriate pH-sensitive dye to be used.

In metal ion-sensitive dyes, the production of pyrophosphate ions during LAMP amplification reduces the free magnesium ions in the solution and the number of magnesium ions interacting with the metal-sensitive dye molecules (Tomita et al., 2008). The absence of metal ions within the structure of the dye results in a conformational change which causes a visual colour change to the solution. Several metal indication dyes have been previously described to be compatible with LAMP, including calcein, which changes from dark to light yellow, requiring the addition of manganese II chloride tetrahydrate. HNB changes from violet to blue (Goto et al., 2009), and Eriochrome Black T (EBT) changes from purple to blue (Oh et al., 2016b). HNB has previously been used to detect SIV, i.e., all influenza A strains and the swine-specific hemagglutinin (H3) subtype (Bakre et al., 2021). A schematic depicting how pH and metal-sensitive dyes work is shown in **Figure 6.2**.

The simplicity of being able to detect end-point LAMP amplifications in a reader-free environment makes colourimetric detection very amenable to PoC use. Therefore, in this Chapter, I sought to assess different colourimetric detection methods with, because of covid restrictions, a SARS-CoV-2 assay being as an exemplar.

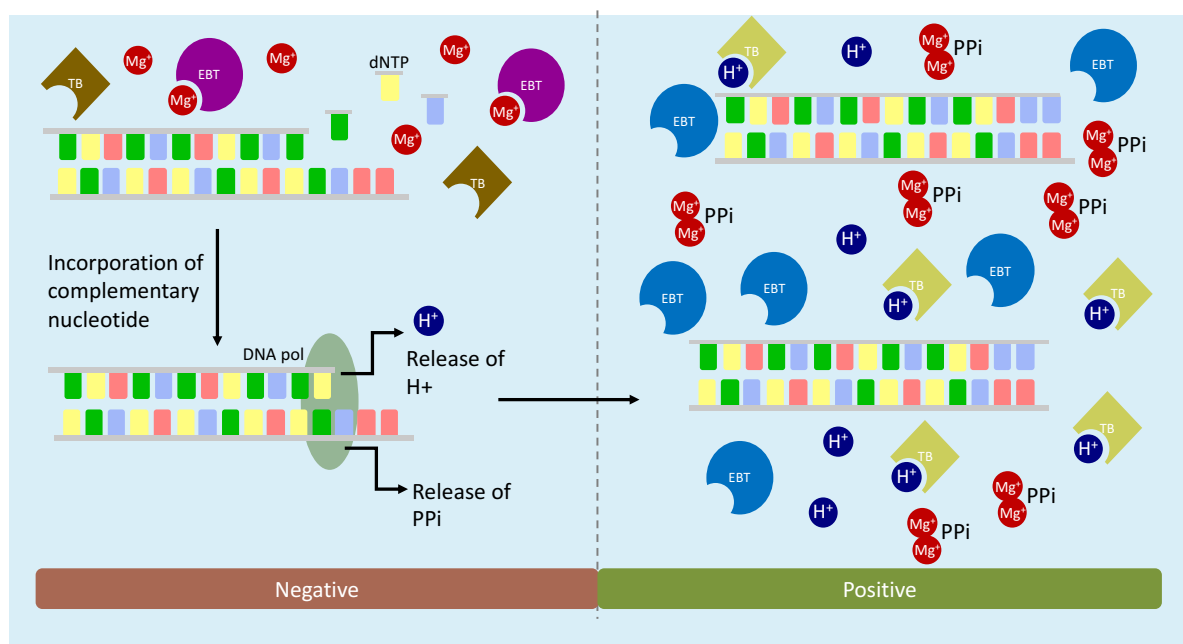


Figure 6.2. Schematic of pH- and metal-sensitive detection of LAMP amplification. During LAMP amplification, protons are released, and the pH of the solution increases. The increase in protons released during DNA amplification causes an increase in protons interacting with the dye, such as Thymol Blue (TB). With metal indicator dyes, such as Eriochrome Black T (EBT). The release of pyrophosphate (PPi), that occurs during the LAMP amplification sequesters magnesium ions away from the metal dye. These structural changes are due to the addition or loss of ions resulting in a conformational change in the dye structure, and a visible colour change of the reaction occurs. Figure created in BioRender.

6.3. CMOS-ISFET sensors

ISFET-based pH-detection of ion concentrations within a solution has long been reported (Purushothaman et al., 2006, Pourmand et al., 2006, Sakata and Miyahara, 2006). The worlds of electronic engineering and molecular biology collided once it was determined that DNA detection could be utilised on chip for sequencing platforms to detect SNPs or sequence DNA via the iterative addition of nucleotides. The CMOS-ISFET sensor was historically used to detect a pH change, could also be implemented to detect amplification with DNA polymerase extending the primer in the presence of the correct nucleotide. The concept of using ISFET in DNA sequencing was subsequently capitalised on by Ion Torrent systems, who were the first to demonstrate de-novo sequencing on-chip (Rothberg et al., 2011).

In 2013, the CMOS-ISFET platform was used to create an integrated circuit combining the ISFET sensing array with embedded heaters and temperature sensors to form a scalable, low-cost and disposable lab-on-chip platform (Toumazou et al., 2013), capable of detecting not only PCR but also LAMP amplification. A major advantage of utilising electrochemical-based sensing is that no additives, which may interfere with the amplification process, are required i.e., it is a label-free approach.

The principle of the label-free detection of amplification is the release of protons liberated from the terminal hydroxide during the addition of incoming nucleotides to the DNA backbone. The increase in protons in the

solution is detected via a charge change to the surface of the ISFET sensor. The ISFET sensor surface consists of a material with a high dielectric constant (also referred to as high-k), usually silicon nitride (Si_3N_4), applied during the typical semiconductor manufacturing process. The Si_3N_4 surface is protonated by the free protons to form silanol (SiOH), which in turn causes an increase in the threshold voltage required to maintain the electron flow across the ISFET (described in **Chapter 1**). The SEN of the sensor is therefore determined by the available binding sites for protons at the solution interface (Bergveld, 2003). The fabrication of ISFETs in unmodified CMOS technology, such as that used in the CMOS-ISFET sensor used in this Thesis, suffers from the accumulation of trapped charge on the floating gate, which causes large and unpredictable threshold voltages (Milgrew and Cumming, 2008). As such, several alternative deposits can be made during fabrication to reduce the amount of trapped charge and increase the binding sites available at the solution interface of the sensor, leading to reduced drift and greater SEN. In this Chapter, I evaluated the use of two alternative fabrication layers and their compatibility with the CMOS-ISFET sensor detection of LAMP amplification.

6.4. Aims

In this Chapter, to determine optimal protocols, I analysed the use of alternative detection methods to real-time intercalating dyes for LAMP detection at the PoC, including the implementation of LF LAMP detection, colourimetric approaches including pH- and metal ion-sensitive dyes, and the CMOS-ISFET label-free detection approach.

Methods

6.5. Bacterial isolates

Laboratory reference strains used in this Chapter include APP serovar 8 MIDG 2220 (405), MH MH232, SS P1/7, GPS SRV5 and PM MIDG 3290. APP, SS, PM and GPS isolates were grown up at 37°C for 14-16 hours in 5% CO₂ on BHI broth (BD, Cat 2237500) supplemented with 15 g/L agar (Sigma-Aldrich, Cat W201201), 0.01% NAD (MP Biomedicals, Cat 100319), and 10% DHB (TCS biosciences, Cat HB035). Isolated bacterial gDNA was extracted from cultured organisms via a FastDNA Spin Kit (MP Biomedicals, Cat 116540600-CF) according to the manufacturer's instructions as previously described in **Chapter 2**. Purified bacterial gDNA from MH was supplied by Professor Andrew Rycroft (Royal Veterinary College).

6.6. LAMP conditions

6.6.1. LAMP primers

Previously validated and optimised LAMP primer sets were used (listed in **Table 6.1**). Primer sets for LFT based detection of LAMP amplification included the addition of appropriate 5' moiety. Opposing primers were produced with either FAM/FITC or biotin for opposing APP LAMP primers and DIG or FAM/FITC for opposing IC LAMP primers.

Table 6.1. LAMP primer sets used in Chapter 6.

Target Species	Primer ID	Sequence (5' – 3')	Reference
APP	APP_F3_A	CTGAAAGTTTCGTCAGCAC	(Ji et al., 2012)
	APP_B3_A	TCTGAAACTGAAAAGTATCCAC	
	APP_LF_A	TTTTTGTGGTAACGTAC	
	APP_LB_A	GACGAACTATTGCAAAAAATCGCG	This Thesis
	APP_FIP_A	GCTCGAAATCCGGGTATATCCT-ATTTAATTGATAAACACGGTGTG	(Ji et al., 2012)
	APP_BIP_A	TCGTTTAAAAGCGTTACAAGAGGA-AACTGAAGACAAGCGGTA	
	APP_LF_A*	FAM-TTTTTGTGGTAACGTAC	This Chapter
	APP_LB_A*	FITC-GACGAACTATTGCAAAAAATCGCG	
	APP_LB_A*v2	Biotin-GACGAACTATTGCAAAAAATCGCG	
	APP_FIP_A*	FAM-GCTCGAAATCCGGGTATATCCT-ATTTAATTGATAAACACGGTGTG	
	APP_BIP_A*	Biotin-TCGTTTAAAAGCGTTACAAGAGGA-AACTGAAGACAAGCGGTA	
PM	PM_F3_A	GGTGCCATCTTGCTTCG	(Bhimani et al., 2015)
	PM_B3_A	TCGATCGCTAGCACCACA	
	PM_LF_A	CGACCATCGGTGCAATTC	
	PM_LB_A	TGGCATTGCATGGCTATCA	
	PM_FIP_A	TGCCGTAGCAGAACTGGACTTTTAGGTGAGCCATGTGAGCT	
	PM_BIP_A	CACACCGAAGCCAGGACTTTTTTACCAATGTACTGGTTGCTTC	
SS	SS_F3_A	TGTCGATGATGTTTGGACTA	(Arai et al., 2015)
	SS_B3_A	GCTTCTCCATATACAAGTCTTG	
	SS_LF_A	AGATAAATCATTGCCT	
	SS_LB_A	GGTCAGCTCAGCCAATCACGC	(Arai et al., 2015)
	SS_FIP_A	TTGCGAGTCCGTAGAGAACGCCAT-CTGACCTTGATGCC	
	SS_BIP_A	CGCTGGTGGTGTAGCTGTAT-TTCACGAGTCCATGACAAG	
MH	MH_F3_B	TGTTGAGATCGCATCAAGAA	(Li et al., 2013)
	MH_B3_B	CCTACCAACAAAACAAGGTAGT	
	MH_LF_B	CCAAACACTAAATTATGCC	
	MH_LB_B	GGAGGCTTTTAAAAGGG	
	MH_FIP_B	GGCGCTCATGAATATGGTCATCTTTTCTGGATCGGAAATATCCTG	
GPS	MH_BIP_B	TTCCCGTAGTTTTTAAAAATGGCGTTTTCCAATCATCCCGAATTCCTT	This Thesis
	GPS_F3_F	CTATCCTTGTTCATCAGGTAC	

	GPS_B3_F	TATAACGCCACTTCACGC	
	GPS_LF_F	GCAGGTGATGAAGCGACAG	
	GPS_LB_F	CACGGCCATATTCAAAGCCA	
	GPS_FIP_F	CATTTTCATCACGCATTGCACG-AGGCGACATCGTACTTTG	
	GPS_BIP_F	TTCTGTGGAAGTATTAGGACTTTC-TTTTCTCATCACGCACCA	
<i>S. scrofa</i>	IC_F3_D	ATTCATTGACCTCCCAGC	This Thesis
	IC_B3_D	GTTTGCATGTAGATAGCGAAT	
	IC_LF_D	CAAGATTAGGCAGATGCCTAAGAG	
	IC_LB_D	ACAACAGCTTCTCATCAGTTACAC	
	IC_FIP_D	AAGAACAGGCCTGTTAGGATTG-ATCATGATGAAACTTCGGTTC	
	IC_BIP_D	AGCAATACATTACACATCAGACACA-ATCCGTAATTACGTCTCGA	
	IC_LF_D*	FIITC-CAAGATTAGGCAGATGCCTAAGAG	This Chapter
	IC_FIP_D*	DIG-AAGAACAGGCCTGTTAGGATTG-ATCATGATGAAACTTCGGTTC	
SARS-CoV-2	CoV_F3	ACCAATGCAGTCCAGATGA	(Rodriguez-Manzano et al., 2021)
	CoV_B3	CACGATTGCAGCATGTGTTAGC	
	CoV_LF	GGACTGAGATCTTTTCATTTTACCGT	
	CoV_LB	ACTGAGGGAGCCTTGAATACA	
	CoV_FIP	TCTGGCCAGTTCCCTAGGTAGTCCAGACAAATTCGTGGTGG	
	CoV_BIP	GGACTTCCCTATGGTGCTAACAACCGGGTCCCAATGTGATCT	
BVS-1	BVS_F3_A	TGCTGCAGGTCTCCATTCA	(Rovira et al., 2009)
	BVS_B3_A	CCCCATCGGAGCTGTACT	
	BVS_LF_A	TCACTGATGTTAGTCCGGGC	
	BVS_LB_A	AGGAGTGGTTAACCTCGTCAA	
	BVS_FIP_A	AGTCTGGTACTAGAGTGCCGTGGTAACCGAGCATACGCT	
	BVS_BIP_A	GGCGGCAACGAGCTGTTAAACTGGCTCTGGTTTTTACCGG	
BVS-2	BVS_F3_H	GATGGCCAGCCAGTCAAT	This Thesis
	BVS_B3_H	CCTTGATTAAAGCGGTCTG	
	BVS_LF_H	CTGGTTTTGCTGAGCGATGAT	
	BVS_LB_H	CCTCTAGCGACTGAAGATGATGTC	
	BVS_FIP_H	CTTTCCCGTCCCTTGCCCTCTTTTGTGCCAGATGCTGGGTAAAG	
	BVS_BIP_H	AACCCGGAGAAGCCCCATTTTTTTAGACACAATTGCCGCTCACT	
SIV	SIV_F3-2_D	GACTGGAAGTGTCTTTGC	(Nakauchi et al., 2011)
	SIV_B3-2_D	TGTTGTTTCGGGTCCCCATT	
	SIV_LF_D	GTCTTGTCTTTAGCCA	
	SIV_LB_D	CAGTGAGCGAGGACTG	
	SIV_FIP_D	TTAGTCAGAGGTGACAGGATTGTTTCAGATCTTGAGGCTCTC	
	SIV_BIP_D	TTGTGTTACAGCTCACCGTGTTTTTTGGACAAAGCGTCTACG	
SIV H3N2	SIV_F3_A	AATTGCTCCTCGGGGTTA	(Gu et al., 2010)
	SIV_B3_A	CCGAATATGCCTCTAGTTTG	
	SIV_LF_A	GCCAATGGGTGCATCTGAC	
	SIV_LB_A	GTCCCAGATATGTTAAGCAAAACAC	
	SIV_FIP_A	GCTTCCATTGGAGTGATGCATTCCGAAGTGGGAAAGCTCAAT	
	SIV_BIP_A	GTAACAGGATCACATATGGGGCGCATCCCTGTTGCCA (A/G) TT	
PCV2	PCV2_F3_D	CAGCACCTCAGCAGCAAC	This Thesis
	PCV2_B3_D	GGGTGTTTCGTCCTTCCTCA	
	PCV2_LF_D	GGTTGGGGTCCGCTTCT	
	PCV2_LB_D	AGAAAATACGGGAGCTTCCAATC	
	PCV2_FIP_D	CAGCGTGAACACCCACCTTTTG-TGCCAGCAAAAAGAATGGA	
	PCV2_BIP_D	ATCCTTCCGAAGACGAGCGCA-TACCCTCCTCGCCAACAA	

*Denotes LFT labelled primers.

6.6.2. LAMP amplification

LAMP reactions were prepared as previously optimised (**Chapter 4**). LAMP primers were present in the LAMP reactions at final concentrations of 0.25 μM F3, 0.25 μM B3, 1 μM LF, 1 μM LB, 2 μM FIP and 2 μM BIP. Duplex LAMP reactions contained twice the total concentration of primers (2x), such that the primer concentrations for each assay remained as detailed above (1x). The duplex assays were tested at different ratios via mixing relevant 10x primer stock preparations to give a 5x working solution diluted into the LAMP reaction to give a final concentration of 2x. LAMP reactions consisted of 1x isothermal buffer without Tris, 6 mM MgSO_4 (NEB, Cat B1003S), 1.4 mM dNTPs (Thermo Fisher Scientific, Cat S34854), 0.5 mg/mL of glycerol-free BSA (Sigma-Aldrich, Cat A9418), 5 mM NaOH (Honeywell, Cat 011-022-00-6) and 0.5 U/ μL of WS *Bst* 2.0 DNA polymerase (NEB, Cat

M0538M). LAMP reactions for the detection of RNA were supplemented with 0.45 U/ μ L of RTx reverse-transcriptase (NEB, Cat M0380). Monitoring of amplification using fluorescent methods included 0.25 μ M of SYTO 9 dye (Thermo Fisher Scientific, Cat S3485). Real-time reactions were run at 63°C in a CFX Connect Real-Time PCR thermocycler (Bio-Rad, Cat 1855201) with fluorescence data acquired every 30 seconds for 40 minutes. For LAMP reactions detecting SARS-CoV-2, incubation and data acquisition took place with a LightCycler 96 (Roche, Cat 05815916001) using identical cycling conditions to that used in the CFX Connect Real-Time PCR thermocycler (Bio-Rad).

6.7. LAMP detection methods

6.7.1. Lateral flow test

LAMP amplification was performed in a total reaction volume of 10 μ L devoid of SYTO 9 dye and amplified at 63°C for 40 minutes in a CFX Connect Real-Time PCR thermocycler (Bio-Rad), with no data acquisition. Completed LAMP reactions were applied in 1-8 μ L volumes directly onto the sample pad of HybriDetect 2T (Milenia Biotec, Cat MGHD21) LFT strip. LAMP product was chased up the LFT strip via placing it, with the sample pad submerged, into a microcentrifuge tube containing 80 μ L of HybriDetect 2T assay buffer (Milenia Biotec, Cat MGCB). The LFT strip was kept in the microcentrifuge tube until the liquid was visible on the absorbent pad located on the top of the strip. This ensured the adequate flow of liquid up the strip and typically took 5-10 minutes. The presence of bands on the LFT strip was documented via photography with a cellular telephone camera.

6.7.2. Gel electrophoresis

LAMP products were separated by gel electrophoresis with 2.0% (W/V) agarose gels at 90 V for 3 hours. 5 μ L of the completed LAMP reaction was combined with 1 μ L of 6x loading dye (NEB, Cat B7024S), and the whole volume run alongside a GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Cat SM0321). Gels were stained with EtBr, and banding patterns were visualised and photographed using a Gel Doc Ez imager (Bio-Rad, Cat 1708270).

6.7.3. Colourimetric

Optimisation of colourimetric LAMP reactions took place using the SARS-CoV-2 primer set, with the inclusion of 200 copies/ μ L of SARS-CoV-2 synthetic RNA (Twist Bioscience, Cat 102024). A panel of pH- and metal-sensitive dyes were titrated into LAMP reactions between 50-200 μ M. pH-sensitive dyes included: BB (Sigma-Aldrich, Cat 114413-5G), TB (Sigma-Aldrich, Cat 14545-5G), NR (Sigma-Aldrich, Cat 72210-5G) and PR (Sigma-Aldrich, Cat P3532-5G). Whilst metal indicating dyes included: EBT (Sigma-Aldrich, Cat 858390-100G), Calmagite (Cal, Sigma-Aldrich, Cat C204-10G-A), hydroxynaphthol blue (HB, Sigma-Aldrich, Cat 1.04593.0025) and xylydyl blue (XB, Sigma-Aldrich, Cat 248266-1G). Colourimetric LAMP reactions included excipients at 4% mannitol and 2% Ficoll for a final reaction volume of 20 μ L. Real-time colourimetric reactions were supplemented with 0.25 μ L of SYTO 9 (Thermo Fisher Scientific) and incubated in a LightCycler-96 (Roche) for 40 minutes at 63°C, with data

acquisition taking place every 30 seconds. The colourimetric LAMP reactions were incubated in a heat block (Yooning, Cat MiniH-100L) at 63°C for 35 minutes.

6.8. Lyophilisation of LAMP reactions

Initially, LAMP reactions were lyophilised as described in **Chapter 4; Section 4.4.2**. Lyophilised reactions were reconstituted in 20 µL of nuclease-free water or nuclease-free water containing various concentrations of NaOH. Reactions were incubated for 35 minutes in a heat block (Yooning) set to 63°C. Lyophilised reactions that were monitored in real-time were rehydrated with the addition of 1.0 µM of SYTO 9 (Thermo Fisher Scientific) and incubated in a LightCycler-96 (Roche) at 63°C for 40 minutes, with data acquisition taking place every 30 seconds.

6.9. Clinical samples

6.9.1. Human clinical samples

A total of 77 clinical nose and throat swabs were obtained as part of routine asymptomatic SARS-CoV-2 screening at Imperial College Charing Cross hospital between April-December 2021, when the most prevalent variant was B.1.1.7 (UK Health Security Agency, 2021). All studies were conducted in accordance with ethical approval (20/HRA/1561; DOCUMAS 20SM5875).

Swabs were supplied in viral transport medium (VTM) and subsequently inactivated under Category 3 laboratory conditions by the addition of equal volumes of eNAT medium (Copan, 6U072S) (performed by Dr Ivanna Pennesi). Genomic extractions were performed with the QIAamp Viral RNA Mini Kit (Qiagen, Cat 52904) following the manufacturers' instructions with the addition of 140 µL inactivated samples and eluted into 80 µL of elution in 4 mM NaOH (Honeywell). Samples had previously been characterised as SARS-CoV-2 positive and negative in RT-qPCR using as described in **Section 6.10**.

6.9.2. Swine clinical tissues

A small subset of 10 swine lungs originating from farms in Portugal (n=4) and England (n=6), which had previously been characterised as pathogen positive or negative by qPCR (**Chapter 5**), were re-amplified in this Chapter. Additionally, five fresh nasal swabs were obtained fresh from a BQP farm in the UK and pooled into a single tube containing 2 mL of eNAT medium (Copan, Cat 6U072S). The samples were extracted via the optimised SmartLid protocol (**Chapter 5**). Briefly, lung tissue was homogenised with CB2 in a lysing matrix A (MP Biomedicals, Cat 116910050-CF) for 30 seconds using a benchtop vortex. 700 µL of the homogenised sample was added to Tube 1 containing 320 µL of a proprietary solution which included TurboBeads and ≤61% IPA. The mixture was mixed by vigorous inversion for 30 seconds, followed by the addition of the SmartLid and magnet, and the magnetic TurboBeads were collected with gentle inversion. The SmartLid was then transferred to Tube 2 containing 400 µL of 80% tripropylene glycol (Sigma-Aldrich, Cat 187593-1L) where the magnet was removed from the SmartLid, and the Tube 2 was mixed via vigorous inversion for 30 seconds. The TurboBeads were once again collected with

the addition of the magnet into the SmartLid and 10x gentle inversions. The SmartLid was then transferred to Tube 3 containing 200 µL of nuclease-free water (Thermo Fisher Scientific, Cat AM9939), where the magnet was removed, and the sample eluted into the water via vigorous inversion for 30 seconds. Lastly, the TurboBeads were collected with the addition of the magnet into the SmartLid and 10x gentle inversions of the tube, after which the SmartLid and magnet were discarded.

6.9.3. Efficiency of sample extraction

The extraction efficiency of using various concentrations of NaOH was performed using both SmartLid and QIAamp Viral RNA Mini extraction protocols. Negative nose and throat swabs (Copan, Cat 6U072S) were spiked with 700 copies/µL of SARS-CoV-2 synthetic RNA (Twist Bioscience), with 140 µL used in the QIAamp Viral RNA Mini Kit (Qiagen) performed as per manufacturer's instructions, eluted into 80 µL of various concentrations (0-4.5 mM) NaOH, and Qiagen AVE was used as an extraction efficiency control. The SmartLid protocol used 200 µL of sample input using the standard SmartLid extraction method (non-optimised), detailed in **Chapter 5**, eluted into 200 µL of various concentrations of (0-4.5 mM) NaOH (Honeywell). The extraction efficiency was assessed using the CDC qPCR assays for SARS-CoV-2, N1 and N2 assays and human RNaseP extraction control (**Section 6.10**).

6.9.4. Elution stability

The stability of RNA in the extraction was assessed using negative nose and throat swabs extracted with the SmartLid standard protocol and eluted into 4 mM NaOH (Honeywell). The sample eluent was pooled and spiked with synthetic SARS-CoV-2 RNA (Twist Bioscience) to a final concentration of 200 copies/µL. The sample was then aliquoted into 25 µL per time point and kept at either: 21°C, 4°C or -80°C. Each condition was tested in qPCR at time intervals of 0, 10, 30, 60, 120, 240, 360 and 480 minutes. The qPCR assays were the CDC-recommended assays for the detection of SARS-CoV-2 and the human *RNaseP* gene, as detailed in **Section 6.10**.

6.10. qPCR conditions

qPCR reactions were carried out using the three CDC recommended assays for the detection of SARS-CoV-2. Two of the assays, named N1 and N2, target regions of the SARS-CoV-2 nucleocapsid gene. The third assay targets a region of the human *RNaseP* gene. The primers were supplied as pre-mixes containing primers and probes (IDT, Cat 10006606) and ran in GoTaq probe RT-qPCR kit (Promega, Cat A6121). The 20 µL qPCR reactions consisted of 1x GoTaq probe qPCR MM, 5 µL of primer mix and 5 µL of the sample. RT-qPCR reactions for SARS-CoV-2 RNA were supplemented with 0.4 µL of GoScript RT. Reactions were amplified via 1 cycle at 45°C for 15 minutes, 1 cycle at 95°C for 2 minutes, followed by 45 cycles of 95°C for 3 seconds and 55°C for 30 seconds, during which fluorescent readings were taken. Reactions were run in a LightCycler-96 (Roche) for the N1 and N2 assays, and a CFX Connect Real-Time PCR thermocycler (Bio-Rad) for the RNaseP assay.

6.11. pH-LAMP detection on the CMOS-ISFET platform

One-well CMOS-ISFET cartridges were produced to contain LAMP reactions for on-chip detection. The one- or eight-well manifold was laser cut out of Perspex, and a silver reference electrode was secured to the underside of the manifold with laser-cut double-sided tape such that the electrode protruded into the well. The manifold was bonded to the printed circuit board (PCB) housing the CMOS-ISFET sensor via two m4 bolts and nuts, which clamped the manifold to the PCB trapping the reference electrode between the two surfaces. The reference electrode was then soldered to the PCB prior to the wells being filled with nuclease-free water and sealed with PCR sealing film (StarLabs, Cat E2796-9793). The cartridge was then placed into the device, and the pixels were mapped based on their sensing potential and heated to 63°C for 300 seconds. Once this time had elapsed, the nuclease-free water was removed with the immediate addition of LAMP reactions at total volumes of 20 µL. The cartridge was re-sealed with PCR sealing film, and the reactions remained at 63°C for 35 minutes, with the output voltage measured every 10 seconds. Data was analysed using a custom MatLab script created and run by Dr Nick Moser (ProtonDx), manifolds were prepared by Matthew Cavuto (ProtonDx), and CMOS-ISFET microchips were bonded to the PCB by Dr Nick Moser.

6.11.1. Reaction quantification

Completed LAMP reactions on the CMOS-ISFET chip were verified to have amplified via quantification with a Qubit fluorimeter in combination with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Cat Q32850). LAMP reactions were diluted 1:10 in nuclease-free water, with 1 µL added to 199 µL of the Qubit working solution, consisting of 1 µL Qubit dsDNA HS Reagent and 198 199 µL of Qubit dsDNA HS Buffer. The Qubit working solution was vortexed for 30 seconds and read in a Qubit 4.0 fluorometer (Thermo Fisher Scientific, Cat Q33238).

Results

6.12. Lateral flow test detection of LAMP amplification

The adaption of LAMP amplification for LFT detection took several optimisation steps. My aim was to produce a LFT for the detection of APP and use the IC primers, as an IC, in a duplex. The duplex of these primer sets was determined to result in no FP amplification, with no amplification found in either real-time or gel electrophoresis analysis. However, the real-time data did suggest that in the duplex, the APP assay was delayed by approximately 3 minutes compared to when the APP primer set was used in a singleplex (**Figure 6.3A**). Additionally, the IC assay was very rapid, taking less than 5 minutes to amplify, and therefore, amplification in the presence of both templates greatly favoured the IC over the APP assay, which was confirmed in gel electrophoresis, as when both templates are present in the duplex the striation pattern seen is only that of the IC (**Figure 6.3B**). To overcome the lack of signal seen in APP LFT, the IC and APP primer concentrations were cross-titrated with the IC showing strong amplification with as little as 20% of the original primer concentration (**Figure 6.3C**). However, little improvement was seen in the signal produced from the APP assay when the primer concentration was increased (**Figure 6.3D**).

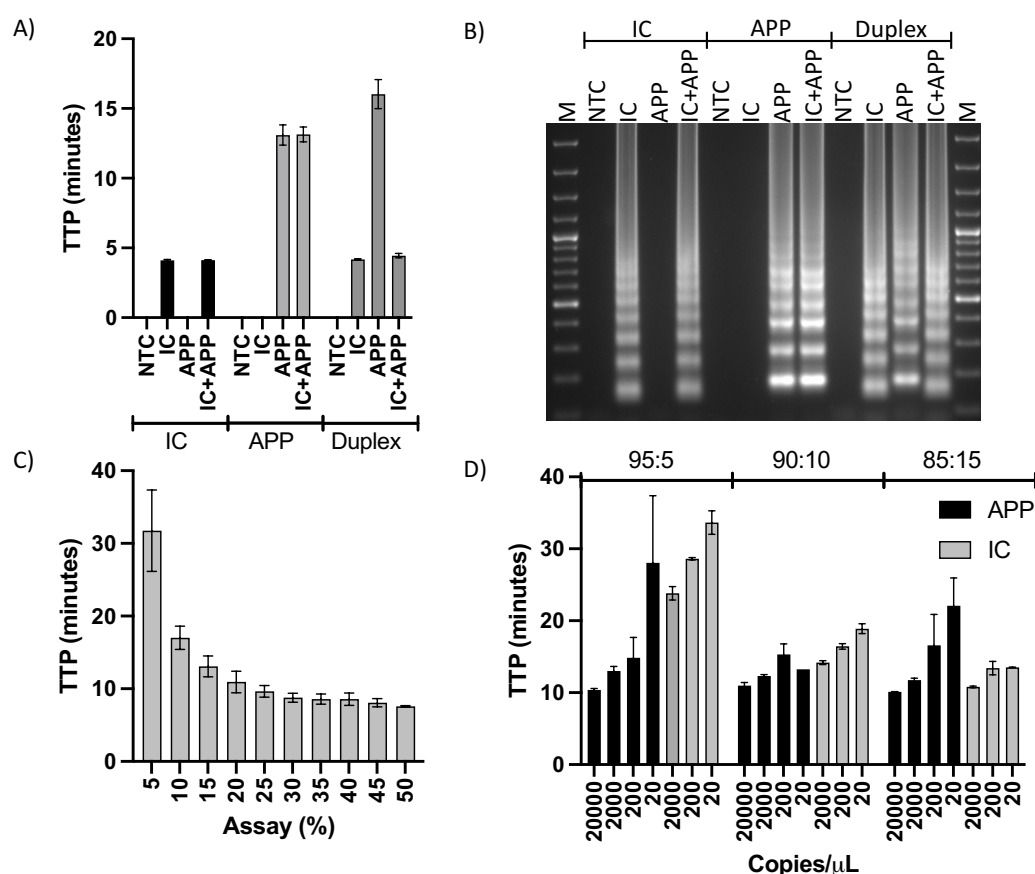


Figure 6.3. Duplex APP and IC LAMP assays. The products of singleplex and duplex APP and IC assays in the presence of either APP (1,000 copies/ μ L), IC (200 copies/ μ L) or a mixture of both were added, detected in real-time (**A**) and via gel electrophoresis (**B**). The effects on TTP caused by decreasing the percentage of IC assay in the duplex when amplified in the presence of 200 copies/ μ L of IC template (**C**). The change in TTP caused by altering the APP:IC ratio in the presence of different copy numbers of APP or IC (**D**). M signifies the addition of GeneRuler 100 bp plus molecular marker (Thermo Fisher Scientific).

Initially, the LFT primers for APP and IC assays were produced with the LB and BIP primers labelled for APP and the LF and FIP primers labelled for the IC assay. However, this labelling strategy resulted in poor signal from the APP assay on the LFT strip, even in a singleplex (data not shown). It was confirmed that the poor signal was not due to the inclusion of LFT primers with a strong amplicon present when the LAMP products were visualised by gel electrophoresis (**Supplementary Figure 22A**). Altering the concentration of labelled primers applied to the LFT strip from 2.4-12.0 pmol did not increase the signal of the APP primer set, even in singleplex amplification reactions (**Supplementary Figure 22B**). When performed with the IC assay, in singleplex, the strongest band was observed when the labelled primers were present at 60% of their un-labelled counterparts, equating to 0.6 μM of LF primer and 1.2 μM FIP for a total of 1.8 μM of labelled primers in the LAMP reaction and 4 μL of the amplicon applied to the LFT strip at 7.2 pmol of labelled primers. A positive IC band was still visible when as little as 0.6 pmol of labelled product applied to the LFT strip (**Supplementary Figure 22B**). This suggested that inefficient incorporation of the labelled primers in the APP assay resulted in the poor detection of amplification with LFT strips. In order to confirm this, the two labelled primers in the APP assay, LF and FIP, were cross-titrated against their amplification pair, LB and BIP, respectively. The results indicated that the amplification speed was more dependent on the inclusion of LF and LB, with 0 μM LB showing the minimal difference to the TTP compared to 0.25, 0.50, 1.00, 1.50 and 2.00 μM (**Supplementary Figure 22C**). The FIP and BIP primer set are required for amplification, and therefore 0 μM of each was not tested. The inclusion of 0.5-4 μM of BIP had little effect on the TTP, whilst 0.5-2 μM FIP resulted in decreased TTP compared to when 3 or 4 μM were used (**Supplementary Figure 22D**). Therefore, it is unclear if the FIP or BIP primer contributes more to the amplification of APP, all that could be determined was that FIP caused greater inhibition to the amplification at high concentrations than was observed with BIP.

In order to determine which labelling strategy resulted in the greatest signal on the LFT strip, labelled primers were trialled in different combinations (**Supplementary Figure 22E**). The combination of FIP and BIP labelled primers was determined to produce a strong signal when both 8 and 16 pmol of label was applied to the strip, with no band observed in the negative control (**Figure 6.4A**). A subsequent cross-titration between APP and IC LFT assays showed that although 20% of the IC primers gave a strong signal for both APP and IC bands on the LFT strip in samples spiked with high concentrations of APP template (**Figure 6.4B**). When 20% IC and 80% APP was trialled with two clinical samples, one designated as containing a high and the other as a medium bacterial load by qPCR, the amplification of APP could not be detected on the LFT strip against a medium bacterial load sample (**Figure 6.4C**). Therefore, duplex detection resulted in reduced SEN of the test assay (APP). When a LoD was performed with the APP LFT-LAMP assay, containing FIP and BIP labelled primers in a single-plex reaction, the LFT detection reached a detection limit of 5 copies/ μL . One replicate produced a visible band on the LFT strip at 2.5 copies/ μL (**Figure 6.4D**) – mirroring the results obtained when the same dilutions of gDNA were performed in real-time LAMP with the unlabelled APP assay (**Supplementary Figure 22F**).

A small subset of 10 clinical lung samples which had previously been characterised by qPCR were re-extracted in the SmartLid protocol, optimised for use with lung tissue (**Chapter 5**) and run with the APP singleplex LFT

assay, containing FIP and BIP labelled primers. Completed reactions were run up LFT strips, with the clinical lung samples previously positive for APP by qPCR producing a visible band on the LFT strip, apart from in lung sample 8 (**Figure 6.4E**). Therefore, the LFT APP assays produced a SEN of 90%, and a SPE of 100%, in this limited subset of clinical samples.

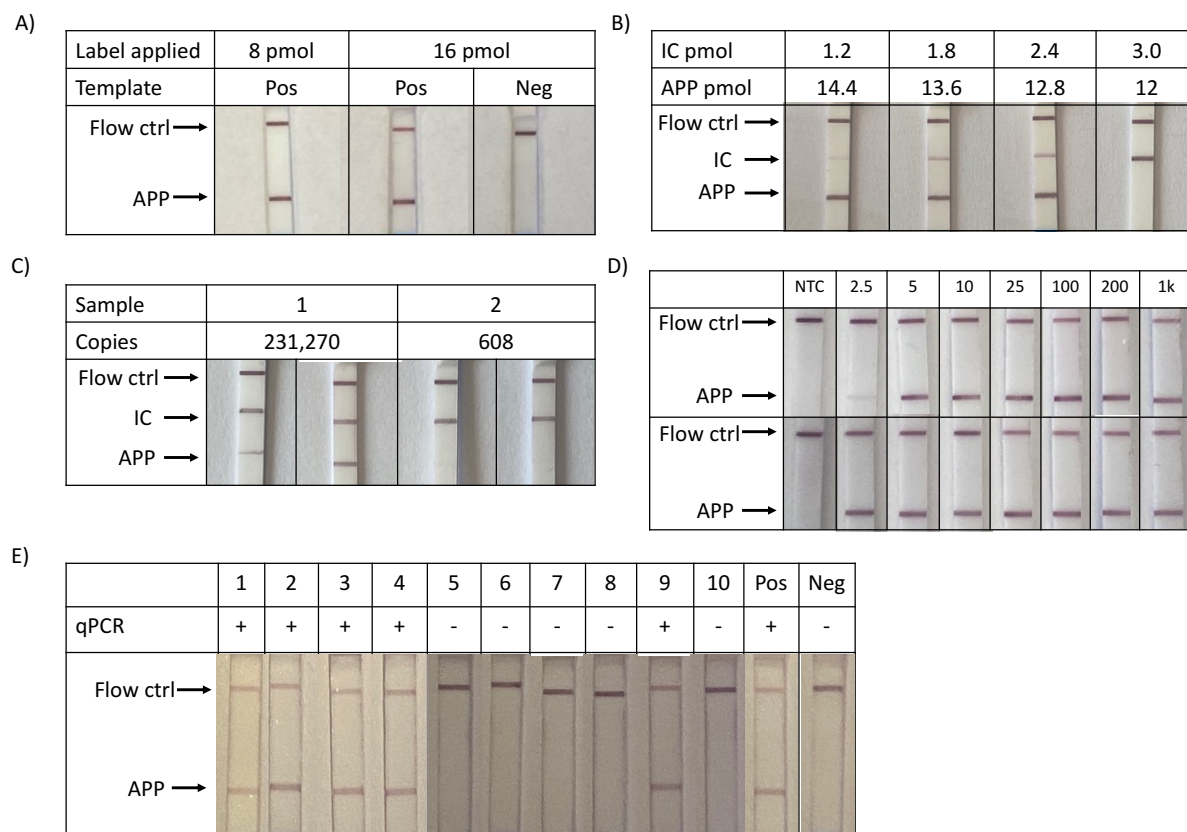


Figure 6.4. Development of APP LFT-LAMP. The use of a singleplex APP LAMP reaction where the LF and FIP primers were labelled with FAM and biotin, respectively. The LAMP reaction was amplified using 2,000 copies/ μ L of APP gDNA (Pos) or nuclease-free water (Neg), with either 2 μ L (8 pmol) or 4 μ L (16 pmol) of the completed reaction was run on the LFT strip (**A**). The inclusion 10, 15, 20 and 25% of IC-LFT primers with the remaining primer concentration consisting of the APP-LFT primers, with the APP-FIP and BIP labelled with biotin and FAM, respectively. Reactions were amplified in the presence of 2,000 copies/ μ L of gDNA APP and 200 copies/ μ L of synthetic IC DNA. 2 μ L of the completed reactions were run up LFT strips (**B**). The amplification of two clinical isolates, in duplicates, using 20% IC 80% APP LFT assay, with 2 μ L of the completed reaction ran up the LFT strip, equating to 2.4 pmol of IC and 12.8 pmol of APP labelled primers (**C**). The analytical SEN achieved with a singleplex APP-LFT with the FIP and BIP labelled with biotin and FAM, respectively. Serial dilutions of APP gDNA (serovar 5) were made in nuclease-free water, and 4 μ L of the completed reaction ran up the LFT strip (**D**). The amplification of 10 lung samples were extracted with the optimised SmartLid protocol for the lung and ran in the APP-LFT assay, detected on strip (**E**).

6.13. Colourimetric detection of LAMP amplification

Previously, in **Chapter 4**, I described the lyophilisation of LAMP reactions using SARS-CoV-2 as an exemplar. In this Section, I describe the combination of the lyophilised RT-LAMP reactions with a colourimetric dye - to create a simple equipment-free readout of LAMP reactions. The work in this section was performed in collaboration with Dr Kenny Malpartida-Cardenas. First, a range of different pH (**Figure 6.5A**) and metal-ion dyes (**Figure 6.5B**) were titrated into RT-LAMP reactions, with EBT producing a colour change after 35 minutes of incubation from

violet to blue. However, the real-time detection of LAMP amplification EBT had a delayed TTP compared to the other dyes, indicating an inhibitory effect of this dye (**Figure 6.5C**). Next, we sought to determine if using EBT in combination with any of the other dyes resulted in an enhanced colour change compared to when EBT was added alone. A colour change from red to green was observed using EBT in combination with TB (**Figure 6.5D**).

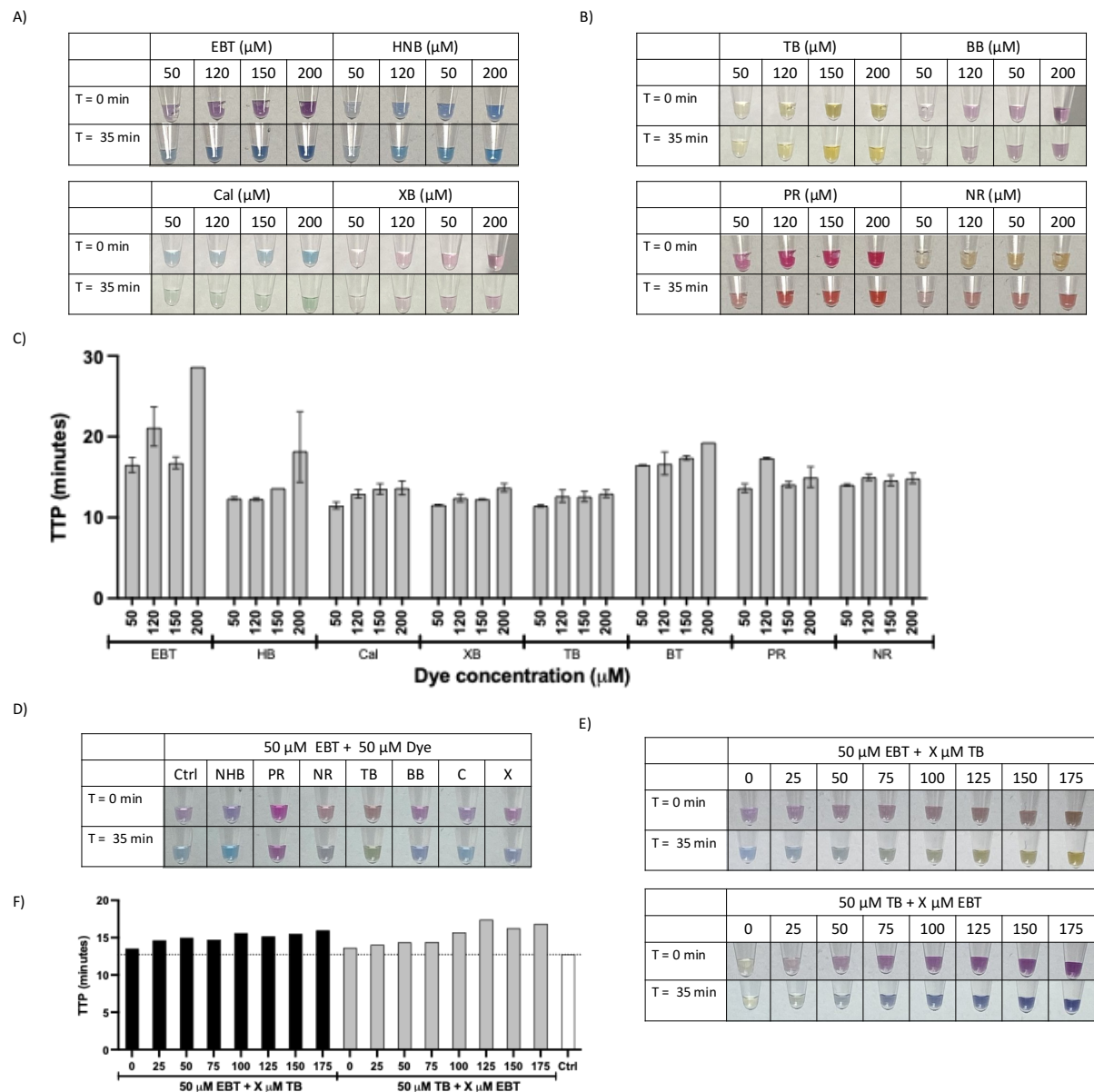


Figure 6.5. Optimisation of colourimetric LAMP. The colour change achieved with different concentrations of metal indicating (A) or pH-sensitive dyes (B) into RT-LAMP reactions, The titration of colourimetric dyes in real-time via reactions being supplemented with SYTO 9 fluorescent dye (C). The cross-titration of EBT with the other colourimetric dyes in RT-LAMP reactions (D). The cross-titration of either 50 μM EBT with increasing concentrations of TB, or 50 μM TB with increasing titrations of EBT in colorimetric RT-LAMP (E) or supplemented with SYTO 9 and amplification detecting in real-time (F). All RT-LAMP reactions included SARS-CoV-2 specific primers and SARS-CoV-2 at 200 copies/ μL . Pictures detailing the colourimetric LAMP reactions were taken pre- (T=0 min) or post-incubation (T=35 min).

A subsequent cross-titration of EBT and TB determined that EBT has little effect on the starting colour but strongly altered the hue of the reaction after amplification, which ranged from blue to green to yellow, whilst

increasing TB only increased the saturation of the reactions (**Figure 6.5E**). Real-time analysis of the amplification of SARS-CoV-2 in the presence of EBT and TB indicated that EBT was slightly more inhibitory than TB (**Figure 6.5F**). Therefore, the concentration of colourimetric dyes used in future experiments was 50 μM of EBT and 125 μM of TB.

One of the benefits of using lyophilised reactions is that the total rehydration of the reaction can be performed using the sample. However, the sample may contain carry-over from the wash buffer or other constituents, which may be inhibitory when the reaction is rehydrated with a high proportion of sample. Therefore, the SARS-CoV-2 RNA input per reaction was kept constant, and the proportion of sample eluent used to rehydrate the reaction was increased. The LAMP reactions displayed a 2-minute (25% increase) in TTP when 100% of the sample was added compared to when there was no sample (**Supplementary Figure 23A**). When the lyophilised reactions were rehydrated with extracted clinical samples obtained from Qiagen kits, it was observed that there was colour variation – with some samples resulting in a colour that was similar to the end-point green (data not shown). In order to increase the pH of the reaction to give a strong red colour on sample rehydration, the pH of the sample eluent was increased with the addition of various concentrations of NaOH, with 4 mM NaOH being the lowest concentration which displayed a red colour on rehydration of the lyophilised colourimetric reactions along with a vivid green colour on amplification (**Figure 6.6A**). Next, the extraction efficiency was investigated to ensure that it was not negatively impacted by the additional NaOH in the elution. Mock clinical samples were extracted with the QIAamp kit or SmartLid and eluted in various concentrations of NaOH or the Qiagen AVE elution buffer, with the QIAamp or nuclease-free water for the SmartLid, as extraction controls. A qPCR of the eluents displayed there was no difference in the TTP (**Supplementary Figure 23B** and **Supplementary Figure 23C**). Additionally, the use of 4 mM NaOH had little impact on the amplification time in RT-LAMP reactions (**Figure 6.6B**). Therefore, 4 mM of NaOH in nuclease-free water was chosen for the eluent of clinical samples. Implementation of increasing the pH of the sample eluent produced positive results in Qiagen kits, with the lyophilised colourimetric reactions achieving a higher analytical SEN than the liquid RT-LAMP reactions, with a LoD down to 10 copies/ μL (**Figure 6.6C**), increasing to 20 copies/ μL in the presence of background human gDNA extracted from negative swabs (**Figure 6.6D**).

A small scale clinical validation was performed in the lyophilised colourimetric LAMP reactions, detecting SARS-CoV-2. Across 77 clinical nose and throat swabs, the lyophilised colourimetric RT-LAMP reactions detected 50 of the 57 SARS-CoV-2 positive samples, as determined by qPCR, a SEN of 86% (**Table 6.2**). Furthermore, the results achieved with the lyophilised colourimetric RT-LAMP reactions were identical to those achieved with fresh colourimetric LAMP reactions (data not shown). However, when clinical samples were extracted with the SmartLid extraction protocol and eluted into 4 mM NaOH nuclease-free water, no colour change occurred. It was confirmed that this was due to magnetic bead carry-over into the sample eluent, which inhibited the metal ion-sensitive dye from changing colour (**Supplementary Figure 24A**), with amplification occurring when the reactions were supplemented with SYTO 9 and observed in real-time (**Supplementary Figure 24B**).

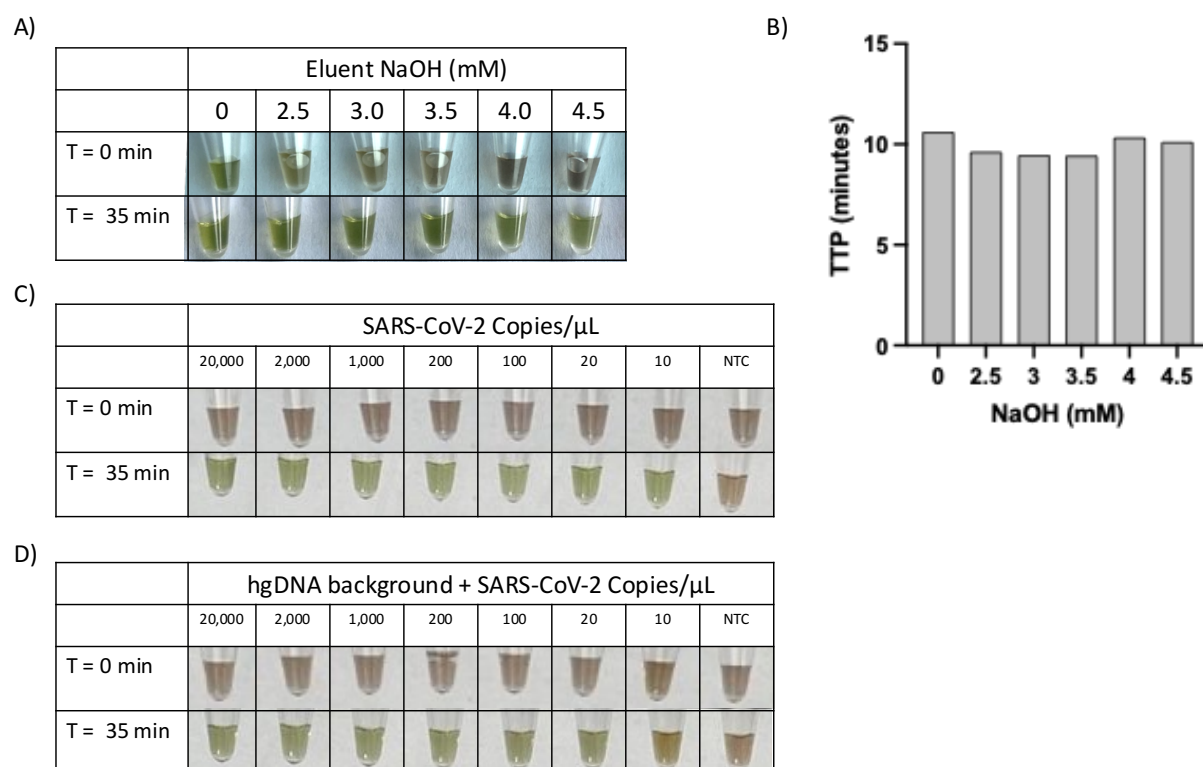


Figure 6.6. Performance of lyophilised colourimetric LAMP. The titration of NaOH into lyophilised colourimetric RT-LAMP reactions (A) and colourimetric RT-LAMP reactions supplemented with SYTO 9 fluorescent dye (B), detecting 200 copies/μL of SARS-CoV-2 RNA. The LoD of the lyophilised colourimetric RT-LAMP reactions performed via serial dilutions of SARS-CoV-2 RNA in the absence (C) and presence (D) of background hgDNA. Reactions were rehydrated in eluent produced from Qiagen extraction using sterile eNAT buffer, or negative swabs stored in eNAT buffer and spiked with SARS-CoV-2 RNA. Pictures detailing the colourimetric LAMP reactions were taken pre- (T=0 min) or post-incubation (T=35 min).

Table 6.2. Results obtained with colourimetric lyophilised LAMP detecting SARS-CoV-2. 77 clinical samples categorised as containing high, medium, low or negative viral load of SARS-CoV-2 based on the qPCR C_t of the n1 gene. Samples were extracted with QIAamp kit and eluted into 4 mM NaOH, and subsequently run in lyophilised colourimetric LAMP reactions in single replicates. Reactions were considered positive if a colour change from red to green was visible after 35 minutes of incubation at 63°C in a heat-block

Cat.	qPCR N1 C _t	Sample (n)	TP (n)	FN (n)	TN (n)	FP (n)	SEN (%)	SPE (%)
H	14-20	18	17	1	/	/	94.4	/
M	20-26	17	17	/	/	/	100	/
L	26-34	22	15	7	/	/	68.2	/
N	/	20	/	/	20	/	/	100
Total	/	77	49	8	20	/	86	100

It was subsequently determined that RNA underwent rapid deterioration in the presence of 4 mM NaOH SmartLid eluent at 21°C (Supplementary Figure 24C). The deterioration of RNA was much slower when stored at 4°C (Supplementary Figure 24D), and no deterioration was observed when the RNA was stored at -80°C (Supplementary Figure 24E). Therefore, the reactions were re-optimised to increase pH in the pre-lyophilisation process. An initial increase of the isothermal buffer pH was performed, which indicated that in wet reactions, a pH above 9.0 greatly increased the TTP of the LAMP reactions (Figure 6.7A), equating to a total reaction pH of 9.31 (Figure 6.7B). The pH of the reactions was optimised with several conditions altered, including the

excipients and the freeze-drying process, to create a proprietary LAMP mixture, which resulted in a pre-lyophilised reaction pH of 9.59 and a pH of 8.0 on rehydration without the need to alter the pH of the eluent (**Figure 6.7C**). Additionally, the dye was changed to PR, as the colour change was the most striking compared to the other dyes within our target LAMP reaction pH (**Figure 6.7D**). The use of PR dye also resulted in reduced inhibition of the real-time LAMP reactions, compared to other dyes - including the previously used metal-indicator dye, EBT (**Figure 6.7E**).

6.14. Colourimetric LAMP PRDC detection

For the detection of the PRDC pathogens, it was determined that isothermal buffer pH 8.0 with the addition of 1 mM NaOH in the colourimetric LAMP reaction resulted in a reaction pH similar to that achieved through lyophilisation (**Figure 6.7F**). Therefore, the LAMP primers for the detection of the bacterial PRDC pathogens were implemented into these 'mock' colourimetric LAMP reactions to assess analytical SEN. Serial dilutions were run in the 'mock' colourimetric LAMP reactions in duplicate (**Figure 6.8**). The analytical SEN achieved by each of the assays in the colourimetric LAMP reactions was very similar to the results of their paired real-time LAMP counterparts, which did not contain any dye or excipients (**Supplementary Figure 25**). The APP LAMP assay achieved a SEN of 5 copies/ μ L in both, with one replicate amplifying of 2.5 copies/ μ L in both detection methods. For MH, a detection limit of 20 copies/ μ L in the colourimetric LAMP and 50 copies/ μ L in the real-time amplification, with one replicate of 20 copies/ μ L amplifying and both replicates of the 10 copies/ μ L, was found. The SS LAMP assay detected all replicates of 10 copies/ μ L in the colourimetric detection and only one replicate in the real-time amplification. The PM LAMP assay achieved a LoD of 20 copies/ μ L in the colourimetric LAMP, with both replicates amplifying at 10 copies/ μ L in the real-time results. The GPS assay achieved 10 copies/ μ L detection in both replicates for both detection methods. The IC achieved down to 10 copies/ μ L in both detection methods, with both also not amplifying at 20 copies/ μ L, indicative of an issue with the 20-copy dilution.

The clinical performance of the colourimetric reactions was assessed in 10 clinical samples that were extracted with the optimised SmartLid protocol for lung tissue (**Chapter 5**), with paired samples extracted in parallel with the gold-standard homogenisation and extraction protocol. The PRDC bacterial LAMP assays were incorporated into each tube across an eight-tube strip, with the first tube not containing any primers, nor DNA polymerase, to act as a colour control. The seventh tube included the LAMP primers for the IC to ensure the validity of the sample extraction process. Six of the 10 colourimetric LAMP reactions were positive for APP, five were positive for MH, five were positive for SS, three were positive for GPS and three were positive for PM (**Figure 6.9A**). In comparison to the results of the SmartLid-real-time LAMP results obtained in **Chapter 5**, the colourimetric LAMP performed with identical results, apart from two samples. Lung sample 8 produced a FP in the colourimetric detection for APP, and lung sample 6 detected MH, which was previously negative by real-time LAMP.

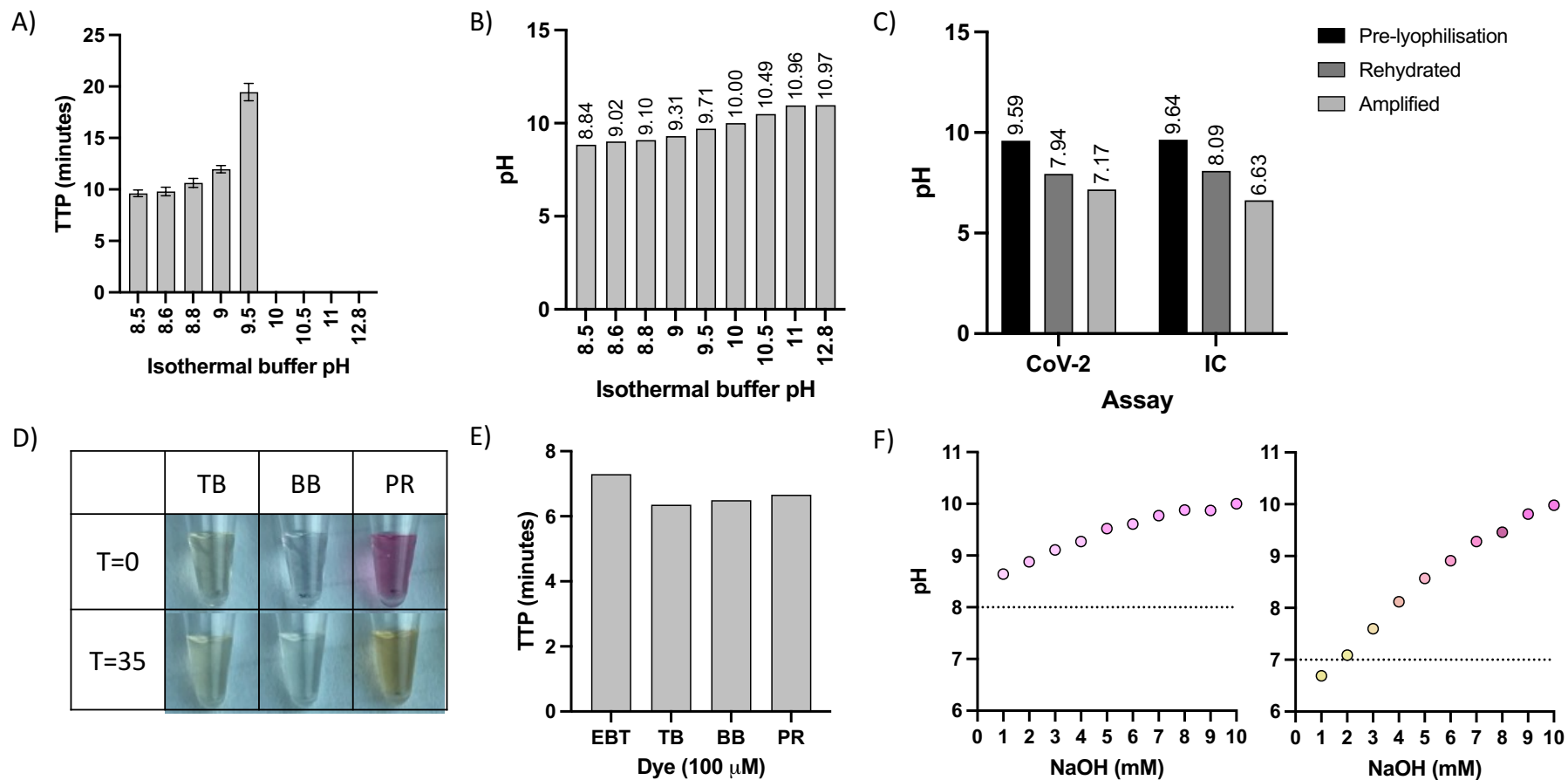


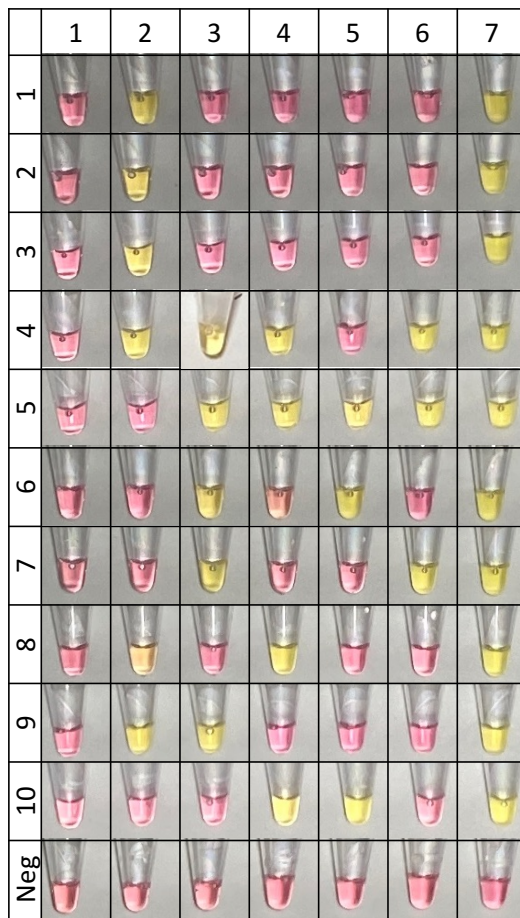
Figure 6.7. Optimisation of lyophilised colourimetric SARS-CoV-2 RT-LAMP. The increase in pH by the addition of NaOH into the isothermal buffer effect on amplification (A) and pH (B) of the LAMP reactions. The reaction pH of the pre-lyophilised, post-lyophilised (rehydrated) and amplified colourimetric reactions, using the optimised lyophilisation formula (C). The colour change (D) and the TTP of the real-time amplification (E) were achieved by different pH-sensitive dye supplemented at 100 µM into an optimised lyophilised formula. The pH of pre- (left) and post- (right) amplification of liquid reactions across a range of NaOH concentrations, with the dotted lines depicting the target pH and the colour of the nodes, the colour of the colourimetric LAMP reaction (F). Pictures detailing the colourimetric LAMP reactions were taken pre- (T=0) or post-incubation (T=35).

	Copies/ μ L															
	2,000	1,000	200*	100*	50*	20*	10*	NTC	2,000	1,000	200*	100*	50*	20*	10*	NTC
APP																
MH																
SS																
PM																
GPS																
IC																

Figure 6.8. Analytical sensitivity of 'mock' lyophilised colourimetric LAMP reactions. Serial dilutions of DNA were performed in nuclease-free water of APP serovar 5, MIDG 2220, MH isolate MH232, SS P1/7, PM isolate HVR5, GPS isolate HRV5 and a synthetic DNA of the IC (*cytB*) target sequence. Images were taken after 30 minutes of incubation in a heat block set to 63°C, with yellow indicative of positive LAMP amplification and pink, the absence of amplification. *APP was performed at concentrations of 2,000, 1,000, 100, 20, 10, 5, 2.5 and 0 copies/ μ L.

A subsequent farm visit was performed to determine the usability pen-side of the Dragonfly diagnostic kit which combines the SmartLid sample preparation device and lyophilised colourimetric LAMP reactions. The Dragonfly diagnostic kit was developed at Imperial College London and is licenced for use by ProtonDx. Nasal swabs were taken from five piglets and pooled into an eNAT tube for extraction with the SmartLid (**Figure 6.9B**). Whilst the sample eluent was used in the Dragonfly test panel for the detection of human respiratory pathogens, including SARS-CoV-2, influenza A, influenza B, respiratory syncytial virus (RSV) and human rhinovirus. None produced amplification with the Dragonfly kit. However, in the laboratory, the nasal swabs were re-extracted with SmartLid and run through the 'mock' lyophilised colourimetric reactions for the detection of both the bacterial and viral pathogens of the PRDC. The pooled samples were positive for SS, GPS and PM, and no viral pathogens were detected (**Figure 6.9C**).

A)



B)



C)

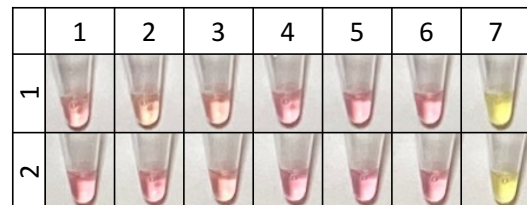
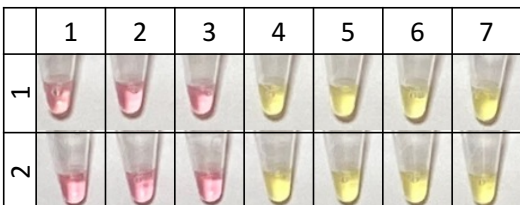


Figure 6.9. The use of colourimetric LAMP reactions in clinical extractions. 10 lung samples (rows) were extracted with the SmartLid protocol optimised for use with lung tissue and evaluated in colourimetric LAMP against the five bacterial pathogens and an IC (A). Columns 1-7 depict the colour control (1), APP (2), MH (3), SS (4), GPS (5), PM (6) and IC (7). The farm visit where nasal swabs were taken and a demonstration of the SmartLid extraction procedure and lyophilised colourimetric LAMP workflow using reactions produced for human respiratory pathogens (B). The five pooled nasal swabs were extracted with the standard SmartLid procedure and used against the bacterial (left) and viral (right) pathogens of the PRDC (C). Rows 1 and 2 signify duplicate extractions on the same swabs. The left columns signify colour control (1), APP (2), MH (3), SS (4), GPS (5), PM (6) and IC (7). The right columns signify colour control (1), BVS1 (2), BVS2 (3), SIV (4), H3N2 (5), PCV2 (6) and IC (7). All reactions were incubated on a heat block for 30 minutes at 63°C prior to an image being taken.

6.15. CMOS-ISFET detection of LAMP amplification

Previously, in **Chapter 4**, I demonstrated that the formulated lyophilised pH-LAMP reactions were capable of a strong pH change and, therefore are compatible with their implementation on the CMOS-ISFET chip platform.

The current chip surface contains a 1 μm surface layer of SiN_4 on top of a 1 μm layer of SiO_2 – named alpha. Two alternative surface materials were trialled, beta and gamma. the beta surface consisted of a 2 μm layer of only SiO_2 , and gamma, a thin (100 nm) layer of a proprietary high-k dielectric material (**Supplementary Figure 26**). Initial testing of the CMOS-ISFET surfaces using pH buffers performed by Dr Nick Moser suggested that the beta and gamma materials exhibited much lower output drift of the sensor, with the gamma surface having a much higher pH SEN (**Table 6.3**).

Table 6.3. Characterisation of different CMOS-ISFET surfaces. The performance of different surface layers of the CMOS-ISFET sensor. Alpha uses the standard CMOS-ISFET architecture containing 1 μm SiO_2 and 1 μm Si_3N_4 atop of the floating gate. Beta has a modification where the sensing layer is 2 μm SiO_2 . Gamma is the beta architecture with an additional 100 nm layer of high-k dielectric material. A pH buffer was applied to the surface of the chip and used to determine the % of pixels which were in range, the drift of the sensor over time and the SEN of the surface. Table reproduced with permission of Dr Nick Moser.

	Alpha	Beta					Gamma			
Pixels in range (%)	58.8	43.5	51.3	48.1	43.7	42.8	23.5	38.5	35.1	40.8
Output drift rate (mV/minute)	8.54	-2.2	-2.3	-1.4	-1.2	-1.4	-1.6	-2.1	-1.4	-1.6
Output pH SEN (mV/pH)	5	5.2	5.1	5.3	6.8	7.5	19.9	18.8	14.5	17.5

Subsequently, pH-LAMP reactions were performed on four of each of the beta and gamma chips using the APP-LAMP assay, with all LAMP reactions containing 20,000 or 2,000 copies/ μL of APP gDNA. The output results supported Dr Moser's results. The sensor output from the gamma chips had a trace that was visibly different to the sensor drift, whilst the beta surface often had indistinguishable traces between the sensor output and interpolated drift rate of that sensor (**Figure 6.10**). Therefore, the increase in SEN of the gamma surface produced much more notable amplification curves on-chip. The gamma sensor surface was chosen for use in future experiments.

It was my intention to perform a sample-to-result validation on-chip with 10 clinical lung samples. This was to be performed using the gamma sensor surface in combination with a six well manifold, where each well contained LAMP primers specific to each of the five bacterial PRDC pathogens, as well as the IC primers in the sixth well. However, due to the ongoing microchip shortage, the production of these chips and therefore validation was delayed and became infeasible to complete within the timeframe of this work.

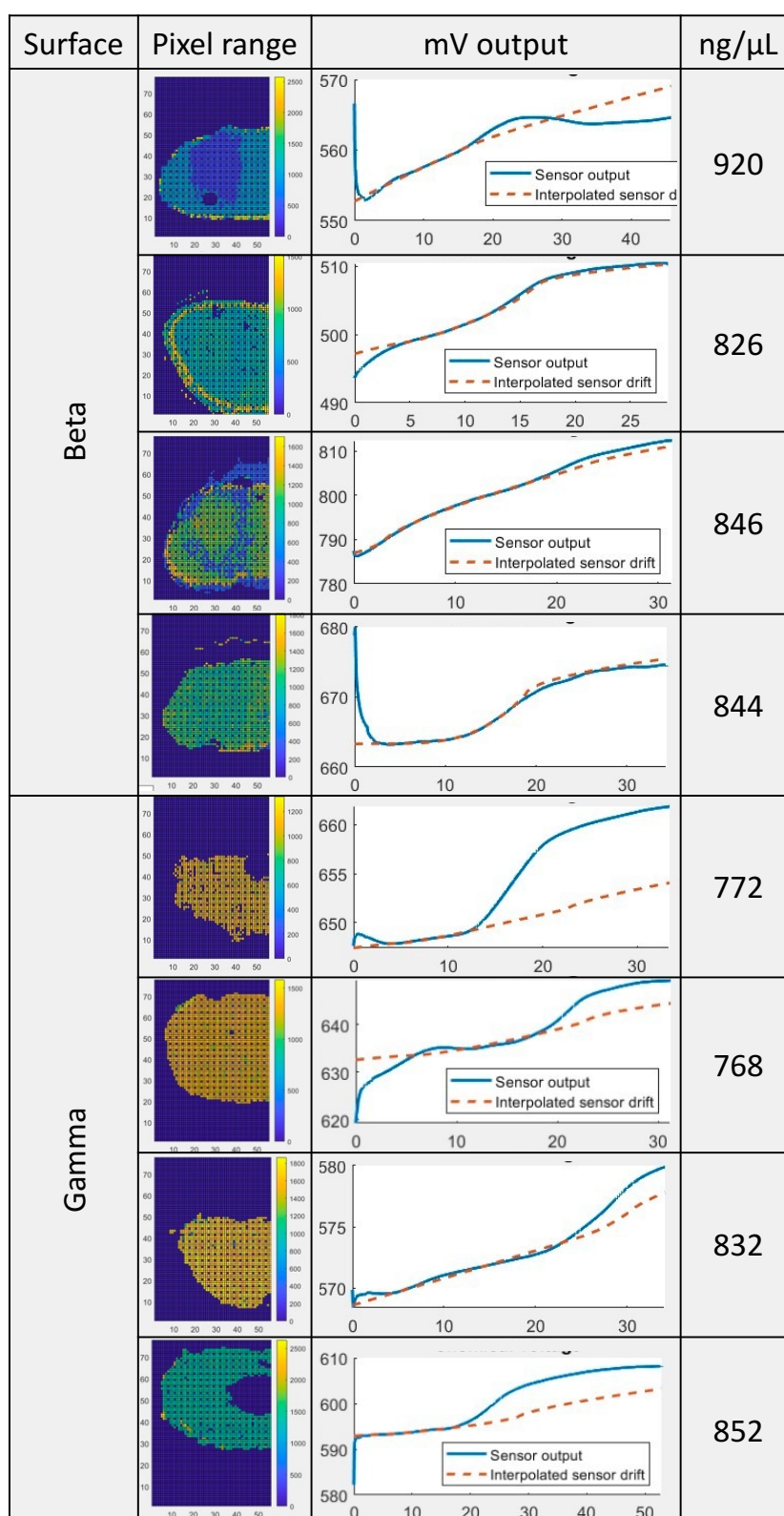


Figure 6.10. LAMP amplification detected on CNOS-ISFET chip. The amplification of the APP assay was detected using two different chip surfaces, beta consisting of a SiO₂ and gamma, a high-k dielectric surface coating. All APP-LAMP reactions contained APP gDNA (serovar 8), at 20,000 or 2,000 copies/ μ L. The pixel range is a visual representation of the max derivative for the pixels in the X axis against the Y axis (55x75 pixels total). The heat map on the right shows the range, with pixels in the high range (green-yellow) considered in range and pixels with a low derivate (blue) out of range. The mV output shows the sensor output (blue trace) against the interpolated sensor drift (dashed line) as calculated with the MatLab script by Dr Nick Moser. The ng/ μ L column depicts the amount of DNA as quantified by Qubit of LAMP reactions which were amplified on-chip.

6.16. Results summary

In this Chapter, I have demonstrated three different methods of detecting LAMP amplification, LFT, colourimetric and CMOS-ISFET detection. The amalgamation of all of the work in this Thesis has resulted in a sample-to-result comparison using 10 lung samples and different LAMP-detection methods. The results of the sample-to-result compared to the gold-standard are listed in **Table 6.4**.

Table 6.4. Sample to result validation using 10 clinical lung samples. 10 clinical lung samples extracted with the gold-standard (Qiagen) technique and ran in qPCR assays for the bacterial PRDC pathogens compared against the SmartLid (SL) extraction method developed in Chapter 5 and ran in LAMP reactions with three different methods of detection, real-time LAMP (LAMP), colourimetric, and LFT.

Assay	Lung	Qiagen-qPCR		SmartLid		
		Copies	Result	LAMP	col-LAMP	LFT-LAMP
APP	1	214,675	Pos	Pos	Pos	Pos
	2	636,644	Pos	Pos	Pos	Pos
	3	261,307	Pos	Pos	Pos	Pos
	4	240,399	Pos	Pos	Pos	Pos
	5	0	Neg	Neg	Neg	Neg
	6	0	Neg	Neg	Neg	Neg
	7	0	Neg	Neg	Neg	Neg
	8	0	Neg	Neg	Pos	Neg
	9	408,480	Pos	Pos	Pos	Pos
	10	0	Neg	Neg	Neg	Neg
MH	1	3,446	Neg	Neg	Neg	-
	2	7,681	Neg	Neg	Neg	-
	3	0	Neg	Neg	Neg	-
	4	1,568	Pos	Neg	Neg	-
	5	78,533	Pos	Pos	Pos	-
	6	3,573	Pos	Neg	Pos	-
	7	105,734	Pos	Pos	Pos	-
	8	53	Neg	Neg	Neg	-
	9	40,293	Pos	Pos	Pos	-
	10	0	Neg	Neg	Neg	-
SS	1	0	Neg	Neg	Neg	-
	2	0	Neg	Neg	Neg	-
	3	0	Neg	Neg	Neg	-
	4	10,666	Pos	Pos	Pos	-
	5	3,435	Pos	Pos	Pos	-
	6	124	Pos	Pos	Pos	-
	7	0	Neg	Neg	Neg	-
	8	4,332	Pos	Pos	Pos	-
	9	55	Pos	Neg	Neg	-
	10	21,037	Pos	Pos	Pos	-
PM	1	0	Neg	Neg	Neg	-
	2	0	Neg	Neg	Neg	-
	3	0	Neg	Neg	Neg	-
	4	113535	Pos	Pos	Pos	-
	5	168813	Pos	Pos	Pos	-
	6	0	Neg	Neg	Neg	-
	7	63195	Pos	Pos	Pos	-
	8	0	Neg	Neg	Neg	-
	9	0	Neg	Neg	Neg	-
	10	0	Neg	Neg	Neg	-

GPS	1	0	Neg	Neg	Neg	-
	2	0	Neg	Neg	Neg	-
	3	0	Neg	Neg	Neg	-
	4	0	Neg	Neg	Neg	-
	5	57	Pos	Pos	Pos	-
	6	1,973	Pos	Pos	Pos	-
	7	0	Neg	Neg	Neg	-
	8	0	Neg	Neg	Neg	-
	9	0	Neg	Neg	Neg	-
	10	14,746	Pos	Pos	Pos	-

Discussion

With the 2020-present global shortage of microchips, progress on the development of the CMOS-ISFET detection device was temporarily halted, and efforts to create a PoC diagnostic device were subverted to other methods of detecting LAMP amplification. Fortunately, LAMP is amenable to several methods of detection. Therefore, in this Chapter, I have successfully assessed the use of three different strategies to detect LAMP amplification, LFT, colourimetric and label-free detection via the CMOS-ISFET sensor. Each detection method was assessed for compatibility to the previously developed SmartLid-lung protocol using 10 clinical lung samples for a complete sample-result performance comparison.

The implementation of LFT strips to detect LAMP amplification appears promising, with several publications reporting identical SEN with LFT-LAMP compared to the real-time detection with fluorescent dyes and gel electrophoresis visualisation of LAMP products (Xue et al., 2021, Zhang et al., 2019b). My results support previous studies, with identical SEN between the APP-LAMP assay detected by LFT compared to the same assay detected in real-time-LAMP using DNA intercalating dyes. Additionally, a small-scale clinical validation of the LFT detection chemistry showed 100% SEN across the 10 clinical lung samples extracted with the SmartLid-lung protocol (**Chapter 5**), compared to the gold-standard homogenisation and Qiagen extraction process detected in qPCR. Therefore, the APP-LFT-LAMP assay combined with the SmartLid-lung extraction process would enable very rapid (<40 minutes) detection of APP-infected animals. However, there are limitations to the LFT-detection method. Firstly, I found that the implementation of duplexing of both IC and APP assays was difficult. A known contributor to poor detection on LFT strips is the hook effect, which is a phenomenon where the concentration of analytes, such as labelled amplicon, is very high, and there can be a decrease in binding efficiency, although this was found not to be the cause of the weak LFT-line banding, as determined by a pmol titration of the target amplicon. Instead, the weak APP-LFT band was most likely due to the IC assay outcompeting the APP assay, with only the IC banding pattern visible only when the duplex reaction was visualised with gel-electrophoresis. The optimisation of the biochemistry to bias primers towards the weaker APP assay did not rectify the problem, especially if APP was present at a low copy number in a sample. Therefore, due to time constraints, I validated the APP-LFT assay alone. Other single-tube multiplex LAMP reactions have been validated to be compatible with LFT detection, such as the triplex assay, *Entamoeba histolytica* and two non-pathogenic *Entamoeba* species (Foo et al., 2017). However, to accomplish the triplex detection, the authors produced their own LFT strips and altered the ratio of Ab and amplicon to produce test lines of the same intensity. Additionally, capture Abs had to be added strategically on the LFT strip to prioritise the detection of certain targets over others. To increase the signal produced by the APP assay in the APP-IC LAMP duplex, alternative LFT detection methods could have been evaluated. It has been reported that use of HRP labelled primers can be used, with detection occurring through the flow of the HRP substrate up the strip, which can increase the signal by 10x, compared to traditional gold-conjugated-Ab detection (Parolo et al., 2013). However, the use of HRP requires two additional user steps, the washing of the strip and the addition of the substrate, which would require greater hands-on time for the user. A more user-friendly approach is to increase the number of binding sites at the capture line on the LFT strip. A recent method which has resulted in an increase in 100x SEN is the use of gold-platinum nanoflowers instead of

gold conjugated-Ab conjugated to a chromogenic substrate, allows for not only a greater density of capture molecules but also amplifies the signal, thus, creating a higher signal at the test line (Zhang et al., 2019c). An alternative strategy, which was not evaluated in this Thesis, is the use of RPA-LFT. RPA-LFT has recently been described with high SEN using an APP-SS duplex reaction (Zhang et al., 2022b). Additionally, the LFT detection of APP combined with RPA-Cas9 has been shown to detect down to 10 copies (Luan et al., 2022).

In this Chapter, I have adapted the previously described (**Chapter 4**) low- or un-buffered LAMP and RT-LAMP lyophilisation methodology to be compatible with colourimetric detection. The initial lyophilised colourimetric LAMP reaction resulted in a novel colour change from red to green using a combination of the metal chelating dye, TB, and the pH-sensitive dye, EBT. Using SARS-CoV-2 as an exemplar, a detectable colour change occurring down to 10 copies μL was achievable. Additionally, the lyophilised colourimetric LAMP reactions demonstrated they were highly sensitive and specific, with 100% SEN and SPE across 77 human nose and throat swabs, compared to my standard LAMP reactions, which found 86% and 100%, respectively, when compared against the results obtained by qPCR. This small-scale clinical validation of the lyophilised colourimetric reaction using human nose and throat swabs used a Qiagen extraction protocol and therefore was used to verify the detection chemistry rather than a true end-to-end PoC workflow. The identical SEN and SPE when the lyophilised colourimetric LAMP reactions were compared to the liquid LAMP reactions is promising. However, it would be hoped that increasing the amount of sample in the LAMP reaction would result in increased SEN in the lyophilised LAMP reactions, as they contained 100% (20 μL) sample, whilst the liquid LAMP reactions only contained 25% (5 μL) sample. The lack of increase in SEN of the lyophilised reactions may be due to the sample containing inhibitors to LAMP, which counteract the benefit of inputting higher sample volume and, therefore greater amounts of target DNA/RNA. This is supported by my data showing no improvement in the amplification of the lyophilised LAMP reaction when the sample input was increased. Therefore, the benefit of rehydrating the lyophilised reaction completely with the sample may require further evaluation.

The combination of pH- and metal-indicating dyes chosen for this Chapter resulted in a colour change from green to red, which might be difficult to distinguish for individuals with colour-limited vision. Although not presented in this Chapter, Dr Kenny Malpartida-Cardenas has developed a MatLab script algorithm to differentiate the reaction status unambiguously for the user. However, this requires pre- and post-incubation images to be taken of the reactions, and in its current form, the requirement for both images to be captured under identical lighting and spatial conditions.

In addition, the metal-indicating dye used in the SARS-CoV-2 study was subsequently determined to be incompatible with the SmartLid rapid extraction protocol due to the carry-over of magnetic particles during the process. An additional unexpected result of lyophilisation was the loss of pH during the process, such that on rehydration, the pH had decreased to the lower end of the active range, resulting in a near-end-point green colour for some samples. This phenomenon has been recently reported to occur when lyophilised RT-LAMP reactions were stored for more than five days, after which the pH had to be corrected with the addition of 10

mM KOH (Lim et al., 2021). We found that increasing the pH of the elution buffer did not affect the nucleic acid extraction efficiency of SmartLid or Qiagen protocols. However, I found RNA to be unstable in 4 mM NaOH at room temperature, which is in agreement with the literature that alkaline solutions can “clean” RNA-binding from silica membranes (Biswal et al., 2021). Whilst under alkaline conditions, RNA undergoes rapid hydrolysis, free hydroxyl groups deprotonate the ribonucleotides 2’hydroxide group forming H₂O and a ribonucleotides 2’oxygen⁻ which performs a nucleophilic attack on the phosphodiester bond of the adjacent ribonucleotide (Li and Breaker, 1999).

To overcome these challenges, the RT-LAMP reactions were further optimised, with the incorporation of a different pH-dye indicator (PR), and negation of the requirement for pH correction on rehydration. The optimised LAMP reactions were tested with the bacterial PRDC pathogens with near-identical correlation with real-time LAMP SEN. In the APP and GPS assays, the LOD was identical, whilst in the SS assay, colourimetric detection was 2x more sensitive, and the PM assay, the real-time amplification was 2x more sensitive. In the MH assay, the real-time results failed to detect 20 copies/μL, i.e., the lowest concentration of DNA detected by the colourimetric MH-LAMP assay. However, illogically the real-time MH-LAMP did detect both replicates of 10 copies/μL. Taken together, these results suggest that differences seen between the colourimetric and real-time LAMP detection methods are due to slight differences in dilution factors, causing sporadic amplification of LAMP near the LOD for each assay, rather than differences in sensitivities of the individual detection methods.

Next, I sought to validate the sample-to-result detection of the bacterial pathogens in clinical samples, using 10 lung samples which were previously extracted and characterised by the gold standard, Qiagen extraction followed by qPCR, and SmartLid -qPCR and SmartLid -real-time LAMP. The colourimetric LAMP proved to be highly sensitive, with one lung sample previously negative by SmartLid-real-time LAMP being positive by SmartLid-colourimetric LAMP. This same sample (sample 6) was positive in SmartLid -qPCR for MH, and the relative quantification of this sample, as determined by Qiagen-qPCR was 3,573 copies/μL (**Chapter 5**). However, one FP did occur in the colourimetric LAMP reactions in the detection of APP in sample 8. The assays on either side of this FP event were not positive, so it is unlikely to be carry-over contamination of the adjacent assays, and all lung samples were prepared and run in the same experiment. Therefore, the FP could have arisen from cross-contamination between samples rather than an intrinsic FP caused by the assay itself. However, due to time constraints, this sample was not repeated to confirm the source of this FP result.

A usability demonstration was performed on a BHQ farm visit. A technical demonstration was given to a panel of 10 veterinarians and demonstrated the usability of the SmartLid extraction process and lyophilised colourimetric reactions on farm. The veterinarians produced highly positive feedback, with great value seen in implementing the rapid testing for the PRDC pathogens pen-side. The farm visit also yielded nasal swabs for piglets, which were pooled and tested in ‘mock’ Dragonfly panels for both the bacterial and viral PRDC pathogens. The pooled nasal swabs were positive for SS, PM and GPS in the Dragonfly panels and validated with qPCR data.

Overall, my results suggest that colourimetric LAMP is a highly sensitive detection method, and an optimised protocol, including the use of LAMP components that can be stored at room temperature without the need for a cold chain, has been formulated. This suggests the Dragonfly diagnostic kit (**Figure 6.11**), originally developed for human respiratory viruses, is a viable product for the detection of PRDC pathogens on farm.

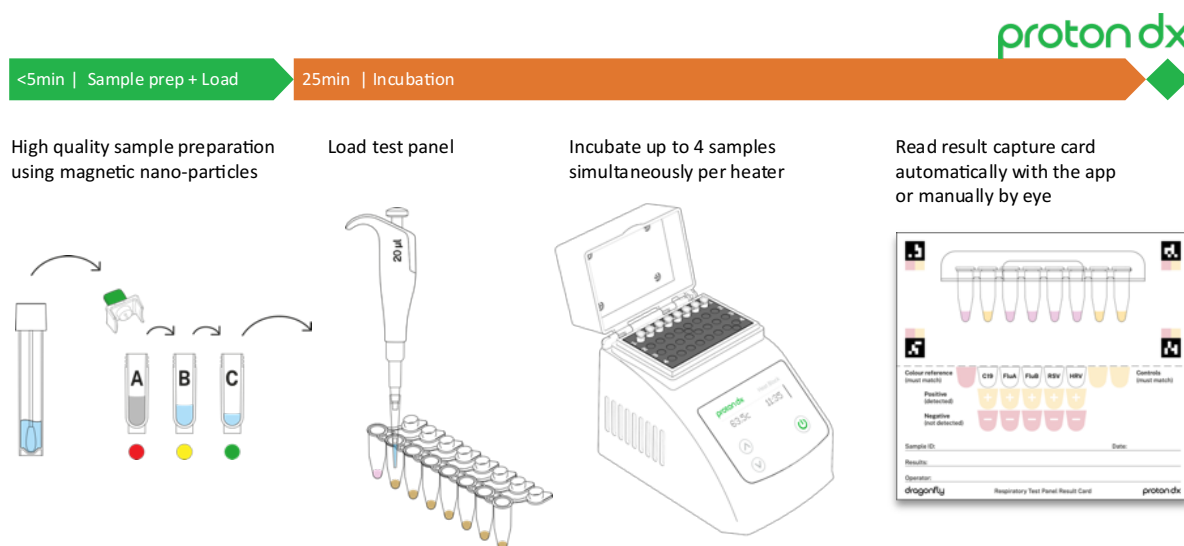


Figure 6.11. The ProtonDx Dragonfly respiratory test panel kit. The colourimetric-LAMP detection of human respiratory pathogens, SARS-CoV-2, influenza A, influenza B, RSVab and human Rhinovirus by the Dragonfly system. Consisting of the single-use SmartLid extraction from human nose and throat swabs followed by the rehydration of colourimetric-LAMP reactions and incubation in a heat-block. Reproduced with permission from the Copyright holder, ProtonDx Ltd.

To my knowledge, the Dragonfly platform is the first marketed colourimetric LAMP test capable of multiplex detection and is delivered in a true PoC, lyophilised format, although others, e.g. for such as the fully automated colourimetric device using a centrifugal force and read by a smartphone application (Oh et al., 2016a) have been described. In recent years there have been several reports of the NEB colorimetric kit being used for home testing, such as the USD \$51 lab-in-a-backpack developed by Queen Mary University researchers (Lin et al., 2022) and SnapDx, a thermos-based RT-LAMP that combines colourimetric RT-LAMP and equipment free extraction currently being developed by Stanford University (Li et al., 2020a). It is of note that the colourimetric kit provided by NEB has a catalogue price of GBP £1.73 per reaction, whilst the core reagents for our lyophilised col-LAMP reaction can be obtained for less than GBP £1.00 per assay (GBP £1.27, if reverse transcriptase is required), and providing a more cost-effective means of implementing colourimetric LAMP assays.

The ability to rapidly adapt Dragonfly panels to different pathogens will aid significantly in the development of future tests, with the PR pH-based detection utilised in Dragonfly being directly transferable to the pH change observed in the CMOS-ISFET sensor. However, I envisage the PRDC bacterial and viral panel on the Dragonfly platform will be produced prior to adaptation to the CMOS-ISFET detection platform.

A major focus of this Thesis has been the implementation of CMOS-ISFET detection of LAMP amplification. The Pantelis lab group has already shown that this approach results in high SEN with the detection of several

pathogens, antimicrobial resistance genes and SNPs (Kalofonou et al., 2020, Rodriguez et al., 2020, Rodriguez-Manzano et al., 2021, Malpartida-Cardenas et al., 2019). However, the issue with trapped charge created during ISFET fabrication onto the CMOS resulted in sensor drift. To decrease the effect of sensor drift, two different sensor surface-interface materials were trialled, and the use of a proprietary high-k dielectric surface layer resulted in higher SEN to pH buffers, as well as easily differentiable amplification curves as less noise is present due to decreased drift. The use of a high-k sensing material has previously been reported to increase pH sensing due to increased surface area density i.e., proton binding sites (Lai et al., 2006, Ortiz-Dosal et al., 2018).

The new surface material was intended to be validated using 10 clinical lung samples. The 10 lung samples were extracted with the optimised SL-lung protocol, however the global chip shortage prevented to final validation to be performed. My previous data from **Chapter 2** demonstrated that a pH change occurred with all bacterial PRDC LAMP assays. Taken together, with the data from pH-based colourimetric detection confirmed in this Chapter, I envisage no biochemical difficulties will be encountered in transferring these assays to the CMOS-ISFET chip.

6.17. Conclusion

In this Chapter I have developed the single-plex detection of APP using a LFT, which detected down to 5 copies/ μ L. Therefore, APP-LFT has the potential to provide rapid, sensitive, and cost-effective detection of this devastating respiratory pathogen. The multiplex detection of all PRDC bacterial pathogens may not be appropriate for use with LFT, as we have previously demonstrated the high instance of multiple bacteria being present in a single sample (**Chapter 3**). However, an alternative detection method, colourimetric LAMP may provide a more appropriate bacterial respiratory panel as no additional processing steps are required to determine the end-point presence of LAMP amplification. In this Chapter, we have developed both a metal-ion- and pH-sensitive colourimetric detection methods, both compatible with lyophilisation enabling their storage and transportation at ambient temperatures. Although the metal ion-sensitive colourimetric detection proved not to be compatible with the SmartLid extraction method, the pH-based phenol-red colourimetric dye provided very encouraging results. The LoD achieved with colourimetric LAMP across all PRDC bacterial pathogen assays was equivalent to that achieved in quantitative LAMP. Additionally, when an end-to-end sample to result was performed with 10 clinical samples, the colourimetric LAMP detected samples which were not detected with the real-time fluorescent detection method. Furthermore, the use of SmartLid extraction and colourimetric LAMP proved popular in a demonstration performed to a group of Swine veterinarians. Therefore, the use of colourimetric LAMP is a versatile detection method which can be easily implemented at the PoC. Lastly, we optimised the CMOS-ISFET detection determining that the proprietary high-k dielectric surface layer resulted in increased sensitivity to the standard SiO₂ layer, therefore the proprietary high-k dielectric will be implemented in all future iterations of the CMOS-ISFET chip.

Chapter 7: Conclusions and future prospects

7.1. Overview

The overall aim of the work described in the Thesis was to develop a PoC diagnostic device for aetiological agents of the PRDC based on the cutting-edge pH-sensitive “lab-on-chip” CMOS-ISFET array platform. At the start of the project, a panel of experts within the porcine sector were consulted as to the bacterial and viral PRDC pathogens of current relevance to the UK, i.e., where the PoC device would be tested. That panel included Dr Susanna Williamson (APHA pig expert group and UK pig disease scanning surveillance leads), Martin Smith (Head of Veterinary Services, BQP UK (Pilgrims)), Paul Penny (UK Swine Manager, CEVA UK), Eduardo Velazquez (Swine Veterinary Service Manager, CEVA UK), and Professor Dan Tucker (Professor of Veterinary Public Health, University of Cambridge Veterinary School). Upon their recommendations, work focused on the detection of five bacterial pathogens: APP, MH, PM, SS, and GPS; and three viral pathogens: PCV2, SIV, including the pandemic H3N2 strain, and the causative agents of PRRS, BVS-1 and BVS-2. Isothermal techniques (such as LAMP, HDA, PSR, RPA) are more amenable to implementation in PoC settings due to the device only requiring an uncomplex heating element calibrated to reach a single temperature, in contrast to expensive heating elements that must withstand very precise temperature cycles required for PCR.

My first objective (**Chapter 2**) was to compare different isothermal amplification methods, LAMP, RPA, HDA and PSR. Although primer sets were identified to detect PRDC pathogens using LAMP, HDA and RPA, PSR failed to produce sensitive amplification and was therefore not investigated further. The HDA primer sets were highly sensitive during analytical sensitivity. However, the HDA amplification became unspecific when testing a panel of other bacterial pathogens (negative controls). Therefore, out of the four isothermal methods trialled, only LAMP and RPA proved to produce sensitive and specific amplification. To my knowledge, this is the first instance of systematically comparing these four isothermal techniques, directly comparing their analytical sensitivity, specificity, and rapidity (TTP). Next, the RPA and HDA amplification methods were optimised to assess their compatibility with the label-free detection required for the CMOS-ISFET. However, amplification with both techniques in a minimally buffered environment resulted in inconsistent pH changes. It was determined that the high degree of primer noise was a persistent limitation to the ability to detect RPA amplification with a label-free approach. Additionally, both RPA and HDA contain much less primer than used in LAMP reactions, and thus it was determined that the quantity of product produced in LAMP was $>400\text{ ng}/\mu\text{L}$, whilst in RPA and HDA it was ~ 3.55 and $17.78\text{ ng}/\mu\text{L}$, respectively.

The selection of the most appropriate isothermal chemistry was central in determining the versatility of the CMOS-ISFET device to other isothermal chemistries, as only LAMP amplification had previously been validated within the Pantelis Georgiou research group. Therefore, this was the first instance of attempting to produce a label-free real-time detection of amplification using a range of isothermal chemistries. In finding that LAMP was the only isothermal chemistry tested to produce a reproducible pH change, ensuring the most appropriate amplification chemistry is being implemented on chip, by the entire laboratory group.

Having determined that LAMP was the only compatible isothermal amplification method with the CMOS-ISFET platform, optimised LAMP primer sets were identified for each of the PRDC pathogens of interest (**Chapter 2**). With two published (MH_B, PM_A), two published but undergoing primer additions (APP_A, SS_A) and one designed in this work (GPS_F) LAMP primer sets. Additionally, LAMP primer sets were designed for the SS divergent isolates, but their incorporation into a triplex assay decreased the sensitivity of the non-divergent SS detection and were not pursued further. Divergent SS are rare isolates in the UK, and the result was not considered to compromise any potential PoC device.

A clinical validation of the LAMP primer sets across 162 clinical samples (**Chapter 3**) indicated that the clinical sensitivity and specificity of previously described primer sets was lower than previously reported in the literature. High clinical sensitivity was achieved among the high bacterial load samples, as determined by qPCR to contain >2,000 copies/μL, with sensitivities of 100% across all LAMP assays, apart from APP, which achieved 95.83%. Among the medium bacterial load samples containing 200-2,000 copies/μL, the sensitivity ranged from 40%, 50% or 53.85% for the PM-, SS- and MH-LAMP assays, respectively, to 86.96% and 89.47% for the APP- and PM-LAMP assays, respectively. Unsurprisingly, samples characterised as containing low bacterial loads (<200 copies/μL) were poorly detected, with SS unable to detect any low copy number samples, MH detected only 28.57%, APP 45.48%, and PM 64.29% of qPCR positive samples. The overall LAMP sensitivity and specificity results are summarised in **Table 7.1**.

Table 7.1. PRDC-LAMP primers identified in this Thesis. The performance of the optimal LAMP primer sets identified in this Thesis in the analytical (Chapter 2) and clinical (Chapter 3) sensitivities and specificities.

Target pathogen	Primer set ID	Target gene	Analytical sensitivity (copies/μL)	Analytical specificity (%)	Clinical sensitivity (%)	Clinical specificity (%)	Reference
APP**	APP_A	<i>dsbE</i> -like	80	100	76.81	97.62	(Ji et al., 2012)
MH	MH_B	<i>mhp165</i>	8	100	71.05	100.00	(Li et al., 2013)
PM	PM_A	<i>kmt1</i>	8	100	80.00	98.70	(Bhimani et al., 2015)
SS	SS_A	<i>recN</i>	8	100	45.24	100.00	(Arai et al., 2015)
GPS	GPS_F	<i>infB</i>	20	100*	79.66	98.08	This Thesis
PCV2	PCV2_D	<i>orf1</i>	200	100	-	-	This Thesis
SIV	SIV_D	<i>M</i>	800	**	-	-	(Nakauchi et al., 2011)
SIV H3	SIV_A	<i>HA</i>	100	**	-	-	(Gu et al., 2010)
BVS-1	BVS_A		200	**	-	-	(Rovira et al., 2009)
BVS-2	BVS_H	<i>nsp2</i>	20	**	-	-	This Thesis

*cut-off for amplification at 30-minutes, otherwise 9/38 isolates amplified (76.32% specificity)

From a clinical standpoint, it is unknown how the bacterial burden relates to the onset of disease, or for how long bacterial or viral nucleic acids remain in the tissue after recovery from an infection. Analysis of our clinical tissues found that the average bacterial load in tissues was 34,976 copies/μL for APP, 19,266 copies/μL for MH, 19,4567 copies/μL for PM, 1,348 copies/μL for SS and 16,890 copies/μL for GPS. The high quantity of APP within the tissues may be reflective of active disease, as the Langford laboratory specialises in APP serotyping. Therefore, the tissue is likely to be sent to the Langford laboratory if APP is suspected, above any other disease.

In **Chapter 4**, the LAMP conditions were optimised to result in more rapid and more sensitive amplification. The optimisation of several components of LAMP and RT-LAMP reactions resulted in an increase in reaction speed, with targets amplifying, on average, 44% faster than the original protocol and a 10-fold increase in sensitivity seen in two of the three RT-LAMP assays tested. The optimised protocol was also incorporated into a formulation compatible with lyophilisation. The ability to store and transport the assay in the absence of a cold chain is a key requirement to fulfil the WHO 'ASSURED' criteria that PoC diagnostics should be deliverable to end users (Kettler et al., 2004). Unexpectedly, the optimised lyophilisation procedure resulted in an increased pH change over the liquid formulation, a finding that was not only critical to the CMOS-ISFET detection platform but also has the potential to increase the sensitivity of the CMOS-ISFET device. This is not only important for the detection of PRDC pathogens but also is a significant finding for all PoC assays designed for the CMOS-ISFET platform, as they will all ultimately be marketed with dried reagents, utilising the formulation produced as part of this work.

For the development of an end-to-end PoC system, the previously designed sample preparation device, SmartLid, was optimised for use on lung tissue. The SmartLid was combined with a simple and rapid homogenisation of tissue using a portable vortex in combination with the MP Biomedicals lysing tube A, which produced comparable homogenisation, but was more user-friendly than homogenisation with a dedicated hand-held homogeniser, the PowerMasher II. The SmartLid protocol was optimised to maximise lung sample input and minimise user steps by altering the wash buffer to contain the polyol, tripropylene glycol – no longer requiring an evaporation step to remove residual wash buffer, which may inhibit downstream amplification processes. The overall time taken to extract DNA from a complex lung sample was less than 5 minutes and proved to be a user-friendly option when trialled with a team of veterinarians. In a LoD experiment performed on lung samples spiked with intact bacterial isolates, the SmartLid protocol did result in a decreased DNA yield, as determined by qPCR, compared to the laboratory-based gold-standard method. The SmartLid extraction increased the qPCR C_t by 3-14 cycles, and the detection limit decreased with using SmartLid, on average, by 75-fold. However, when 10 paired clinical lung samples were extracted with each method, the qPCR C_t difference between the extraction protocols was 3.67 cycles, with only three MH-positive and one SS-positive not detected when extracted with SmartLid and detected with qPCR. Overall, the time taken to complete the SmartLid tissue extraction is < 5 minutes, which is significantly less than the gold-standard extraction protocol, which takes at least 78 minutes (**Chapter 5**).

In **Chapter 6**, three different methods for the detection of LAMP amplification were assessed, LFT, colourimetric, and the CMOS-ISFET lab-on-chip technology. Initial LFT detection using a LAMP duplex reaction detecting both APP and a porcine housekeeping gene, *cytB*, as an IC, proved unachievable without compromising on the sensitivity of the APP assay. When the single-plex APP LAMP assay was detected by LFT, a LoD produced identical results as the APP-LAMP reaction detected in real-time with fluorescent dye. However, a comparison of the 10 clinical lung samples extracted with SmartLid did result in a single false-negative to give an overall clinical sensitivity of 90%, compared to real-time LAMP results. Due to the complexity of performing single-tube LAMP multiplexing when several pathogens may be present in the sample, frequently seen with the PRDC, LFT strips

were determined to be inappropriate for a PRDC PoC test. During colourimetric detection, several dyes and optimisation steps were performed, ultimately resulting in the production of a lyophilised colourimetric RT-LAMP reaction, the first instance of lyophilisation of an un-buffered LAMP reaction, with no pH correction taking place during rehydration of the reaction. The lyophilised colourimetric reactions used the pH-sensitive dye, PR, with a colour change from pink to yellow, indicative that amplification had occurred. Data from the lyophilised reactions for the detection of several human viral pathogens were used to produce liquid reactions, which mimicked the lyophilised reactions as closely as possible in terms of the achieved colour change and the starting and end pH values. The 'mock' lyophilised colourimetric LAMP reactions detecting the bacterial PRDC pathogens achieved identical sensitivity to that achieved in the LAMP reactions detected by real-time using a fluorescent dye. Additionally, the 10 clinical samples extracted with the SmartLid produced a sensitivity and specificity of 100%, compared to the real-time LAMP results. Lastly, the CMOS-ISFET detection platform was optimised to contain an ion-sensing layer consisting of a proprietary high-k dielectric which produced a higher degree of sensitivity. This new surface will be implemented in future iterations of the CMOS-ISFET chip, and it is expected to translate to a higher sensitivity when detecting LAMP amplification events.

7.2. Conclusions

In this Thesis, I have identified LAMP primer sets to detect all PRDC targets identified by swine veterinary experts to be relevant to the UK. I have optimised and successfully lyophilised the LAMP biochemistry in a minimally buffered environment, ensuring compatibility with pH-based sensing of LAMP amplification. I have built upon the already developed SmartLid extraction process to extract DNA from lung tissue, resulting in increased sensitivity compared to the original SmartLid protocol, all in under 5 minutes. Furthermore, I have validated, on a small clinical sample set (n=10), the sample-to-result ability to detect all bacterial PRDC pathogens of interest. Lastly, I have performed user feedback, showcasing our results to swine veterinarians and performing a colourimetric test at a farm, ensuring the PoC in development remains end-user-focused. The progress made in this Thesis creates the initial foundations for the timely diagnosis of key PRDC pathogens, enabling the appropriate treatment, reduction of antimicrobial usage and altering herd management to combat disease outbreaks, limiting the economic losses incurred.

7.3. Future work and translation prospects

Whilst great progress was made during this work, due to time constraints, several aspects of the work described in this Thesis were not explored as comprehensively as initially intended. Optimal primer sets were identified for all PRDC targets outlined by a focus group of swine professionals. However, because of the limited availability of viral samples and time constraints due to the SARS-CoV-2 pandemic - the focus was mainly on PRDC-associated bacterial pathogens. Therefore, further validation is required to ensure that the viral pathogens are detected in clinical settings. Out of the five viral primer sets identified, three have been previously described, including clinical validation of both SIV primer sets, SIV_D for the generalised detection of SIV achieving 96.3% sensitivity and with one FP across 45 human nasal swabs (Nakauchi et al., 2011). For the H3N2 specific LAMP

primers set (SIV_A) across 27 swine lung samples, the RT-LAMP assay produced a higher number of positive results than the PCR assay of the same target (Gu et al., 2010). A larger clinical study was performed on the BVS-1 specific primer set (BVS_A) with experimentally infected boars. However, only 52% of the PCR-positive serum samples were detected by the BVS-1 RT-LAMP assay (Rovira et al., 2009). Thus, further testing of each of the viral pathogens is required to ensure their clinical relevance. It may be advantageous to re-visit the original primer design of all viral targets, with the published sequences being at least 10 years old and distinct diversity continually being reported across viral phylogenies (Sun et al., 2020, S et al., 2022, Liu et al., 2017).

Another area of increasing importance to the UK is the threat of ASF. ASF is a viral pathogen which only infects swine and wild boars, causing internal bleeding, sometimes seen under the skin. Rapid sudden death whole herds, can with a fatality rate of 80-100% (Moennig, 2000). ASF originated in Africa, however, outbreaks outside of that continent have occurred. The latest in 2018 in China, was estimated to result in a loss of 38 million pigs and an economic impact of USD \$111.2-billion (You et al., 2021), but continues to be a problem for Chinese and other Asian producers. Currently, the disease is rapidly spreading across Europe, with the latest outbreaks on the French-Germany border (Department for Environment Food & Rural Affairs, 2022a). The difficulty in containing ASF is that it is very easily transmitted through contaminated meat products - remaining viable and infectious for extended periods of time (Mazur-Panasiuk et al., 2019). Therefore, in an attempt to safeguard the UK, the government has implemented strict guidelines on importing pig products from the EU (Department for Environment Food & Rural Affairs, 2022b). However, the difficulty in implementing an ASF test into our panel, is that ASF is a notifiable disease, and currently, even suspicion of ASF is a notifiable event (APHA, 2019), as is any testing for the disease. That also means such animals would not be able to be moved from the farm. Therefore, under current regulations, the inclusion of ASF into a diagnostic panel is not welcomed by end-users (personal correspondence, Dr Susannah Williamson, APHA). However, given the concern of importation of ASF, there may be a role for a PoC that detects ASF at not only farms but also at airports/ports where pork products enter the UK.

During the course of this Thesis, Professor Pantelis Georgiou, Dr Nick Moser and Dr Jesus Rodriguez-Manzano founded a start-up company, ProtonDx. ProtonDx has since validated and licences a human respiratory test panel using the colourimetric LAMP conditions detailed in this Thesis, with the platform named Dragonfly. The Dragonfly human respiratory test panel has recently been CE-IVD marked, therefore in compliance with the European IVD Device Directive (IVDD 98/79/EC), required for use as an in vitro diagnostic throughout European member states. Still in development by Imperial College London and ProtonDx, is the CMOS-ISFET sensor-based lab-on-chip device, Lacewing. The commercialisation of Dragonfly by ProtonDx offers the PRDC pathogen panel a clear route to market, requiring the simple switch of the human respiratory pathogen primer sets for the PRDC pathogen primer sets identified in this Thesis. Whilst the reception to the implementation of Dragonfly on farm was very positive from veterinarians, more work will be required to adapt the current product for use pen-side, for example, the development of a rechargeable battery for the heat block or a portable external power source that can also run the vortex for tissue homogenisation. Additionally, it would be advantageous to supply the kit

in a hardened plastic casing that can contain all components required to run the Dragonfly test in a hyper-portable and rugged package, more appropriate for rapid set-up in less-than-ideal conditions. This lab-in-a-suitcase idea is nothing novel, being previously implemented by researchers detection diseases in resource-limited settings such as the 2015 Ebola outbreak in Guinea (Faye et al., 2015). However, unlike the previous lab-in-a-suitcase, Dragonfly is a true PoC diagnostic, requiring only minimally trained personnel to use, with no previous laboratory experience.

The benefit of bringing to market the PRDC panels as a Dragonfly product, in the first instance, has several advantages. Firstly, it is the fastest route to market, with the platform already readily available, resulting in a lead-time for production of a few weeks – enabling veterinary access to the product as soon as possible. Secondly, the product offers high value, offering all six targets in a single test at a current list price of GBP £20/test panel, compared to current diagnostic pricings i.e., the APHA offer a single qPCR diagnostic service for GBP £25-30 per viral pathogen, with a turnaround time of up to 7 days (APHA, 2022a). The affordability of the test panel will not only provide maximum value to the customer but also ensures the greatest market penetration. Thirdly, the lack of regulation in veterinary diagnostics enables the product to be field-tested by veterinarians who can determine first-hand the detection rate of each pathogen. Veterinarian feedback will be invaluable to align diagnostic results with the clinical symptoms, not only to reduce the risk to the CMOS-ISFET platform (as the Dragonfly test and Lacewing both rely on pH change detection methods) but also to enable the rapid alterations to primer sets without regulatory resolutions or costs. Lastly, the use of Dragonfly can significantly improve the tools available to veterinarians through the correct diagnosis and appropriate treatment (i.e., antibiotics) in a timely fashion - as little as 30 minutes, the time it takes for a typical farm visit.

As the Lacewing device is still in development, only a limited number of experiments were performed on an early-stage concept device in this Thesis. A large amount of work is still required to develop Lacewing into a working sample-to-result device, with the current prototype geared towards the sample input being a swab, for human respiratory pathogens driven by the COVID-19 pandemic. In its current form, the Lacewing device consists of a reader device with a compact footprint, stackable design and interchangeable single-use lacewing cartridges and a companion application that is used to track and share the test results (**Figure 7.1**). The advantage of using Lacewing over Dragonfly is that although the test time should not significantly change, the hands-on time will be drastically reduced – owing to the integrated sample preparation steps. Additionally, the ability of the CMOS-ISFET chip to detect amplification in real-time as opposed to the end-point detection of Dragonfly, allows Lacewing to provide a wealth of data, the use of the IC assay as a reference to determine the

sample input, it may be possible to report the relative pathogenic load, and therefore Lacewing is much more amenable for use during surveillance or even treatment programmes.



Figure 7.1. The Lacewing device. The current prototype of the Lacewing device consisting of a single-use Lacewing cartridge, the Lacewing reader, and the Lacewing companion application. Image used with the permission of the Copyright holder, ProtonDx Ltd.

In early 2022, ProtonDx and Imperial were awarded a joint grant from the Department of Environment, Food and Rural Affairs' Innovation Programme, part of Innovate UK, to continue the work detailed in this Thesis and produce a PRDC Lacewing prototype, including a small-scale field validation of the device on farms.

In conclusion, the work described in this Thesis has surpassed several of its key objectives. Not only has strong progress been made in the development of a PoC diagnostic device for the PRDC pathogens, but the work presented here is also likely highly translatable to other veterinary pathogens. Building upon this work, the availability of a commercial (Lacewing or Dragonfly) device that detects eight PRDC pathogens would be a world first, and -has the potential to transform the veterinary diagnostic market.

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Appendix I

Supplementary Table 1. Negative species used in Chapter 2. A panel of 41 bacterial isolates used to test the cross-reaction and SPE of amplification assays.

Isolate I.D.	Pathogen
MIDG 1560	<i>A. suis</i>
MIDG 1579	<i>A. suis</i>
MIDG 3256	<i>A. suis</i>
MIDG 1573	<i>A. equuli</i>
MIDG 1574	<i>A. equuli</i>
MIDG 1575	<i>A. equuli</i>
MIDG 1564	<i>A. equuli</i>
APP sero2	<i>A. pleuropneumoniae</i>
APP Sero5b	<i>A. pleuropneumoniae</i>
APP Sero8	<i>A. pleuropneumoniae</i>
MIDG 2226	<i>A. pleuropneumoniae</i>
MIDG 2205	<i>A. pleuropneumoniae</i>
MIDG 3182	" <i>A. porcitisillarum</i> "
MIDG 3248	" <i>A. porcitisillarum</i> "
MIDG 3249	" <i>A. porcitisillarum</i> "
MIDG 3250	" <i>A. porcitisillarum</i> "
MIDG 3805	<i>G. parasuis</i>
MIDG 3305	<i>G. parasuis</i>
MIDG 3330	<i>G. parasuis</i>
MIDG SVR5	<i>G. parasuis</i>
MH232	<i>M. hyopneumoniae</i>
ms40	<i>M. hyopneumoniae</i>
ms34	<i>M. hyopneumoniae</i>
MIDG 1568	<i>P. multocida</i>
MIDG 1570	<i>P. multocida</i>
MIDG 1584	<i>P. multocida</i>
MIDG 3263	<i>P. multocida</i>
MIDG 3264	<i>P. multocida</i>
MIDG 3265	<i>P. multocida</i>
MIDG 3267	<i>P. multocida</i>
MIDG 3268	<i>P. multocida</i>
MIDG 3269	<i>P. multocida</i>
MIDG 3270	<i>P. multocida</i>
MIDG 3271	<i>P. multocida</i>
MIDG 3272	<i>P. multocida</i>
MIDG 3272	<i>P. multocida</i>
MIDG 1578	<i>p. pneumotropica</i>
MIDG 1586	<i>P. pneumotropica</i>
LSS17	<i>S. suis</i>
LSS19	<i>S. suis</i>
P1/7	<i>S. suis</i>
SS1003	<i>S. suis</i>

Supplementary Table 2. Synthetic DNA constructs used in Chapter 2. Synthetic constructs produced by IDT. The T7 sequence is highlighted in purple and the reverse complement for the T3 sequence is highlighted in blue, used to produce ssDNA or to transcribe + or – strand RNA.

Sequence ID	Sequence
SS_9588 divergent1	TAATACGACTCACTATAGGGAAAGATTGCGCGTCATATCTATGAACTGTTCTATCACTTTTACCAGATAAAAGCAGAAA TTCGGCATCATCAAAAGTCAAATTTAAGAAGAACCCTGTTTATTCGGTTTGTGGAAGATGGGGTCAATGAGATTCT TCAATGATCTCCATCTGGCTGACAGTTTTTTTGGTTTGGATACAGGGATTTACCAAGAGTTTTGGAAAAATGATTCGT GGAGTCAGGCTTATTTACGTGGAGCTTTTTTAGCAAGTGGTCTGTAAAGGATCCTGAAAAGGTTAAATACCAATTGG AAATTGCCTCTGTCTATAGTGATCATGCCTATGACTTGGCCAATTTGATGCAGAAATTTTATTGGATGCAAAGGTTA TAGAGCGGTGCAAGGGAACCATTAACCTACTTGAACGGGCAGAAAGATATTATGGACTTCCTTTTGGTGATGGGGCAG AAGATGCAAAAGACTGAGTTTGAAAACGTGAAATTTGTTACGGGAAGCTCGGAATGATTTAAATCGTGCCACTAATGCTG AAGCGGCCAATATTGCCAAGACAGTCACTTAGTGAGGGTTAATT
SS_9588 divergent2	TAATACGACTCACTATAGGGAAAGATTGCTCGGCATGTCTATGAAATGCTCCAGACCTTTTACCAGATTAAGCGGAA TTCGTCACCAACGCAACGCAAAACCTTAGAAAAGATAGGATTTATCTGTTTCATATCGAGGACGGGGTCAATGATATTC TCAACGACCTCTATCTGGCAGATAGCTTTTTCGGCTTGGAGACGGGGATTTACCGCTGGTATTGGAAAAATGATTCCT GGAGCCAGGCCCTATCTGCGTGGTGCCCTTTTGGCGGCGGGCTCTGTCAAGGACCTGAAAAAGGTAAGTACCAGCTGG AAATTGCCTCGGTCTACAGCGACCATGCTCTTGACTTGGCGGACTTGATGCAGAAATTTCTGCTGGATGCCAAGGTGA TTGAACGAAAAAAGGAGCATTACCTATTTGCAACGAGCCGAGGATATCATGGATTTCTCCTGGTCAATGGGGCAG AAGAGGCAAGACCGAGTTTGAAAATGTCAAGCTCCTGCGCGAGGCCAGAAACGATCTCAATCGTGCGACCAATGCCG AGGCGGCCAATATTGCCAAGACAGTCTTAGTGAGGGTTAATT
PCV2_orf1	TAATACGACTCACTATAGGGACCAGCGCACTTCGGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCAG CAAAAAGAATGGAAGAAGCGGACCCCAACCACACAAAAGGTGGGTGTTACCGTGAATAATCCTTCCGAAGACGAGCG CAAGAAAATACGGGAGCTTCCAAATCTCCTTTTGAATTTTATTGTTGGCGAGGAGGTAATGAGGAAGGACGGAAC ACCCACCTCCAGGGGTTTCGCTAATTTTGTGAAGAAGCAACATTTAATAAAGTGAATGGTATTTTCGGTGCCCGCTG CCACATCGAGAAAGCGAAAGGAACTGATCAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTACTGATCGAATG TGGAGCTCCTAGATCTCAAGGACAACGGAGTGACCTGTCTACTGCTGTGAGTACCTTGTCTGGAGAGCGGGAGTCTGGT GACCGTTGACAGGACGACCCCTGTAACTTTTGTGAGAAATTTCCGCGGGCTGGCTGAACCTTTTGAAGTGAGCGGGAA AATGCAGAAGCGTGATGGAAGACTAATGTACACGTCAATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAAATGGGCTGC TAATTTTGCAGACCCGAAACCACATACTGGAACACCTAGAAACAAGTGGTGGGATGGTTACCATGGTGAAGAAGT GGTTGTTATTGATGACTTTTATGGCTGGCTGCCCTGGGATGATCTACTGAGACTGTGTGATCGATATCCATTGACTGT AGAGACTAAAGTGGAAGTGTACCTTTTTTGGCCCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTA CTCCTCAACTGCTGTCCAGCTGTAGAAGCTCTTATCGGAGGATTACTTCTTGGTATTTTGAAGAATGCTACAGA ACAATCCACGGAGGAAGGGGGCCAGTTTCGTACCCCTTTCCCCCATGCCCTGAATTTCCATATGAAATAAATTACTG AGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCATCTTAGTGAGGGTTAATT
SIV_M	TAATACGACTCACTATAGGGATGAGTCTTCTAACCGAGGTGCAACGTACGTTCTTTCTATCATCCCGTCAGGCCCC TCAAAGCCGAGATCGCGCAGAGACTGGAAGTGTCTTTGTCAGGAAAGAACACAGATCTTGAGGCTCTCATGGAATGGC TAAAGACAAGACCAATCTTGTACCTCTGACTAAGGGAATTTTAGGATTTGTGTTCACGCTCACCGTGCCAGTGAAGC GAGGACTGCAGCTAGACGCTTTGTCCAAAATGCCCTAAATGGGAATGGGGACCCGAACAACATGGATAGCTTAGTG AGCGTTAATT
BVS	TAATACGACTCACTATAGGGATGCCAAATAACAACGGCAAGCAGCAGAGAAGAGAAAGAGGGGGATGGCCAGCCAGTCA ATCAGCTGTGCCAGATGCTGGGTAAGATCATCGCTCAGCAAAACAGTCCAGAGGCAAGGGACCGGGAAAGAAAAATA AGAAGAAAAACCCGGAGAAGCCCATTTTCTCTAGCGACTGAAGATGATGTAGACATCACTTTACCCCTAGTGAGC GGCAATTGTGTCTGTCTCAATCCAGACCGCCTTTAATCAAGGCGCTGGGACTTGCACCTGTGATTCAGGAGGT TGCTGGGTGCAATGATAAAGTCCAGCGCCAGCAACCTAGGGGAGGACAGGCCAAAAAGAAAAAGCCTGAGAAGCCAC ATTTTCCCTGGCTGCTGAAGATGACATCCGGCACCACCTCACCAGACTGAACGCTCCCTCTGCTTGAATCGATCC AGACGGCTTTCAATCAAGGCGCAGGAAGTGCCTGCTTTCATCCAGCGGGAAGGTGAGTTTTCAGGTTGAGTTTATGC TGCCGGTTGCTCATACAGTGCGCTGATTCGCGTGACTTCTACATCCGCCAGTCTTAGTGAGGGTTAATT

Supplementary Table 3. All primers used in Chapter 2. All primer sequences used across all the amplification chemistries used in this Chapter.

Amplification chemistry	Target pathogen	Target gene	Primer ID	Sequence	Length (bp)	GC%	Reference
RPA	APP	<i>apxIVA</i>	RPA_APP1_F	GCAGTGCTTCTGTCTGTTAGAGTCACGCCTTCT	32	53.13	(Li et al., 2019)
			RPA_APP1_R	TATAGCGAATGTCTTTATCCGTCGTGTAAT	30	36.67	
			RPA_APP1_prb	CAATTAAGTAGTATACGCAATGCAAAGCA TGAT CCCTACCGTTATGCA-C3 Spacer	47	38.30	
			RPA_APP2_F1	TGGCGGTAATGGCGATGACACCCCTCATCGCGGCA	35	62.86	This Thesis
			RPA_APP2_F2	TAATGGCGGTAATGGCGATGACACCCCTCATCGCG	35	57.14	
			RPA_APP2_F3	GGTTAATGGCGGTAATGGCGATGACACCCCTCATCG	35	54.29	
			RPA_APP2_F4	TACGGTTAATGGCGGTAATGGCGATGACACCCCTCA	35	51.43	
			RPA_APP2_F5	TGATACGGTTAATGGCGGTAATGGCGATGACACCC	35	51.43	
			RPA_APP2_F6	TAATGATACGGTTAATGGCGGTAATGGCGATGACA	35	42.86	
			RPA_APP2_F7	GGGTAATGATACGGTTAATGGCGGTAATGGCGATG	35	48.57	
			RPA_APP2_F8	TGCGGGTAATGATACGGTTAATGGCGGTAATGGCG	35	51.43	
			RPA_APP2_F9	CGGTGCGGGTAATGATACGGTTAATGGCGGTAATG	35	51.43	
			RPA_APP2_F10	TGCCGGTGCGGGTAATGATACGGTTAATGGCGGTA	35	54.29	
			RPA_APP2_R1	CGGTTATCATTATTGGTATCTTCATAAACGATATC	35	31.43	
			RPA_APP2_R2	GCGCGGTTATCATTATTGGTATCTTCATAAACGAT	35	37.14	
			RPA_APP2_R3	CTTGCGCGGTTATCATTATTGGTATCTTCATAAAC	35	37.14	
			RPA_APP2_R4	TCTCTTGCGCGGTTATCATTATTGGTATCTTCATA	35	37.14	
			RPA_APP2_R5	ATATCTCTTGCGCGGTTATCATTATTGGTATCTTC	35	37.14	
			RPA_APP2_R6	TCGATATCTCTTGCGCGGTTATCATTATTGGTATC	35	40.00	
			RPA_APP2_R7	GTGTCGATATCTCTTGCGCGGTTATCATTATTGGT	35	42.86	
			RPA_APP2_R8	AAGGTGTCGATATCTCTTGCGCGGTTATCATTATT	35	40.00	
			RPA_APP2_R9	TTTAAGGTGTCGATATCTCTTGCGCGGTTATCATT	35	40.00	
			RPA_APP2_R10	AATTTTAAGGTGTCGATATCTCTTGCGCGGTTATC	35	40.00	
			RPA_APP2_R11	GTAATTTTAAGGTGTCGATATCTCTTGCGCGGTT	35	40.00	
			RPA_APP2_R12	TCAGTAAATTTTAAGGTGTCGATATCTCTTGCGCG	35	40.00	
			RPA_APP2_R13	ATATCAGTAAATTTTAAGGTGTCGATATCTCTTGCG	35	31.43	
			RPA_APP2_R14	TTAATATCAGTAAATTTTAAGGTGTCGATATCTCT	35	25.71	
			RPA_APP2_prb	GTGGCTACGGTGCGGACAC CTAT ATCTATAGCAAAGGACACGGACAG-C3 Spacer	47	53.19	
			RPA_APP2_R_LF	CTTGCGCGGTTATCATTATTGGTATCTTCATAAAC	35	37.14	
			RPA_APP_Apx3 ⁺ _F1	CAAGAGATATCTCTCCTCCGTGCTTCTGAGCAAAG	35	48.57	
			RPA_APP_Apx3 ⁺ _F2	ACACAAGAGATATCTCTCCTCCGTGCTTCTGAGCA	35	48.57	
			RPA_APP_Apx3 ⁺ _F3	GCGACACAAGAGATATCTCTCCTCCGTGCTTCTGA	35	51.43	
			RPA_APP_Apx3 ⁺ _F4	CAGGCGACACAAGAGATATCTCTCCTCCGTGCTTC	35	54.29	
			RPA_APP_Apx3 ⁺ _F5	CTTCAGGCGACACAAGAGATATCTCTCCTCCGTGC	35	54.29	
			RPA_APP_Apx3 ⁺ _F6	CCTCTTCAGGCGACACAAGAGATATCTCTCCTCCG	35	54.29	
			RPA_APP_Apx3 ⁺ _F7	TTACCTCTTCAGGCGACACAAGAGATATCTCTCCT	35	45.71	
			RPA_APP_Apx3 ⁺ _F8	GTTTTACCTCTTCAGGCGACACAAGAGATATCTCT	35	42.86	
			RPA_APP_Apx3 ⁺ _F9	ATTGTTTTACCTCTTCAGGCGACACAAGAGATATC	35	40.00	

			RPA_APP_Apx3' _F10	TATATTGTTTTACCTCTTCAGGCGACACAAGAGAT	35	37.14		
			RPA_APP_Apx3' _R1	TCATAAAATAGAAAATATTCGTTTATCGAATGAGC	35	25.71		
			RPA_APP_Apx3' _R2	AGATCATAAAATAGAAAATATTCGTTTATCGAATG	35	22.86		
			RPA_APP_Apx3' _R3	CCAAGATCATAAAATAGAAAATATTCGTTTATCGA	35	25.71		
			RPA_APP_Apx3' _R4	ACACCAAGATCATAAAATAGAAAATATTCGTTTAT	35	22.86		
			RPA_APP_Apx3' _R5	TTCACACCAAGATCATAAAATAGAAAATATTCGTT	35	25.71		
			RPA_APP_Apx3' _R6	GTATTACACCAAGATCATAAAATAGAAAATATTC	35	25.71		
			RPA_APP_Apx3' _R7	TTGGTATTACACCAAGATCATAAAATAGAAAATA	35	25.71		
			RPA_APP_Apx3' _R8	AAATTGGTATTACACCAAGATCATAAAATAGAAA	35	25.71		
			RPA_APP_Apx3' _R9	TTCAAAATTGGTATTACACCAAGATCATAAAATAG	36	27.78		
			RPA_APP_Apx3' _R10	CGGTTCAAAATTGGTATTACACCAAGATCATAAAA	36	33.33		
			RPA_APP_Apx3' _prb	AAACGTTGGTGAGCACTCAGGTGGAGAAGAT TGTT TGAGTCGATGGCCGG-C3 Spacer	49	53.06		
	MH	mhp165		RPA_MH1_F	TCAGAAGTTGATCCAAAACAGGACTAAAA	30	33.33	(Liu et al., 2019)
				RPA_MH1_R	CCCGTAGTTTTAAAAATGGCGAGGAGGCT	30	46.67	
				RPA_MH1_prb	CTACCAAAACAAAACAAGGTAGTTTTATTGGAT TAT TCCCAATCATCCCGA-C3 Spacer	49	36.73	
		p183 (p97)		RPA_MH2_F1	GCAAAAGATAGTTCAACTAATCAATATGTAAGT	33	27.27	This Thesis
				RPA_MH2_F2	AAAGATAGTTCAACTAATCAATATGTAAGTATC	33	24.24	
				RPA_MH2_F3	GATAGTTCAACTAATCAATATGTAAGTATCCAG	33	30.30	
				RPA_MH2_F4	AGTTCAACTAATCAATATGTAAGTATCCAGAAC	33	30.30	
				RPA_MH2_F5	TCAACTAATCAATATGTAAGTATCCAGAACCAA	33	30.30	
				RPA_MH2_F6	ACTAATCAATATGTAAGTATCCAGAACCAAATT	33	27.27	
				RPA_MH2_F7	AATCAATATGTAAGTATCCAGAACCAAATTCCT	33	30.30	
				RPA_MH2_F8	CAATATGTAAGTATCCAGAACCAAATTCCTTCG	33	36.36	
				RPA_MH2_F9	TATGTAAGTATCCAGAACCAAATTCCTTCGCTG	33	39.39	
				RPA_MH2_F10	GTAAGTATCCAGAACCAAATTCCTTCGCTGTTT	33	39.39	
				RPA_MH2_R1	ACTTCATCTGGGCTAGCTAAAATTCACGGGCA	33	45.45	
				RPA_MH2_R2	TGAACTTCATCTGGGCTAGCTAAAATTTACGG	33	42.42	
				RPA_MH2_R3	GGCTGAACTTCATCTGGGCTAGCTAAAATTTCA	33	42.42	
				RPA_MH2_R4	ACTGGCTGAACTTCATCTGGGCTAGCTAAAATT	33	42.42	
				RPA_MH2_R5	ATAACTGGCTGAACTTCATCTGGGCTAGCTAAA	33	42.42	
				RPA_MH2_R6	TTAATAACTGGCTGAACTTCATCTGGGCTAGCT	33	42.42	
				RPA_MH2_R7	ATGTTAATAACTGGCTGAACTTCATCTGGGCTA	33	39.39	
				RPA_MH2_R8	AAAATGTTAATAACTGGCTGAACTTCATCTGGG	33	36.36	
				RPA_MH2_R9	CTTAAATGTTAATAACTGGCTGAACTTCATCT	33	30.30	
				RPA_MH2_R10	AATCTTAAATGTTAATAACTGGCTGAACTTCA	33	27.27	
				RPA_MH2_prb	TCATCTGGGCTAGCTAAAATTTACGGGCAC TTT TGACT AAGATCTGCTTTTA-C3 Spacer	52	40.38	
	PM	kmt1		RPA_PM1_F	TTGCCGCGAAATTGAGTTTTATGCCACTTG	30	43.33	(Zhao et al., 2019)
				RPA_PM1_R	AATAACGTCCAATCAGTTGCGCCGTTGTCA	30	46.67	
				RPA_PM1_prb	GGCTCGTTGTGAGTGGGCTTGTCTCG TACT CTTTTTATTGGCTTG-C3 Spacer	44	50.00	
				RPA_PM2_F1	CACGACGTGCAGAACCGGTGTTAAAGTCACCATAGG	36	52.78	This Thesis
				RPA_PM2_F2	CCGCACGACGTGCAGAACCGGTGTTAAAGTCACCAT	36	55.56	
				RPA_PM2_F3	TTGCCGCACGACGTGCAGAACCGGTGTTAAAGTCAC	36	55.56	
				RPA_PM2_F4	CCATTGCCGCACGACGTGCAGAACCGGTGTTAAAGT	36	55.56	

			RPA_PM2_F5	CGACCATTGCCGCACGACGTGCAGAACCGGTGTTAA	36	58.33	
			RPA_PM2_F6	CATCGACCATTGCCGCACGACGTGCAGAACCGGTGT	36	61.11	
			RPA_PM2_F7	GGGTACCATCGACCATTGCCGCACGACGTGCAGAAC	36	61.11	
			RPA_PM2_F8	CCCCAAGATGGGTACCATCGACCATTGCCGCACGAC	36	61.11	
			RPA_PM2_F9	TCATCACCCCAAGATGGGTACCATCGACCATTGCCG	36	55.56	
			RPA_PM2_F10	CAATCATCACCCCAAGATGGGTACCATCGACCATTG	36	50.00	
			RPA_PM2_F11	AAGCAATCATCACCCCAAGATGGGTACCATCGACCA	36	50.00	
			RPA_PM2_F12	GGGAAGCAATCATCACCCCAAGATGGGTACCATCGA	36	52.78	
			RPA_PM2_R1	CTAACCGCCTGAAAGCATTGGGTTAGGCGAAAATG	36	47.22	
			RPA_PM2_R2	ATCCTAACCGCCTGAAAGCATTGGGTTAGGCGAAA	36	47.22	
			RPA_PM2_R3	GTTTGATTTCATCATCCTAACCGCCTGAAAGCATTG	36	41.67	
			RPA_PM2_R4	AAAGTTTGATTTCATCATCCTAACCGCCTGAAAGCAT	36	38.89	
			RPA_PM2_R5	TAAAAAGTTTGATTTCATCATCCTAACCGCCTGAAAG	36	36.11	
			RPA_PM2_R6	CTGTAAAAAGTTTGATTTCATCATCCTAACCGCCTGA	36	38.89	
			RPA_PM2_R7	TCCCTGTAAAAAGTTTGATTTCATCATCCTAACCGCC	36	41.67	
			RPA_PM2_R8	CCTTCCCTGTAAAAAGTTTGATTTCATCATCCTAACCC	36	38.89	
			RPA_PM2_prb	GCAGAACCGGTGTTAAAGTCACCATAGGTA TGAT CCCACTGCAAAGTTT-C3 Spacer	49	44.90	
	SS	recN	RPA_SS_recN_F1	AATAGTCCAAAACATCATCGACAGTTCCACCATAT	35	37.14	
			RPA_SS_recN_F2	TAAAATAGTCCAAAACATCATCGACAGTTCCACCA	35	37.14	
			RPA_SS_recN_F3	TACTAAAATAGTCCAAAACATCATCGACAGTTCCA	35	34.29	
			RPA_SS_recN_F4	TTTTACTAAAATAGTCCAAAACATCATCGACAGTT	35	28.57	
			RPA_SS_recN_F5	TGATTTTACTAAAATAGTCCAAAACATCATCGACA	35	28.57	
			RPA_SS_recN_F6	CGCTGATTTTACTAAAATAGTCCAAAACATCATCG	35	34.29	
			RPA_SS_recN_F7	CTTCGCTGATTTTACTAAAATAGTCCAAAACATCA	35	31.43	
			RPA_SS_recN_F8	ATTCTTCGCTGATTTTACTAAAATAGTCCAAAACA	35	28.57	
			RPA_SS_recN_F9	TGTATTCTTCGCTGATTTTACTAAAATAGTCCAAA	35	28.57	
			RPA_SS_recN_F10	GATTGTATTCTTCGCTGATTTTACTAAAATAGTCC	35	31.43	
			RPA_SS_recN_R1	ACGGCAATCGTCTCATGCAATTGGAAAGTCGTCTG	35	48.57	
			RPA_SS_recN_R2	TTGACGGCAATCGTCTCATGCAATTGGAAAGTCGT	35	45.71	
			RPA_SS_recN_R3	ATTTTGACGGCAATCGTCTCATGCAATTGGAAAGT	35	40.00	
			RPA_SS_recN_R4	TAGATTTTGACGGCAATCGTCTCATGCAATTGGAA	35	40.00	
			RPA_SS_recN_R5	ATCTAGATTTTGACGGCAATCGTCTCATGCAATTG	35	40.00	
			RPA_SS_recN_R6	ATAATCTAGATTTTGACGGCAATCGTCTCATGCAA	35	37.14	
			RPA_SS_recN_R7	TCGATAATCTAGATTTTGACGGCAATCGTCTCATG	35	40.00	
			RPA_SS_recN_R8	TGGTCGATAATCTAGATTTTGACGGCAATCGTCTC	35	42.86	
			RPA_SS_recN_R9	ATGTGGTCGATAATCTAGATTTTGACGGCAATCGT	35	40.00	
			RPA_SS_recN_R10	GCGATGTGGTCGATAATCTAGATTTTGACGGCAAT	35	42.86	
			RPA_SS_recN_prb	CATCATCGACAGTTCCACCATATTTCTTCG TAA TGGTATTGAGCAAATC-C3 Spacer	49	38.78	
Underlined and bold bases within probe (prb) sequences represent where the fluorophore (FAM), a basic site (THF residue) and quencher (BHQ-1) were situated, respectively.							
Amplification chemistry	Target pathogen	Target gene	Primer ID	Sequence	Length (bp)	GC%	Reference
HDA	APP	apxIVA	HDA_APP_apxIVA_F1	ACGTCCCGGGTTATTTGGCGCATTCA	26	53.85	This Thesis
			HDA_APP_apxIVA_R1	TCACCGAAATAATGCCTCCGACCTGA	26	50.00	

		HDA_APP_apxIVA_F2	CGACACGGTTTCAAGCCCATCTCCATCT	28	53.57
		HDA_APP_apxIVA_R2	CGATGTGAATGCGCCAAATAACCCGG	26	53.85
		HDA_APP_apxIVA_F3	AAGCAGCCAACTCCTCAGAAAGTGCG	26	53.85
		HDA_APP_apxIVA_R3	ACGTCGCACAATTAATCTAACCCGGCACC	28	50.00
		HDA_APP_apxIVA_F4	TCAGGTCGGAGGCATTATTTCGGTGA	26	50.00
		HDA_APP_apxIVA_R4	GGAGCAGGAAATAGCAAAGATTGGGGCGG	29	55.17
		HDA_APP_apxIVA_F5	TGTCCGCACCGTAGCCACCTCTTAGA	26	57.69
		HDA_APP_apxIVA_R5	GGCGGTAATGGCGATGACACCCTCA	25	60.00
		HDA_APP_apxIVA_F6	AAGGTGTCGATATCTCTTGCGCGGTT	26	50.00
		HDA_APP_apxIVA_R6	TCTAAGAGGTGGCTACGGTGCGGACA	26	57.69
	dbsE-like	HDA_APP_DbsE_F1	CCGACCGAAGATTGGCGAGATAAAC	26	53.85
		HDA_APP_DbsE_R1	AAGGCTCTTTCGGCAAACCTGTTATTA	26	38.46
		HDA_APP_DbsE_F2	TGAAAGTTTCGTCAGCACCTTCCAGT	26	46.15
		HDA_APP_DbsE_R2	AACGCTTTTAAACGAGGGAGAAAATC	26	38.46
		HDA_APP_DbsE_F3	AATAACAGTTTGCCGAAAGAGCCTTA	26	38.46
		HDA_APP_DbsE_R3	GGCACGCCTTGTTCTTTCAATTTGAGT	27	44.44
		HDA_APP_DbsE_F4	AGATTGGCGAGATAAACCTTTTCCGGA	27	44.44
		HDA_APP_DbsE_R4	ATATAAGGCTCTTTCGGCAAACCTGTT	26	38.46
		HDA_APP_DbsE_F5	AGTTTCGTCAGCACCTTCCAGTTATT	26	42.31
		HDA_APP_DbsE_R5	TCTTGTAACGCTTTTAAACGAGGGAGA	27	40.74
	apfB	HDA_APP_apfB_F1	TTCGTTATTACTTGTCTGGCGCAAC	26	46.15
		HDA_APP_apfB_R1	AAACGCCGATTCTGCCTCTGTAGCCG	26	57.69
		HDA_APP_apfB_F2	CGACGCATTTTGATTTTACTACGTTGGGC	29	44.83
		HDA_APP_apfB_R2	GCCGCAATGCCCGCAACAACCTTTTCC	26	57.69
		HDA_APP_apfB_F3	GGCAGAATCGGCGTTTATCAACTGTT	26	46.15
		HDA_APP_apfB_R3	CCCGTCTTGAATTTCTGTTGCGCACT	26	50.00
		HDA_APP_apfB_F4	GCAAGACGGGCAATTCGGTTTTAAGA	26	46.15
		HDA_APP_apfB_R4	TCGCCCCAATAAGTCGGTAAACTCGA	26	50.00
		HDA_APP_apfB_F5	GCATATTTTGACCGCCGAAGATCCGA	26	50.00
		HDA_APP_apfB_R5	GCCGGGTTTATTTGCGTTTGGATTATGCC	29	48.28
	metP	HDA_APP_Metp_F1	AGCAACAAGTTAGAGCCGGTAGAGGT	26	50.00
		HDA_APP_Metp_R1	ACTGTTTACCACCTTCCCCGCCACCG	26	57.69
		HDA_APP_Metp_F2	GGAATCCGAACGCCAAGAAGAGCAGG	26	57.69
		HDA_APP_Metp_R2	AACTTGTTGCTTTTCTTGCTCGGTGCG	26	46.15
		HDA_APP_Metp_F3	CGACCGAGCAAGAAAAGCAACAAGTT	26	46.15
		HDA_APP_Metp_R3	ACCGAAACTGTTTACCACTTTCCCCGCC	28	53.57
		HDA_APP_Metp_F4	AAATTGAAAGAGCAGCTGGATTCCGC	26	46.15
		HDA_APP_Metp_R4	AGCACTTTCCGACATCAAGCGTTCCA	26	50.00
		HDA_APP_Metp_F5	CCGAACGCCAAGAAGAGCAGGAAATT	26	50.00
		HDA_APP_Metp_R5	GCGAACCTAAACCTCTACCGGCTCTA	26	53.85
MH	mhp165	HDA_MH_mhp165_F1	TGCCTTGGTATGACTGGATCTCTGT	26	46.15
		HDA_MH_mhp165_R1	TGCAAAGGGTCAAAATCTTTCTGCCT	26	42.31

			HDA_MH_mhp165_F2	AAGACGGTCGTTGAATTTTCCATTGT	26	38.46
			HDA_MH_mhp165_R2	ACAAGAGATCCAGTCATACCAAGGCA	26	46.15
			HDA_MH_mhp165_F3	TGCCTTGGTATGACTGGATCTCTTGTCTCC	30	50.00
			HDA_MH_mhp165_R3	TGCAAAGGGTCAAAATCTTTCTGCCTATG	29	41.38
			HDA_MH_mhp165_F4	AGTTAAGACGGTCGTTGAATTTTCCA	26	38.46
			HDA_MH_mhp165_R4	AGAGATCCAGTCATACCAAGGCAGAA	26	46.15
			HDA_MH_mhp165_F5	TTCTGCCTTGGTATGACTGGATCTCT	26	46.15
			HDA_MH_mhp165_R5	AGGGTCAAAATCTTTCTGCCTATGAAT	27	37.04
	PM	kmt1	HDA_PM_kmt1_F1	CGCCGTTGTCAAGGAAGCAGATTGGC	26	57.69
			HDA_PM_kmt1_R1	CGTTGTGAGTGGGCTTGTCCGTAGTCTT	28	53.57
			HDA_PM_kmt1_F2	GGCTCAACACACCAAACCTGCCCCAA	26	53.85
			HDA_PM_kmt1_R2	CGTTGTGAGTGGGCTTGTCCGTAGTCTT	28	53.57
			HDA_PM_kmt1_F3	ACTACCGACAAGCCCACTCACAACGA	26	53.85
			HDA_PM_kmt1_R3	TGCCGCGAAATTGAGTTTATGCCAC	26	46.15
			HDA_PM_kmt1_F4	TCAGACTGACAAGGAAATATAAACCGGCA	29	41.38
			HDA_PM_kmt1_R4	TGCTTCCTTGACAACGGCGCAACTGA	26	53.85
			HDA_PM_kmt1_F5	CAATCAGTTGCGCCGTTGTCAAGGAA	26	50.00
			HDA_PM_kmt1_R5	TGAGTGGGCTTGTCCGTAGTCTTTTA	26	46.15
	SS	recN	HDA_SS_recN_F1	TCCAAAACATCATCGACAGTTCACCA	27	44.44
			HDA_SS_recN_R1	CGTCTCATGCAATTGGAAAGTCGTCTGG	28	50.00
			HDA_SS_recN_F2	AGTCCAAAACATCATCGACAGTTCCA	26	42.31
			HDA_SS_recN_R2	TCGTCTCATGCAATTGGAAAGTCGTC	26	46.15
			HDA_SS_recN_F3	AGCTGAACTTCCAAATCATCTCCAGA	26	42.31
			HDA_SS_recN_R3	TGGAAGTGTGATGATGTTTGGACT	26	42.31
			HDA_SS_recN_F4	TCGACAGTTCCACCATATTCTTCGT	26	42.31
			HDA_SS_recN_R4	CGTCTCATGCAATTGGAAAGTCGTCTGGATT	31	45.16
			HDA_SS_recN_F5	ACATCATCGACAGTTCACCATATTT	26	38.46
			HDA_SS_recN_R5	TGCAATTGGAAAGTCGTCTGGATTGCT	27	44.44

Amplification chemistry	Target pathogen	Target gene	Primer ID	Sequence	Length (bp)	GC%	Reference
LAMP	APP	<i>dfsE</i> -like	APP_F3_A	CTGAAAGTTTCGTCAGCAC	19	47.37	(Ji et al., 2012)
			APP_F3.2_A	TTTCTAATTCAGACGCTGAAAG	22	36.36	This Thesis
			APP_B3_A	TCTGAAACTGAAAAGTATCCAC	22	36.36	(Ji et al., 2012)
			APP_B3.2_A	ACTGAAGACAAGCGGTAAAA	20	40.00	This Thesis
			APP_LF_A	TTTTTGTTGGTAACGTAC	18	33.33	
			APP_LB_A	GACGAACTATTGCAAAAAATCGCG	25	40.00	
			APP_LB.2_A	ACTATTGCAAAAAATCGC	18	33.33	
			APP_FIP_A	GCTCGAAATCCGGGTTATATCCT-ATTTAATTGATAAACACGGTGTG	50	36.00	(Ji et al., 2012)
			APP_BIP_A	TCGTTTAAAAGCGTTACAAGAGGA-AACTGAAGACAAGCGGTGA	44	38.64	This Thesis
			APP_BIP.2_A	TCGTTTAAAAGCGTTACAAGAGGA-ACTGAAGACAAGCGGTAAAA	45	37.78	
		<i>apxIVA</i>	APP_F3_B	TTGATGCCGGTGCGGGTA	18	61.11	(Yang et al., 2009)
			APP_B3_B	CCAAAGTTCGGATAAAT	17	35.29	
			APP_LF_B	TTTGCCGCCGATGAGGGT	18	61.11	
			APP_LB_B	CGTTTATGAAGATACCAA	18	33.33	
			APP_FIP_B	CGTAGCCACCTCTTAGAATATCATTTTTTGGTTAATGGCGGTAATGG	47	40.43	
			APP_BIP_B	ATCTTTAGCAAAGGACACGGACAGTTTTAGGTGTCGATATCTCTTGC	47	42.55	
	MH	<i>p36</i>	MH_F3_A	AGTATTATTGTTGCTAATCCTGT	23	30.43	(Liu et al., 2015a)
			MH_B3_A	GTTCACCCATCACGTAGG	18	55.56	
			MH_A_LF	TTGCAATCGCAAAAAGAGCA	20	40.00	
			MH_A_LB	AAATCCAGAAGCATCCC	17	47.06	This Thesis
			MH_FIP_A	CAC TACCGATAACTTTTTGATCGGA-TGATATAATTACAAGGGCTTACC	49	36.73	(Liu et al., 2015a)
			MH_BIP_A	GAAGTGTTTTAGATACAGCAAGGCTT-GAACCGAATTAGGCGATAC	46	41.30	
		<i>mhp165</i>	MH_F3_B	TGTTGAGATCGCATCAAGAA	20	40.00	(Li et al., 2013)
			MH_F3.2_B	TGAATCTGGATCGGAAATATC	21	38.10	
			MH_B3_B	CCTACCAACAAAACAAGGTAGT	23	39.13	
			MH_LF_B	CCAAACACTAAATTATGCC	19	36.84	
			MH_LB_B	GGAGGCTTTTAAAAGGG	17	47.06	
			MH_FIP_B	GGCGCTCATGAATATGGTCATCTTTTTCTGGATCGGAAATATCCTG	46	43.48	
			MH_FIP.2_B	GGCGCTCATGAATATGGTCATC-TGAATCTGGATCGGAAATATC	44	43.18	
			MH_BIP_B	TTCCCGTAGTTTTAAAAAATGGCGTTTTCCAATCATCCGAATTCCTT	48	37.50	
	PM	<i>kmt1</i>	PM_F3_A	GGTGCTATCTTGCTTCG	18	55.56	(Bhimani et al., 2015)
			PM_B3_A	TCGATCGCTAGCACCACA	18	55.56	
			PM_LF_A	CGACCATCGGTTGCATTTTC	19	52.63	
			PM_LB_A	TGGCATTGCATGGCTATCA	19	47.37	
			PM_LB.2_A	AGGACTTTTGGCATGTCATG	20	45.00	This Thesis
			PM_FIP_A	TGCCGTAGCAGAACTGGACTTTTAGGTGAGCCATGTGAGCT	42	50.00	(Bhimani et al., 2015)
			PM_BIP_A	CACACCGAAGCCAGGACTTTTTTACCAGAAATGTACTGGTTGCTTC	45	46.67	
			PM_F3_B	GTGGTGCGAATGAACCTA	18	50.00	(Sun et al., 2010)
			PM_F3-G_B	GCGGTGCGAATGAACCGA	18	61.11	This Thesis
			PM_B4_B	AACGTCCAATCAGTTGCG	18	50.00	(Sun et al., 2010)
			PM_LF_B	AATGCCATTTCCCATTTCAAGTG	23	39.13	This Thesis
			PM_LB_B	GTGGCAAAGAAAAGCACAGTTT	22	40.91	
			PM_FIP_B	GCCCACTCACACAAGCCATAtttCCGCTAAATTGAGTTTTATGC	46	41.30	(Sun et al., 2010)
			PM_BIP_B	TTGTCGGTAGTCTTTTATTGGCTttttGGATTGGCTCAACACACC	47	40.43	
			PM_F3_C	CCAAGTCATTAACAGAGAGGT	21	42.86	This Thesis
			PM_B3_C	CAAAACCGCACTTTTTCG	18	44.44	
			PM_LF_C	TGTCTTACCAGTGATCAATCACT	23	43.48	

			PM_LB_C	CAAATCCCGGACCGATTTCG	20	55.00	
			PM_FIP_C	ATGTTAGCCGTTATTATTCCACAGTTTTTATATAGCCTGTTTTTAAATAGCACC	54	31.48	
			PM_BIP_C	TCCACCAAATTGTGGCAGTCCTTTTTTATAGGTTATTCCATTTTGTTC	49	36.73	
SS	recN	SS_F3_A	TGTCGATGATGTTTGGACTA	21	38.10	(Arai et al., 2015)	
		SS_B3_A	GCTTCTCCATATACAAGTCTTG	23	39.13		
		SS_LF_A	AGATAAATCATTGCCT	16	31.25	This Thesis	
		SS_LB_A	GGTCAGCTCAGCCAATCACGC	21	61.90		
		SS_FIP_A	AAGCTGAACTTCCAATCATCTCC-CAGCGAAGAATACAATCTATTGAC	49	38.78	(Arai et al., 2015)	
		SS_BIP_A	TAGAGAAAGAATTGGTTGAACGAGC-GCGGATAATATCTTCTAAAACAAC	50	36.00		
	gdh	SS_F3_B	GTACTGTGTGGCTGAAGG	18	55.56	(Intorasoot et al., 2016)	
		SS_B3_B	ATGTTGGCCATGATGTCT	18	44.44		
		SS_LF_B	GCCATTTTCCTTGTAGACTTTGAT	24	37.50	This Thesis	
		SS_LB_B	CTGCCCTTGAAATGAGTCAAAAC	23	43.48		
		SS_FIP_B	TTGCGAGTCCGTAGAGAACGCCAT-CTGACCTTGATGCC	39	53.85		
		SS_BIP_B	CGCTGGTGGTGTAGCTGTAT-TTCACGAGTCCATGACAAG	40	50.00	(Intorasoot et al., 2016)	
	9588	SS_9588_F3_1	CAAGATTGCGCGTCATATCTATG	23	43.48	This Thesis	
		SS_9588_B3_1	CTGACTCCACGAATCATTTTCCAA	24	41.67		
		SS_9588_FIP_1	ACCGAATAAACACGGTTCTTCTTTACTTTTACCAGATAAAAGCAGA	51	31.37		
		SS_9588_BIP_1	GATGGGGTCAACGAGATTCTCAATTTTGGTGAAATCCCTGTATCCAA	47	42.55		
		SS_9588_F3_2	CAAGATTGCTCGGCATGTCTATG	23	47.83		
		SS_9588_B3_2	CTGGCTCCAGGAATCATTTTCCAA	24	45.83		
		SS_9588_FIP_2	ACAGAATAAATCCTATTCTTTCTAATTTTCCTTTTACCAGATTAAGGCGGA	51	31.37		
		SS_9588_BIP_2	GACGGGGTCAATGATATTCTCAATTTTGGTGAAATCCCGTCTCCAA	47	44.68		
	588	SS0588_F3_A	TGGCCTAAGAAACGTCATG	19	47.37		
		SS0588_B3_A	TTCCAAACCATCCGCATG	18	50.00		
		SS0588_LF_A	GGATTTCCCTTGAGGTATCCAAGAA	25	40.00		
		SS0588_LB_A	GGCTAGTTTTTCTCAATATGGCATG	25	40.00		
		SS0588_FIP_A	AGTGGATTCTCCCTTCTCAGGTATTTGCGGTTTGACCTACAAGT	46	43.48		
		SS0588_BIP_A	AACAGATAATCGTGGTCTGTTTGATTTTATCTAGCCAAACCTGCTT	46	36.96		
	588	SS_0588_B_F3	TGGCCTAAGAAACGYCATG	19	47.37		
		SS_0588_B_B3	TCTCYTCATACTCKGTCAT	19	36.84		
		SS_0588_B_B3.2	GTCATAACRTTGCCTTCYAA	20	35.00		
		SS_0588_B_FIP	AAGTGGATTCTCCCTTYTCAGGTTTTCGTCGTTTGACCTAYAAG	45	40.00		
		SS_0588_B_BIP	ACGGATAATCGTGGTYTGTTTGATTTTAGGTCYAACCAGACYTG	44	38.64		
		SS_0588_B_F3	TGGCCTAAGAAACGYCATG	19	47.37		
		SS_0588_B_B3	TCTCYTCATACTCKGTCAT	19	36.84		
		SS_0588_B_B3.2	GTCATAACRTTGCCTTCYAA	20	35.00		
		SS_0588_B_FIP	AAGTGGATTCTCCCTTYTCAGGTTTTCGTCGTTTGACCTAYAAG	45	40.00		
		SS_0588_B_BIP	ACGGATAATCGTGGTYTGTTTGATTTTAGGTCYAACCAGACYTG	44	38.64		
	9588	SS_d9588.1_F3_A	ATGATTTCGTGGAGTCAGG	18	50.00		
		SS_d9588.1_B3_A	TATCTTCTGCCCGTTGCA	18	50.00		
		SS_d9588.1_FIP_A	GCAATTTCCAATTGGTATTTACCTTTTTTTATTTACGTGGAGCTTTTTTAGC	53	32.08		
		SS_d9588.1_BIP_A	ATAGTGATCATGCCTATGACTTGGCTTTTTCGACCGCTCTATAACCTT	48	41.67		

			SS_d9588.1_F3_B	GGTCTGTAAAGGATCCTGAA	21	42.86	
			SS_d9588.1_B3_B	CAATCACCAAAAGGAAGTCC	20	45.00	
			SS_d9588.1_FIP_B	CTGCATCAAATTGGCCAAGTCATAGTTTTAAGGGTAAATACCAATTGGAAAT	52	34.62	
			SS_d9588.1_BIP_B	GGATGCAAAGGTTATAGAGCGGTTTTATATCTTCTGCCCGTTGC	44	45.45	
			SS_d9588.1_F3_C	GCTTTTTTAGCAAGTGGTTCT	21	38.10	
			SS_d9588.1_B3_C	CATAATATCTTCTGCCCGTTG	21	42.86	
			SS_d9588.1_FIP_C	AGGCATGATCACTATAGACAGAGGTTTTGTAAAGGATCCTGAAAAGGGT	49	40.82	
			SS_d9588.1_BIP_C	ATGACTTGGCCAATTTGATGCATTTTCAAGTAGGTAATGGTTCCCT	46	39.13	
			SS_d9588.1_F3_D	CAAATTTAAAGAAGAACCGTGTT	23	30.43	
			SS_d9588.1_B3_D	ATTTCCAATTGGTATTTACCCCT	22	31.82	
			SS_d9588.1_FIP_D	AACCAAAAAAAGTGCAGCCAGATGTTTTGTTTTGTGGAAGATGGGGT	49	38.78	
			SS_d9588.1_BIP_D	GGATTTCACCAAGAGTTTTGGAAAATTTCTTTACAGAACCACTTGCTAA	50	34.00	
			SS_d9588.1_F3_E	CGGTTTTGTGGAAGATGG	19	47.37	
			SS_d9588.1_B3_E	GGTATTTACCCCTTTTCAGGATC	22	40.91	
			SS_d9588.1_FIP_E	GAAATCCCTGTATCCAAACCAAAATTTTGTCAATGAGATTCTCAATGATCT	52	32.69	
			SS_d9588.1_BIP_E	ACCAAGAGTTTTGGAAAATGATTCGTTTTCTTTACAGAACCACTTGCTAA	50	34.00	
			SS_d9588.2_F3_A	TCAACGACCTCTATCTGGCA	20	50.00	
			SS_d9588.2_B3_A	TTCTGCATCAAGTCCGCC	18	55.56	
			SS_d9588.2_FIP_A	CACGCAGATAGGCCTGGCTCTTTTGGCTTGGAGACGGGGATT	42	57.14	
			SS_d9588.2_BIP_A	CGGGCTCTGTCAAGGACCCCTTTTGCTGTAGACCGAGGCAATT	43	53.49	
			SS_d9588.2_F3_B	TTTTCGGCTTGGAGACGG	18	55.56	
			SS_d9588.2_B3_B	CAAGTCCGCCAAGTCAAGAG	20	55.00	
			SS_d9588.2_FIP_B	GCCAAAAAGGCACCACGCAGTTTTATTTACCGCTGGTATTGGA	44	47.73	
			SS_d9588.2_BIP_B	CGGGCTCTGTCAAGGACCCCTTTTGCTGTAGACCGAGGCAATT	43	53.49	
			SS_d9588.2_F3_C	TGGAAATTGCCTCGGTCTAC	20	50.00	
			SS_d9588.2_B3_C	GCGCAGGAGCTTGACATT	18	55.56	
			SS_d9588.2_FIP_C	ATCACCTTGGCATCCAGCAGAATTTTAGCGACCATGCTCTTGACT	45	46.67	
			SS_d9588.2_BIP_C	ACCTATTTGCAACGAGCCGAGTTTTTGCTCTTCTGCCCCAAT	44	50.00	
			SS_d9588.2_F3_D	CACCAACGGCAAAACCTTAG	20	50.00	
			SS_d9588.2_B3_D	GGCAATTTCCAGCTGGTACT	20	50.00	
			SS_d9588.2_FIP_D	CCGTCTCCAAGCCGAAAAAGCTTTTTTTCATATCGAGGACGGGGTC	46	50.00	
			SS_d9588.2_BIP_D	ATGATTCCCTGGAGCCAGGCCTTTTAGGGTCCTTGACAGAGCC	42	54.76	
	GPS	infB	GPS_F3_A	CTGAGAAATTCGGTGGTGATG	21	47.62	(Chen et al., 2010)
			GPS_B3_A	CCTTTATCGAGGTAAGATTCTG	21	42.86	
			GPS_FIP_A	ACTTAATTCTAATACTTCCGATTTTTACTTGAAGCCATTCTTCTT	46	28.26	
			GPS_BIP_A	GTATTAGAATTAAGTGCAGTGTTTTACCGCTTGCCATACCCTCTT	46	41.30	
			GPS_F3_B	CGATTGTGGTTGCGGTAAAC	20	50.00	(Zhang et al., 2012)
			GPS_B3_B	TAAGATTTCGATAACCACACCGC	22	45.45	
			GPS_FIP_B	GAACATCACCAACGAATTCTCAGAATTTTAAACCAGAAGCAAACCCAGAGC	52	42.31	
			GPS_BIP_B	ATGGGGATTGACGACTTACTTGAAGCTTTATACCCTCTTCACTGCACCTTAATTC	55	40.00	
			GPS_F3_C	CGAAAGCAACGGATATCGT	19	47.37	This Thesis
			GPS_B3_C	CAGAAATCACTTCGTGTTGT	20	40.00	
			GPS_FIP_C	TCGCGTGTGGATTGCTTCAA-CTGTAGTAGCAGCTGACG	41	48.78	

			GPS_BIP_C	GATTGTGGTTGCGGTAACAAAA-CTCTTGTTCTACACGCTCTA	44	40.91	
			GPS_F3_D	GCGGCATTACCTCAATG	18	50.00	
			GPS_B3_D	CAGAAATCACTTCGTGTTGT	20	40.00	
			GPS_FIP_D	ATTGCTTCAATGGTTTGTGGCATTAAACGGATATCGTTGTTCTTG	46	36.96	
			GPS_BIP_D	GATTGTGGTTGCGGTAACAAAA-CTCTTGTTCTACACGCTCTA	44	40.91	
			GPS_F3_E	ACTATCCTTGTTCAATCAGGTA	22	36.36	
			GPS_B3_E	TATAACGCCACTTCACGC	18	50.00	
			GPS_LF_E	CGGCCATATTCAAAGCCACAAAGTA	25	44.00	
			GPS_LB_E	CGCAGGTGATGAAGCGACAG	20	60.00	
			GPS_FIP_E	CATTTTCATCACGCATTGCACGA-CGTTAAACAAAGGCGACAT	43	41.86	
			GPS_BIP_E	TTCCTGTGGAAGTATTAGGACTTTC-TTTTCTCATCACGCACCA	44	40.91	
			GPS_F3_F	CTATCCTTGTTCAATCAGGTAC	22	40.91	
			GPS_B3_F	TATAACGCCACTTCACGC	18	50.00	
			GPS_LF_F	CACGGCCATATTCAAAGCCA	20	50.00	
			GPS_LB_F	GCAGGTGATGAAGCGACAG	19	57.89	
			GPS_FIP_F	CATTTTCATCACGCATTGCACG-AGGCGACATCGTACTTTG	41	46.34	
			GPS_BIP_F	TTCCTGTGGAAGTATTAGGACTTTC-TTTTCTCATCACGCACCA	44	40.91	
			GPS_F3_G	GAATATGGCCGTGTTCTGT	18	50.00	
			GPS_B3_G	ACATCACGGAATTTACCTTG	20	40.00	
			GPS_LF_G	GGCCTGCTGATTCAATGTCTTTA	23	43.48	
			GPS_LB_G	GTGCGTGATGAGAAAAAGCG	21	47.62	
			GPS_FIP_G	GTCCTAATACTTCCACAGGAATTGA-GCAATGCGTGATGAAAAATGG	46	41.30	
			GPS_BIP_G	CAGGTGATGAAGCGACAGTG-ACGGTATAACGCCACTTC	39	51.28	
			GPS_F3_H	ATTTCACTTCACTGACTAAAGAGA	23	30.43	
			GPS_B3_H	GGTACGCCTGAAAGTCCTA	19	52.63	
			GPS_LF_H	ATGGTAAAGACATTGAATCAGCAGG	25	40.00	
			GPS_FIP_H	AGTACGATGTCGCCTTTGTTTAA-AATAATGTACTCTGCAACTATCCTT	49	34.69	
			GPS_BIP_H	GTGTTCGTGCAATGCGTGAT-ACTTCCACAGGAATTGATGG	41	46.34	
			GPS_F3_I	AGTGGTGCGTGATGAGAA	18	50.00	
			GPS_B3_I	ATCGCTTCTACAGAGCCT	18	50.00	
			GPS_LB_I	CGATGTTGCAGAATTGAACGTG	22	45.45	
			GPS_FIP_I	TTCGCTTTTGTGTTGGCGAGC-GTGAAGTGGCGTTATACCG	40	50.00	
			GPS_BIP_I	ATATGTTTAGCAATATGGCGGAAGG-TGTACGTCTGCTTTCACAAAT	46	39.13	
PCV2	orf2		PCV2_F3_A	ATGGGCTGCTAATTTGCAGAC	22	45.45	(Chen et al., 2008)
			PCV2_B3_A	TCAATAGGAAATTCAGGGCATG	22	40.91	
			PCV2_FIP_A	GTACAGTTCCACCTTTAGTCTCTTTTGTTTACCATGGTGAAGAAGTGG	48	43.75	
			PCV2_BIP_A	CAACTGCTGTCCAGCTGTAGATTTTCTCCGTGGATTGTTCTGTAG	48	47.92	
	orf2		PCV2_F3_B	CTCCAGTGCTGTTATTCTAGATGA	24	41.67	(Zhou et al., 2011)
			PCV2_B3_B	TTTCCAGCAGTTTGTAGTCTCA	22	40.91	
			PCV2_FIP_B	GAAGGGCTGGGTTATGGTATGGCTTTT-ACAGCCCTCACCTATGACC	47	51.06	
			PCV2_BIP_B	CCGCTACTTTACCCCAAACCTTTT-GCCACAGCTGATTTCTTTTGTG	50	44.00	
	orf1		PCV2_F3_C	GGGAGTCTGGTGACCGTT	18	61.11	This Thesis
			PCV2_B3_C	CCATCCCACCACTGTTTCT	20	50.00	

			PCV2_LF_C	TTCAAAAGTTCAGCCAGCCC	20	50.00	This Thesis
			PCV2_LB_C	TGGGTGTGGTAAAAGCAAATGG	22	45.45	
			PCV2_FIP_C	TCACGCTTCTGCATTTTCCCGCTTTTGTAGCAGCACCCGTGTAACG	45	51.11	
			PCV2_BIP_C	CACGTCATTGTGGGGCCACCTTTTTTCCAGTATGTGGTTTCCGG	44	52.27	
			PCV2_F3_D	CAGCACCTCAGCAGCAAC	18	61.11	
			PCV2_B3_D	GGGTGTTTCGTCTCTCTCA	19	57.89	
			PCV2_B3.2_D	AAAATTAGCGAACCCTGGAG	21	47.62	
			PCV2_LF_D	GGTTGGGGTCCGCTTCT	17	64.71	
			PCV2_LF.2_D	CCCACCTTTTGTGTGGTTGG	20	55.00	
			PCV2_LB_D	AGAAAATACGGGAGCTTCCAATC	23	43.48	
			PCV2_FIP_D	CAGCGTGAACACCCACCTTTTG-TGCCCAGCAAAAAGAATGGA	43	48.84	
			PCV2_BIP_D	ATCCTTCCGAAGACGAGCGCA-TACCCTCTCGCCAACAA	40	55.00	
			PCV2_F3_E	AAAAGGTGGGTGTTACG	18	50.00	This Thesis
			PCV2_B3_E	CTTCTCGATGTGGCAGC	18	55.56	
			PCV2_LF_E	AAAAAGGGAGATTGGAAGCTCC	22	45.45	
			PCV2_LB_E	CGCTAATTTTGTGAAGAAGCAAAC	24	37.50	
			PCV2_FIP_E	CCTCTCGCCAACAATAAAATAATCTTTTGAATAATCCTTCCGAAGACG	50	38.00	
			PCV2_BIP_E	ACCTCCAGGGTTTCGCTAATTTTTCACCGAAATACCATTTCACCT	45	42.22	
	SIV	H3	SIV_F3_A	AATTGCTCCTCGGGGTTA	18	50.00	(Gu et al., 2010)
			SIV_B3_A	CCGAATATGCCTCTAGTTTG	20	45.00	
			SIV_LF_A	GCCAATGGGTGCATCTGAC	19	57.89	
			SIV_LB_A	GTCCCAGATATGTTAAGCAAAACAC	25	40.00	
			SIV_FIP_A	GCTTCCATTGGAGTGATGCATTCCGAAGTGGGAAAAGCTCAAT	44	45.45	
			SIV_BIP_A	GTAAACAGGATCACATATGGGGCGCATCCCTGTTGCCARTT	45	46.67	
		M protein	SIV_F3_B	TTTCTATCATCCCCTCAGGC	20	50.00	This Thesis
			SIV_B3_B	CCCATTCCCATTTAGGGCA	19	52.63	
			SIV_LF_B	GTTCTTTCCTGCAAAAACACTTTCC	25	40.00	
			SIV_LB_B	CCAGTGAGCGAGGACTGCA	19	63.16	
			SIV_FIP_B	GCCATTCCATGAGAGCCTCAAGTTTTCGAGATCGCGCAGAGACT	44	52.27	
			SIV_BIP_B	TTGTGTTTACGCTCACCGTGCTTTTTTTTGGACAAAGCGTCTACG	45	44.44	
		M protein	SIV_F3_C	TTCTAACCGAGGTCGAAAC	19	47.37	(Jung et al., 2015)
			SIV_B3_C	GGACAAAGCGTCTACGC	17	58.82	
			SIV_LF_C	TCTGYGCGATCTCGGCT	17	58.82	
			SIV_LB_C	CACCTCTGACTAAGGG	16	56.25	This Thesis
			SIV_FIP_C	TGTTCTTYCCTGCAAGACATTTTCTATCATCCCGTCAG	40	40.00	(Jung et al., 2015)
			SIV_BIP_C	CTAAAGACAAGACCAATCTTTTACTGGGCACGGTGAGCG	39	48.72	
		M protein	SIV_F3-1_D	GACTTGAAGATGTCTTTGC	19	42.11	(Nakauchi et al., 2011)
			SIV_F3-2_D	GACTGGAAAGTGTCTTTGC	19	47.37	
			SIV_B3-1_D	TGTTATTTGGATCCCCATT	19	36.84	
			SIV_B3-2_D	TGTTGTTCCGGTCCCCATT	19	52.63	
			SIV_LF_D	GTCTTGCTTTAGCCA	16	43.75	
			SIV_LB_D	CAGTGAGCGAGGACTG	16	62.50	
			SIV_FIP_D	TTAGTCAGAGGTGACAGGATTGTTTCAGATCTTGAGGCTCTC	43	44.19	

		SIV_BIP_D	TTGTGTTTCACGCTCACCGTGTTTTTTGGACAAAGCGTCTACG	42	47.62	
BVS-1	European	BVS_F3_A	TGCTGCAGGTCTCCATTCA	19	52.63	(Rovira et al., 2009)
		BVS_B3_A	CCCCATCGGAGCTGTACT	18	61.11	
		BVS_LF_A	TCACTGATGTTAGTCCGGGC	20	55.00	
		BVS_LB_A	AGGAGTGGTTAACCTCGTCAA	21	47.62	
		BVS_FIP_A	AGTCCTGGTACTAGAGTGCCGTTGGTAACCGAGCATACGCT	41	53.66	
		BVS_BIP_A	GGCGGCAACGAGCTGTTAAACTGGCTCTGGTTTTTACCGG	41	53.66	
	Type 1	BVS_F3_B	CAGTTGCTGGGTGCAATGA	19	52.63	This Thesis
		BVS_B3_B	CTGGATGAAAGCGACGCA	18	55.56	
		BVS_LF_B	GGCTTTTTCTTTTTGGCCTG	20	45.00	
		BVS_LB_B	GAACGCTCCCTCTGCT	16	62.50	
		BVS_FIP_B	GCCAGGGGAAAATGTGGCTTCTTTTTTCAGCAACCTAGGGGAGGA	44	52.27	
		BVS_BIP_B	TGAAGATGACATCCGGCACCACTTTTGCCTTGATTGAAAGCCGTCT	46	47.83	
	Type 1	BVS_F3_C	AAAGCCTGAGAAGCCACATT	20	45.00	
		BVS_B3_C	AGGCGCACTGTATGAGCA	18	55.56	
		BVS_LF_C	ACCGGCAGCATAACTCAAC	20	50.00	
		BVS_LB_C	TCCAGCGGAAGGTCAGTTTTT	22	54.55	
		BVS_FIP_C	CAAGCAGAGGGAGCGTTCAGTTTTTCTGGCTGCTGAAGATGAC	44	52.27	
		BVS_BIP_C	CAATCAAGGCGCAGGAACGTTTTACCGGCAGCATAACTCAAC	44	47.73	
BVS-2	NA	BVS_F3_D	GGGAGCAGTAGTTGCACTC	19	57.89	(Rovira et al., 2009)
		BVS_B3_D	GGACGACAAATGCGTGGTTA	20	50.00	
		BVS_LF_D	GATGAATTTCCAGGTTTCT	19	36.84	
		BVS_LB_D	CCTGCCACCACGTGA	17	64.71	
		BVS_FIP_D	GCACAAACGGCATCTGGAGGTTTGGGGGTGTACTCAGC	39	58.97	
		BVS_BIP_D	TAGCCGCAAGTACATTCTGGCCATTTGCCGCAATCGGATG	41	53.66	
	M protein	BVS_F3_E	GGGAGCAGTAGTTGCACTC	19	57.89	(Qin et al., 2009)
		BVS_B3_E	GGACGACAAATGCGTGGTTA	20	50.00	
		BVS_LF_E	ATGAATTTCCAGGTTTCTATG	21	33.33	
		BVS_LB_E	CACGTTGAAAGTGCCGAGG	20	60.00	
		BVS_FIP_E	GCACAAACGGCATCTGGAGGTTTGGGGGTGTACTCAGC	39	58.97	
		BVS_BIP_E	TAGCCGCAAGTACATTCTGGCTTTGCCGCAATCGGATGA	40	52.50	
	nsp2	BVS_F3_F	CAGATCCGATTGCGGCAG	18	61.11	(Zhang et al., 2011)
		BVS_B3_F	TTCTACGCGGTGCAGGAA	18	55.56	
		BVS_LF_F	CAGAACTTTCAGGCATTTGATAGC	24	41.67	
		BVS_LB_F	ATCCCCTTCTCAGCCCA	18	61.11	
		BVS_FIP_F	CATCGGCTCGGATGGTGTGATGGGCGACAATGTCCCTA	39	58.97	
		BVS_BIP_F	ACCCATGAGTGAGCCCGTACTCGACCCACTCAAAGGTTTCA	41	53.66	
	Type 2	BVS_F3_G	CAGAAGAGAAAGAAGGGG	19	52.63	This Thesis
		BVS_B3_G	CTGGATTGACGACAGACACA	20	50.00	
		BVS_LF_G	CCTCTAGCAGCTGAAGATGATGT	23	47.83	
		BVS_LB_G	GATGATCTTACCCAGCATCT	20	45.00	
		BVS_FIP_G	GCCTCTGGACTGGTTTTGCTGATTTTCCAGTCAATCAGCTGTGCC	45	51.11	
		BVS_BIP_G	AACCCGGAGAAGCCCCATTTTTTTCGCTCACTAGGGGTAAAGTG	45	48.89	

	Type 2	BVS_F3_H	GATGGCCAGCCAGTCAAT	18	55.56
		BVS_B3_H	CCTTGATTAAAGGCGGTCTG	20	50.00
		BVS_LF_H	CTGGTTTTGCTGAGCGATGAT	21	47.62
		BVS_LB_H	CCTCTAGCCACTGAAGATGATGTC	24	50.00
		BVS_FIP_H	CTTCCCGGTCCCTTGCCCTCTTTTGTGCCAGATGCTGGGTAAG	43	55.81
		BVS_BIP_H	AACCCGGAGAAGCCCCATTTTTTTAGACACAATTGCCGCTCACT	45	46.67
	Mitochondrial DNA D-loop	mtD_A_F3	AGGCCCTAACACAGTCAA	18	50.00
		mtD_A_B3	GTTATAGGGTGTGTAGAGCATA	22	40.91
		mtD_A_FIP	ACTGAATAGCACCTTGTTTGGAT-TTGTAGCTGGACTTCATGG	43	41.86
		mtD_A_BIP	CGGGACATAACGTGCGTACA-AGTTTAATGGGGGGTAAGG	40	50.00
		mtD_B_F3	CATATCATTATTGATCGTACATAGC	25	32.00
		mtD_B_B3	TCCTGAAGTAAGAACCAGATG	21	42.86
		mtD_B_FIP	GGCATGGTAATTAAGCTCGTGATCT-ACATATCATGTCAAATAACTCCAG	50	38.00
		mtD_B_BIP	TGGCAGGGATCCCTCTTCTC-CCTGTTAAAGTTCATCAATAGAAAC	46	43.48
		mtD_C_F3	AACATGCGTATCACCACC	18	50.00
		mtD_C_B3	GGCGATTTTAGATGAGATGG	20	45.00
		mtD_C_LF	CGGGTTGCTGGTTTCACG	18	61.11
		mtD_C_LB	TGGGGGTTTCTATTGATGAACCTTA	25	36.00
		mtD_C_FIP	GAAGAGGGATCCCTGCCAAG-ATTAGATCACGAGCTTAATTACCA	45	44.44
		mtD_C_BIP	CTCGGGGCCCATAAATCGTG-AAGAACCAGATGCCTGTT	39	51.28
		mtD_D_F3	CCATCTCATCTAAAATCGCC	20	45.00
		mtD_D_B3	GATCATGAGTTCCATGAAGTC	21	42.86
		mtD_D_FIP	TGTGTGAGCATGGGCTGATTA-CTCTTTCCCTTAAATAAGACAT	45	40.00
		mtD_D_BIP	GGATGCTTGGAATCAGCCAT-AGCTACAATTGATTTGACTGTG	43	44.19
		mtD_E_F3	CATCTGGTCTTACTTCAGGA	21	42.86
		mtD_E_B3	CCGGATCATGAGTTCCATG	19	52.63
		mtD_E_FIP	GCTGATTAGTCATTAGTCCATCGAG-ATCTCATCTAAAATCGCCCA	46	41.30
		mtD_E_BIP	GGATGCTTGGAATCAGCCAT-AGCTACAATTGATTTGACTGTG	43	44.19
	12S rRNA	rRNA12_A_F3	AGCTGTAAAAAGCCCTAGT	19	42.11
		rRNA12_A_B3	CGCCAAGTCCTTTGAGTT	18	50.00
		rRNA12_A_FIP	CAGTTTGGGTCCTAGCTATCGTG-AATAAAATAACCCACGAAAGTGA	47	40.43
		rRNA12_A_BIP	GATTAGATACCCCACTATGCCTAGC-AGTTGCGAGTAGTACTCTGG	46	47.83
		rRNA12_B_F3	CCACTATGCCTAGCCCTA	18	55.56
		rRNA12_B_B3	CGGTATATAGGCTGAATTGGC	21	47.62
		rRNA12_B_FIP	AAGTCCTTTGAGTTTTAGGCAGT-TAGTTACATAACAAAATATTCGCC	49	34.69
		rRNA12_B_BIP	CGGTGCTTCACATCCACCTA-GGTTGGTAAGGTCTATCGG	40	52.50
		rRNA12_C_F3	ATAAAATAACCCACGAAAGTGA	22	31.82
		rRNA12_C_B3	GGGGTTTATCGATTATAGAACAG	23	39.13
		rRNA12_C_LF	CCCAGTTTGGGTCCTAGCTA	20	55.00
		rRNA12_C_LB	CTCGCAACTGCCTAAAATCTAAA	23	43.48
		rRNA12_C_FIP	AGGCATAGTGGGGTATCTAATCC-CTCTAATAATCCTGACACACGA	46	43.48
		rRNA12_C_BIP	AACAAAATATTCGCCAGAGTACT-CCTCTAGTGGATGTGAAG	44	43.18
		rRNA12_D_F3	GTGAGAATGCCCTCCAGA	18	55.56

			rRNA12_D_B3	GGTTAATCGTATGACCGCG	19	52.63	
			rRNA12_D_FIP	AAGGCGTTATGAGCTACCGT-TCCTAAAGATCAAAGGAGCA	42	42.86	
			rRNA12_D_BIP	CACGGGAAACAGCAGTGATAAAA-GCACGAGATTACCAACTC	43	44.19	
			rRNA12_E_F3	CAAACTATTTCGCCAGAGTAC	21	42.86	
			rRNA12_E_B3	ATTGTGCTTACTATGTTCTCTT	22	31.82	
			rRNA12_E_FIP	CAGGCTCCTCTAGGTGGATG-TCGCAACTGCCTAAACTC	40	52.50	
			rRNA12_E_BIP	AATCGATAAACCCCGATAGACCT-TAGGGTTTGCTGAAGATGG	43	44.19	
		CytB	CytoB_A_F3	ATTCATTGACCTCCCAGC	18	50.00	
			CytoB_A_B3	GTTTGCATGTAGATAGCGAAT	21	38.10	
			CytoB_A_FIP	GTTAGGATTGCAAGATTAGGCAGA-CAAACATCTCATCATGATGAACT	50	36.00	
			CytoB_A_BIP	AGCAATACATTACACATCAGACACA-ATCCGTAATTTACGTCTCGA	46	36.96	
			CytoB_B_F3	GAGCTACGGTCATCACAA	18	50.00	
			CytoB_B_B3	TTGTTGGATCCGGTTTCG	18	50.00	
			CytoB_B_FIP	CGGAAAAGCCCCCTCAGATTC-CTATCAGCTATCCCTTATATCGG	45	48.89	
			CytoB_B_BIP	ACCCTCACACGATTCTTCGC-GGAATAGGAGATGTACGGC	40	52.50	
			CytoB_C_F3	ATCATTCTGAGGAGCTACG	19	47.37	
			CytoB_C_B3	TTGTTGGATCCGGTTTCG	18	50.00	
			CytoB_C_FIP	AAGCCCCCTCAGATTCATCTAC-GTCATCACAAATCTACTATCAGC	47	42.55	
			CytoB_C_BIP	CAAAGCAACCCTCACACGATTC-TAGGAGATGTACGGCTGC	41	51.22	
			CytoB_D_F3	ATTCATTGACCTCCCAGC	18	50.00	
			CytoB_D_B3	GTTTGCATGTAGATAGCGAAT	21	38.10	
			CytoB_D_LF	CAAGATTAGGCAGATGCCTAAGAG	24	45.83	
			CytoB_D_LB	ACAACAGCTTCTCATCAGTTACAC	25	40.00	
			CytoB_D_FIP	AAGAACAGGCCTGTTAGGATTTG-ATCATGATGAACTTCGGTTC	45	40.00	
			CytoB_D_BIP	AGCAATACATTACACATCAGACACA-ATCCGTAATTTACGTCTCGA	46	36.96	
			CytoB_E_F3	TCCCTCTTAGGCATCTGC	18	55.56	
			CytoB_E_B3	CCAATGTTTCATGTTCTAGGAAT	24	33.33	
			CytoB_E_FIP	ACTGATGAGAAAGCTGTTGTTGTG-TCCTAACAGGCCTGTTCTT	44	43.18	
			CytoB_E_BIP	TTCGCTATCTACATGCAAACGGA-CCGTAGTATAGACCTCGGC	43	48.84	
Amplification chemistry	Target pathogen	Target gene	Primer ID	Sequence	Length (bp)	GC%	Reference
PSR	APP	rpoD	PSR_APP_rpoD_F	ACGAATTCGTACATAGAAGTATAG-GTCGGAAGAATGGATTGAAAAAGC	48	37.50	(Sarkar et al., 2022)
			PRS_APP_rpoD_R	GATATGAAGATACATGCTTAAGCA-TCGATATTTACGCGAATTTGGTCTG	48	37.50	
	MH	mhp183	PSR_Mhp183_F	ACGAATTCGTACATAGAAGTATAG-CCAGAACCAAATTCCTTCGCTG	46	41.30	This Thesis
			PSR_Mhp183_R	GATATGAAGATACATGCTTAAGCA-CTGGCTGAACTTCATCTGGGGCTA	48	41.67	
	PM	toxA	PSR_PM_ToxA_F	ACGAATTCGTACATAGAAGTATAG-GAAATGGCTGGAAAAACCAAGTG	46	39.13	
			PSR_PM_ToxA_R	GATATGAAGATACATGCTTAAGCA-GAAAAGGCGCTGAAATTACTGTATC	49	36.73	

Supplementary Table 4. HDA primer3 conditions. Text file parameter input for the generation of HDA primer pairs using primer3 software.

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Primer3 File - http://primer3.sourceforge.net
P3_FILE_TYPE=settings

PRIMER_FIRST_BASE_INDEX=1
PRIMER_THERMODYNAMIC_OLIGO_ALIGNMENT=1
PRIMER_THERMODYNAMIC_TEMPLATE_ALIGNMENT=0
PRIMER_PICK_LEFT_PRIMER=1
PRIMER_PICK_INTERNAL_OLIGO=0
PRIMER_PICK_RIGHT_PRIMER=1
PRIMER_LIBERAL_BASE=1
PRIMER_LIB_AMBIGUITY_CODES_CONSENSUS=0
PRIMER_LOWERCASE_MASKING=0
PRIMER_PICK_ANYWAY=0
PRIMER_EXPLAIN_FLAG=1
PRIMER_MASK_TEMPLATE=0
PRIMER_TASK=generic
PRIMER_MASK_FAILURE_RATE=0.1
PRIMER_MASK_5P_DIRECTION=1
PRIMER_MASK_3P_DIRECTION=0
PRIMER_MIN_QUALITY=0
PRIMER_MIN_END_QUALITY=0
PRIMER_QUALITY_RANGE_MIN=0
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Supplementary Table 5. All clinical strains used in Chapter 3. Clinical samples characterised by their tissue, originating from England, Ireland, England and Wales or Portugal, tabulated with their disease association and qPCR result for each for the 5 pathogens. Blank cells indicate instances where the qPCR was not performed for that pathogen.

ID	Tissue	Location	Disease association	APP	MH	PM	SS	GPS
1	Lung	Portugal	Unknown	-	-	-	-	-
2	Lung	Portugal	Unknown	-	+	-	-	-
3	Lung	Portugal	Unknown	-	+	-	-	-
4	Lung	Portugal	Unknown	-	+	-	-	-
5	Lung	Portugal	Unknown	-	+	-	-	-
6	Lung	Portugal	Unknown	-	+	-	-	-
7	Lung	Portugal	Unknown	+	+	+	-	+
8	Lung	Portugal	Unknown	-	-	-	-	-
9	Lung	Portugal	Unknown	+	-	-	-	-
10	Lung	Portugal	Unknown	-	-	-	-	-
11	Lung	Portugal	Unknown	+	+	-	-	-
12	Lung	Portugal	Unknown	+	+	-	-	-
13	Lung	Portugal	Unknown	+	+	-	-	-
14	Lung	Portugal	Unknown	+	+	-	-	-
15	Lung	Portugal	Unknown	+	+	+	+	-
16	Lung	Portugal	Unknown	+	+	+	+	-
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21	Lung	Portugal	Unknown	+	+	-	-	-
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23	Lung	Portugal	Unknown	+	+	+	-	-
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31	Lung	Ireland	Unknown	-	+	+	-	+
32	Lung	England	Unknown	-	+	+	+	+
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117	Lung	England	Unknown	+	-	-	-	
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119	Liver	England and Wales	Unknown				-	+
120	Lung	England and Wales	Poly				+	+
121	Liver	England and Wales	Poly				+	+
122	Lung	England and Wales	Poly				+	+
123	Lung	England and Wales	Mucosal				+	+
124	Liver	England and Wales	Enteric				+	+

125	Lung	England and Wales	Respiratory & Poly				+	+
126	Lung	England and Wales	Enteric				+	+
127	Liver	England and Wales	Poly				+	+
128	Liver	England and Wales	trauma				+	+
129	Liver	England and Wales	Enteric & Respiratory				+	+
130	Lung	England and Wales	Enteric				-	+
131	Liver	England and Wales	Poly				-	+
132	Liver	England and Wales	Enteric				+	+
133	Liver	England and Wales	Mucosal				+	+
134	Lung	England and Wales	Enteric & Respiratory				-	+
135	Lung	England and Wales	Enteric & Respiratory				+	+
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137	Liver	England and Wales	Respiratory & Poly				+	+
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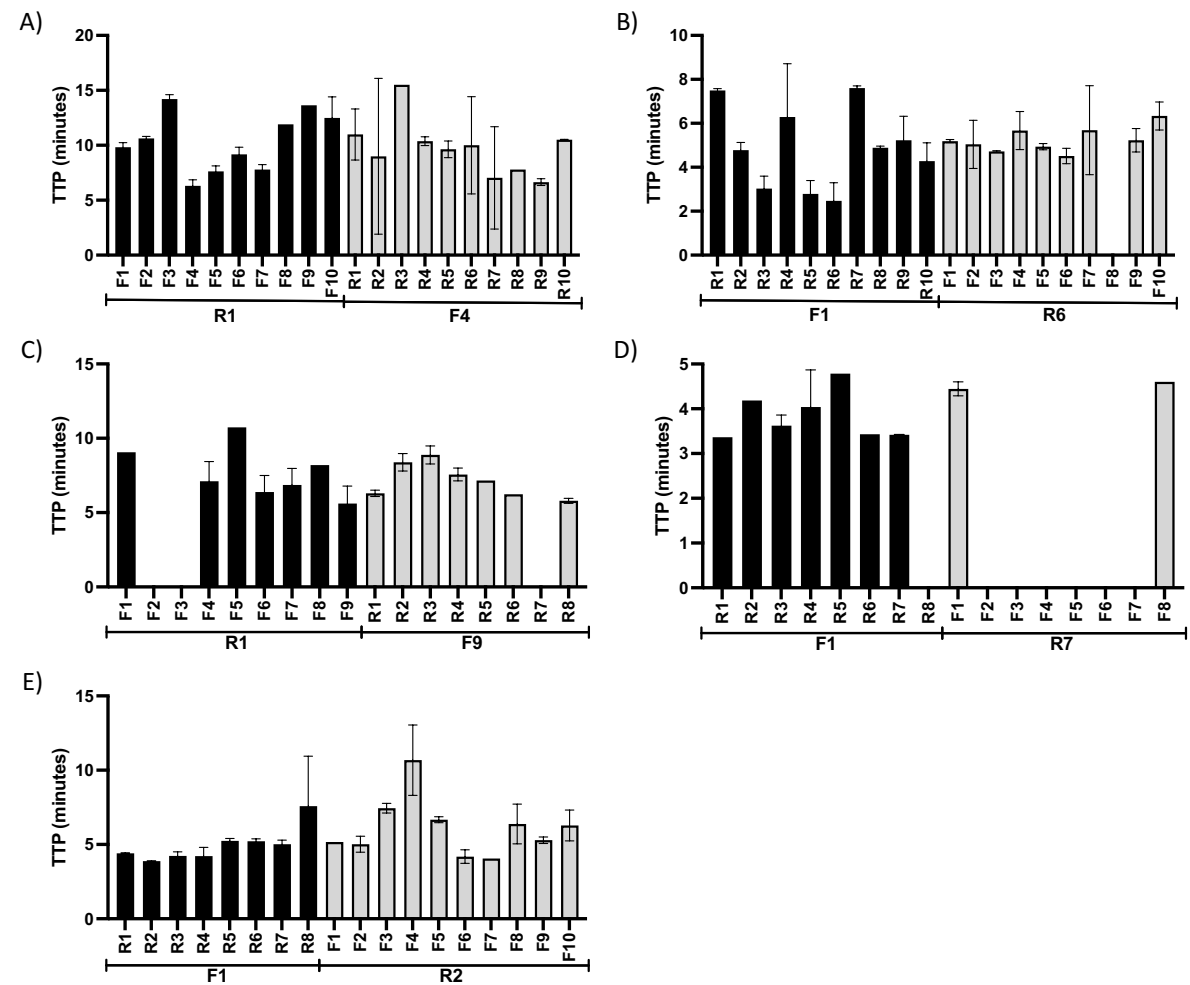
Supplementary Table 6. Python scripts used in this Thesis. Python scripts exported in LaTeX and uploaded to GitHub.

Purpose	URL	Input CSV
Correlation matrix	https://github.com/OliverStringer/Thesis2022/blob/main/qPCR_tissues.zip	coinfection
Venn diagram	https://github.com/OliverStringer/Thesis2022/blob/main/qPCR_tissues.zip	coinfection
Confusion matrices	https://github.com/OliverStringer/Thesis2022/blob/main/qPCR_tissues.zip	coinfection
Clinical tissue agreement	https://github.com/OliverStringer/Thesis2022/blob/main/Clinical_amplification.zip	results_SS

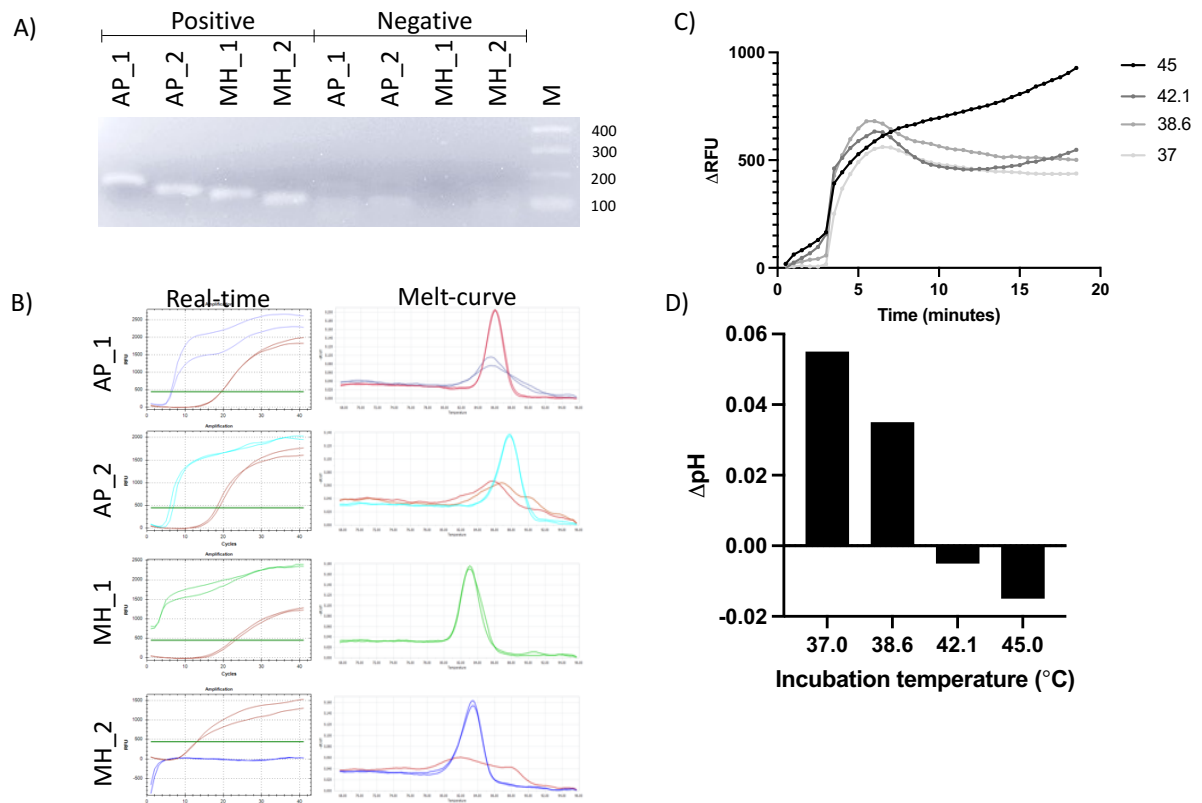
Supplementary Table 7. Amplification of Qiagen and SmartLid extractions. The C_t and delta C_t values were obtained when serial dilutions of bacterial isolates, APP, SS and PM were spiked into homogenised lung tissues and extracted with either Qiagen or SmartLid (optimised) protocols. Each C_t value represents a replicate in the qPCR with amplification with primers specific for APP, SS and PM. The Delta C_t represents the difference between the means, with a negative value is indicative that the mean SmartLid extraction resulted in a higher C_t value than the mean Qiagen extraction.

	OD ₆₀₀	Qiagen extraction		SmartLid extraction		ΔC_t
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	
APP	1	15.57	15.17	19.07	19.34	-3.835
	0.1	20.27	19.68	34.49	34.54	-14.54
	0.01	23.03	22.45	40.44	37.7	-16.33
	0.001	27.05	26.21	N.D.	N.D.	N.D.
	0.0005	27.56	27.16	N.D.	N.D.	N.D.
	0.0001	30.44	29.65	N.D.	N.D.	N.D.
	0.00005	32.14	32.1	N.D.	N.D.	N.D.
	0.00001	32.44	32.53	N.D.	N.D.	N.D.
SS	1	16.14	16.24	23.62	23.67	-7.455
	0.1	19.18	19.16	28.89	28.89	-9.72
	0.01	22.55	22.41	34	33.24	-11.14
	0.001	25.95	25.75	36.81	35.73	-10.42
	0.0005	27.45	27.32	37.03	36.25	-9.255
	0.0001	29.32	29.21	N/D.	N/D.	N/D.
	0.00005	30.44	30.14	N/D.	N/D.	N/D.
	0.00001	32.47	32.37	N/D.	N/D.	N/D.
PM	1	4.01	3.78	16.13	16.19	-12.265
	0.1	16.01	15.85	25.01	25.71	-9.43
	0.01	19.52	19.72	33.87	33.48	-14.055
	0.001	22.72	22.88	34.96	36.38	-12.87
	0.0005	23.5	23.5	35.45	35.49	-11.97
	0.0001	26.22	26.12	36.09	35.02	-9.385
	0.00005	27.4	27.28	34.91	36.16	-8.195
	0.00001	29.97	29.41	34.62	34.41	-4.825

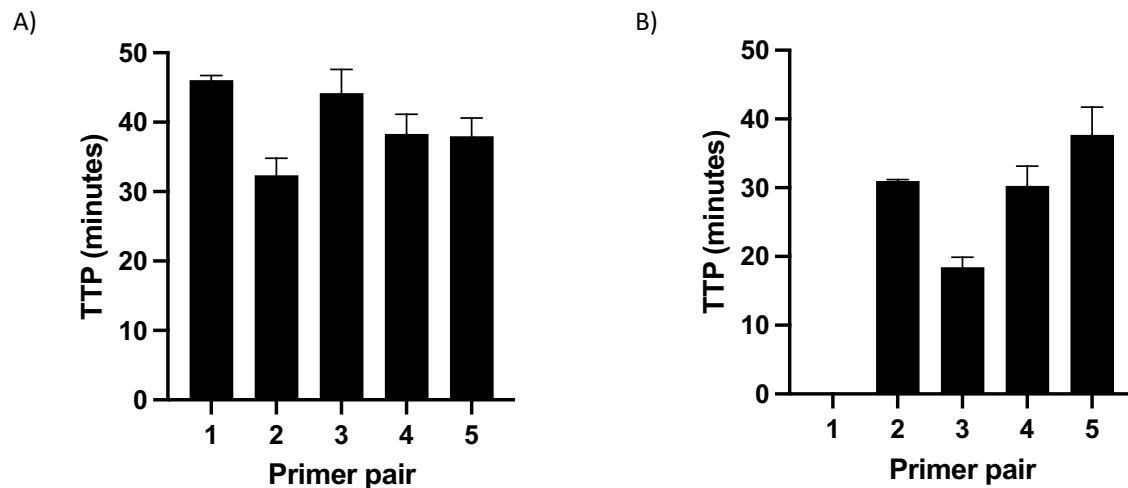
Appendix II



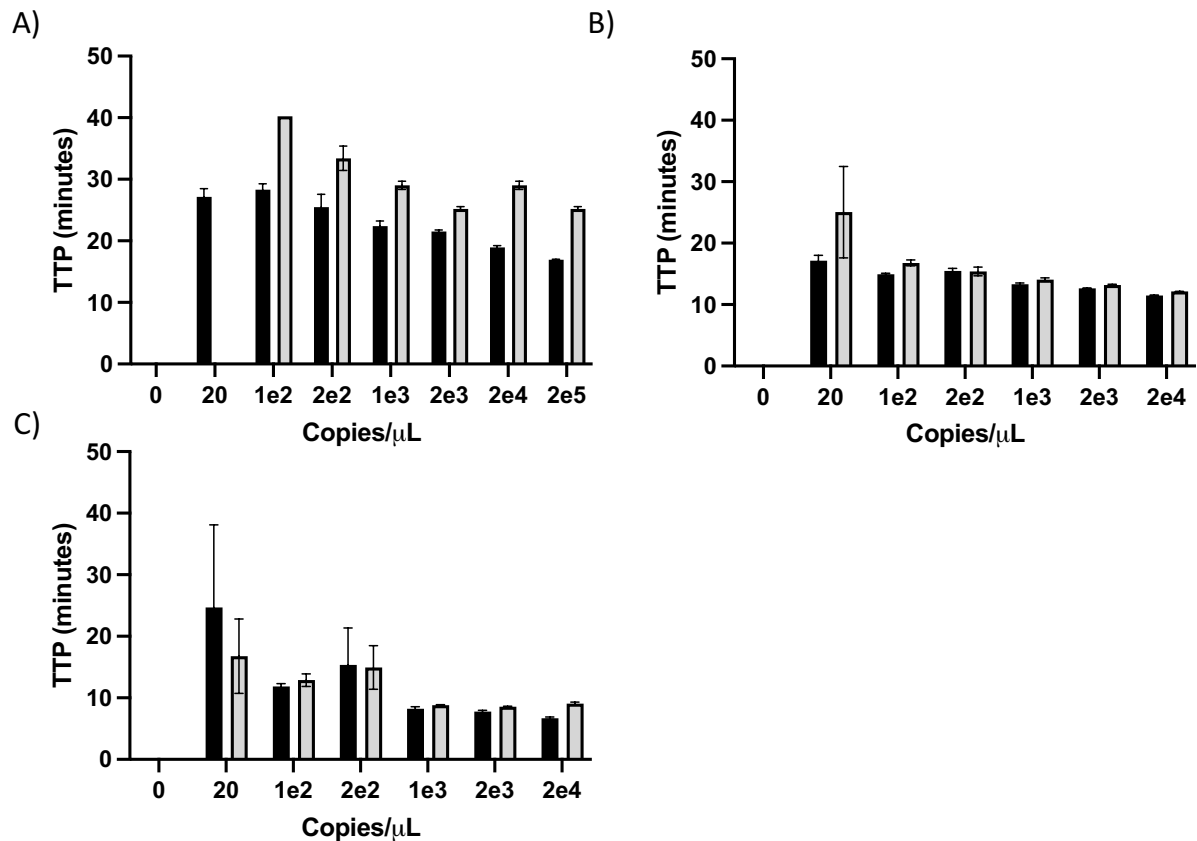
Supplementary Figure 1. RPA primer screen. Primer screens performed in RPA to achieve the lowest TTP for each variable primer in the detection of: APP with F4R9 chosen (A), APP 3' with F3R6 chosen (B), MH with F9R8 chosen (C), PM with F1R7 chosen (D) and SS with F6R2 chosen (E). Either the forward or reverse primer was screened against a static opposing primer (black columns) and the primer with the lowest TTP was used against all opposing primers (grey columns). Columns represent the mean of two replicates and error bars the S.D. between replicates.



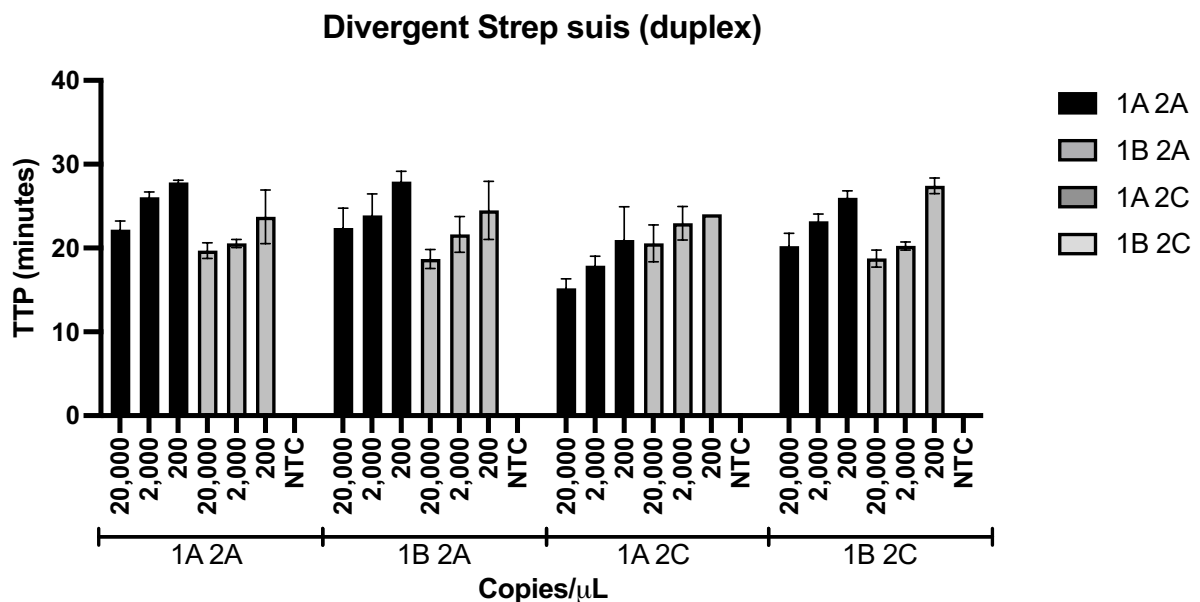
Supplementary Figure 2. RPA primer-dimerization. The products of RPA amplification performed in the presence and absence of target DNA ran on a 2% agarose gel (**A**). The difference in amplification curves of four RPA assays achieved with EvaGreen dye for the real-time and melt-curve. Red traces signify negative reactions and alternative colours signify positive reactions (**B**). The difference in RFU between positive and negative APP_2 RPA reactions across a range of temperatures, with amplification detected with EvaGreen dye (**C**). APP_2 pH difference between pre- and post-amplification achieved across a range of incubation temperatures (**D**).



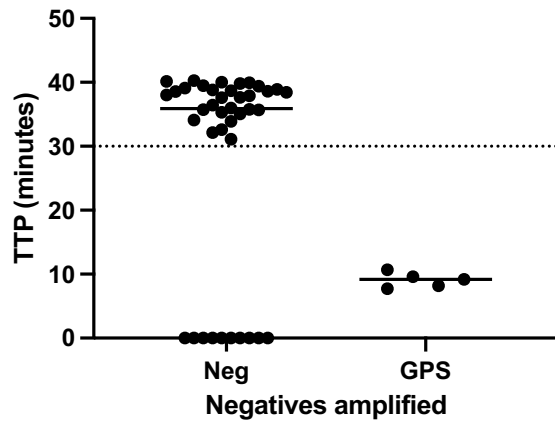
Supplementary Figure 3. HDA assay optimisation. HDA primer screen achieved with five primer pairs targeting MH (**A**) and five primer sets targeting PM, *kmt1* (**B**). Columns represent the geographical mean of four replicates and error bars the S.D.



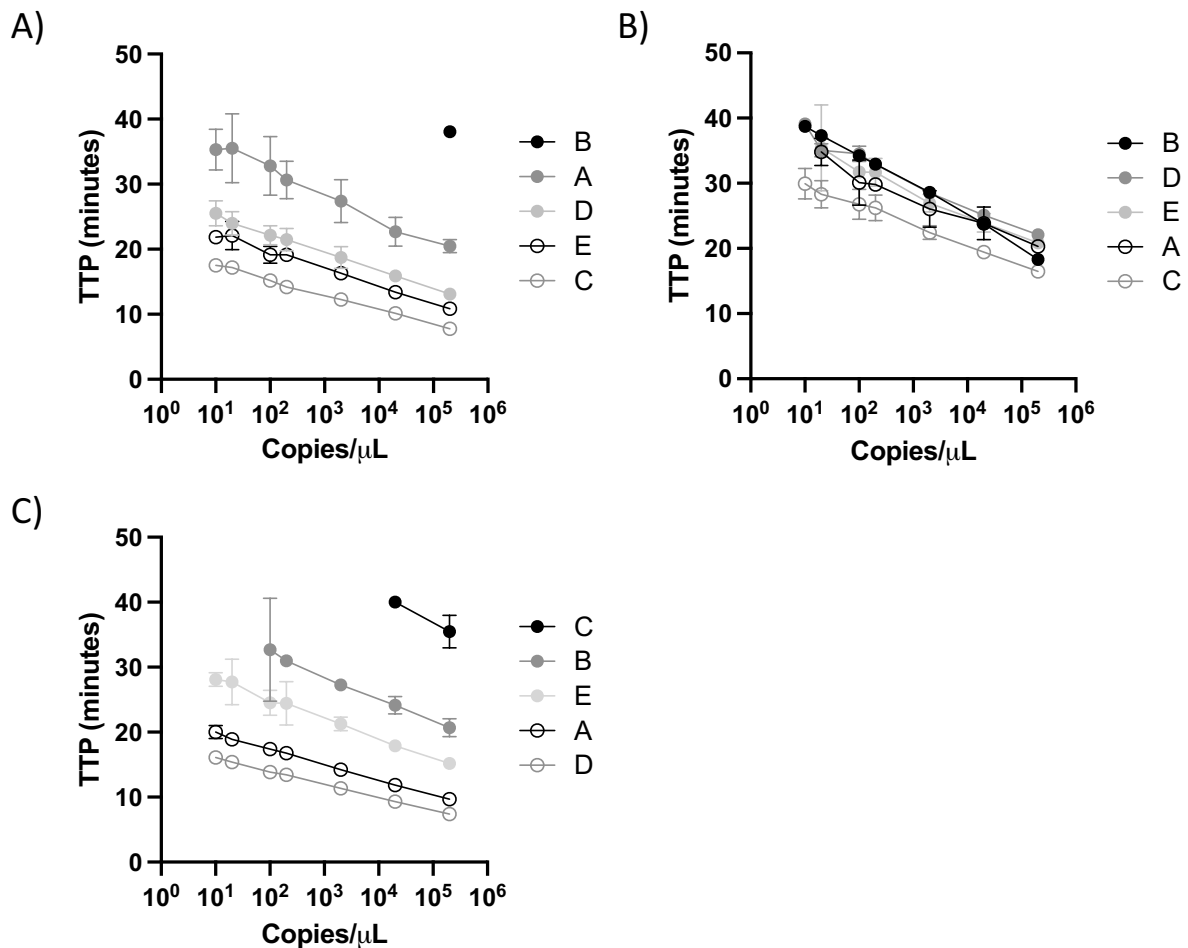
Supplementary Figure 4. Optimisation of LAMP primer sets. Re-designed LAMP primers (grey columns) produced containing common SNPs or matching T_m of opposing primers, compared against the original (black columns) primer sequences using serial dilutions of gDNA from APP (A), MH (B) and PM (C). Columns represent the geometrical mean of three replicates and error bars the S.D.



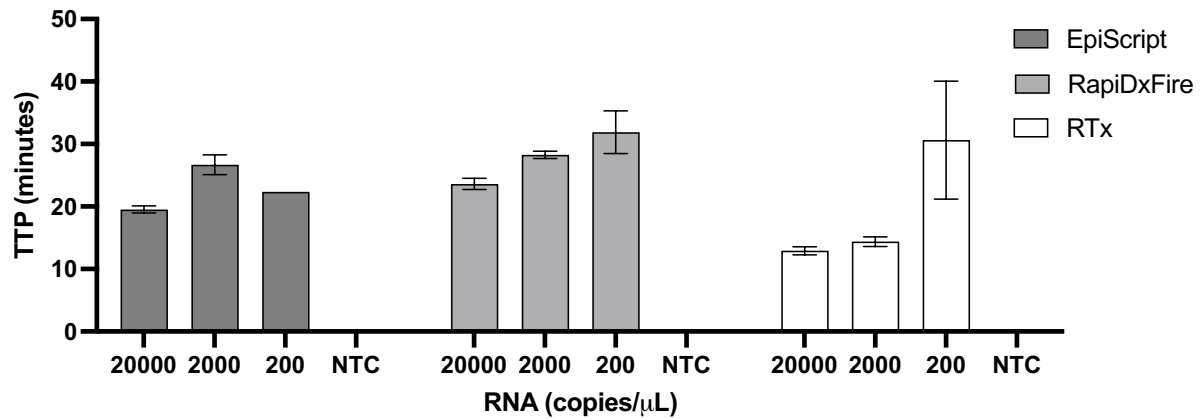
Supplementary Figure 5. Divergent SS primer screen in duplex. Combination of SS divergent primers targeting divergent clade 1 (1A and 1B) and divergent clade 2 (2A and 2C) against a serial dilution of synthetic DNA containing sequences specific for divergent SS 1 and divergent SS 2. Columns represent the geometrical mean of three replicates and error bars the S.D.



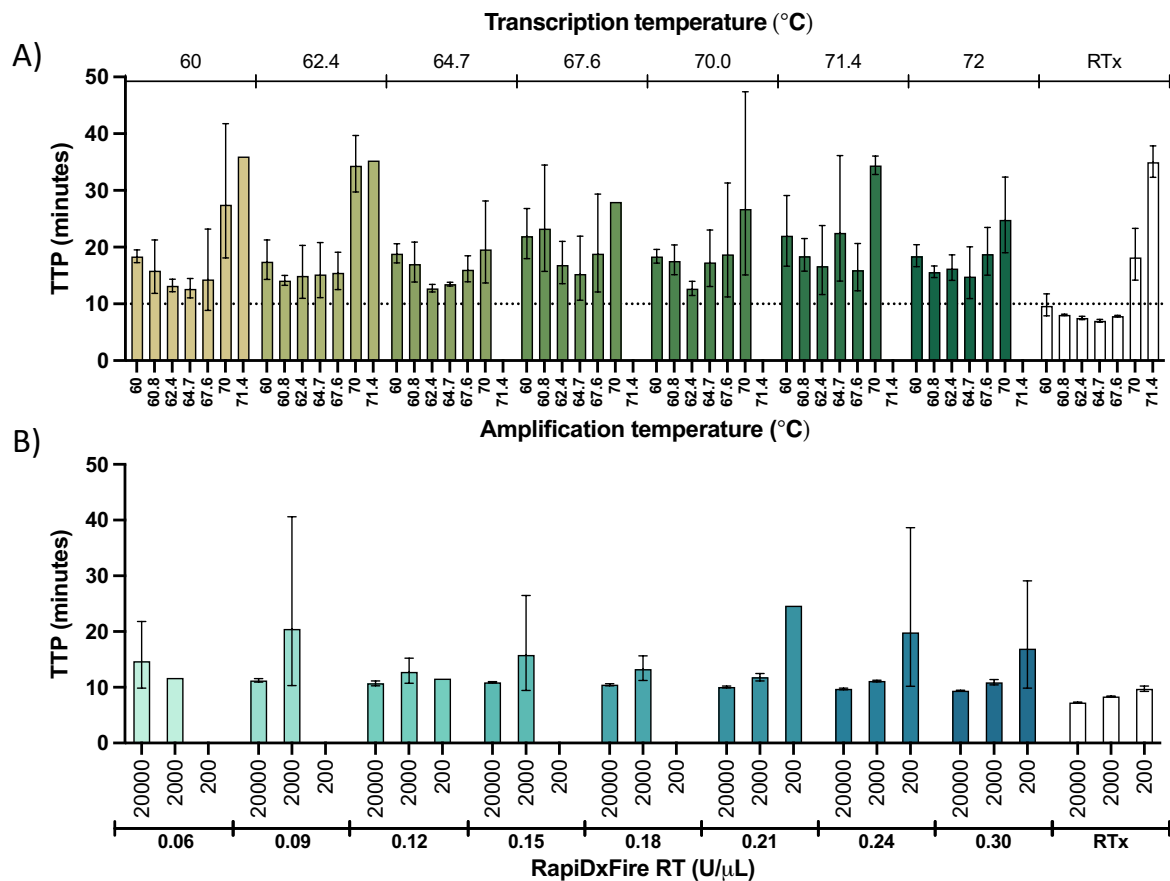
Supplementary Figure 6. False positive amplification with the GPS LAMP assay. The TTP that was achieved across 38 isolates of negative species using the optimal primers of the GPS assay. Each point represents the mean of duplicates performed on each isolate.



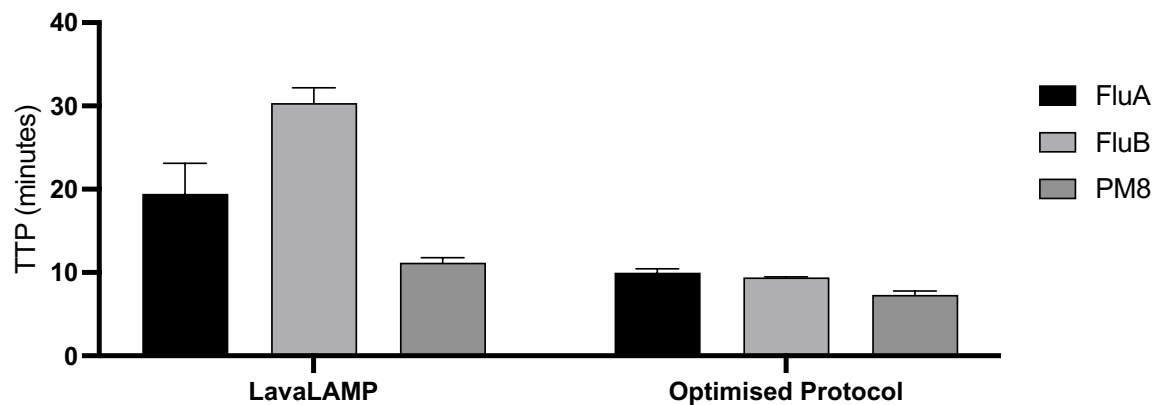
Supplementary Figure 7. Internal control primer screen. Primer screen on LAMP assay primers designed against the 12S rRNA (A), mitochondrial D-loop (B), and *cytochrome B* (C) genes. LAMP reactions were performed with serial dilutions of the IC synthetic DNA. Datapoints represent the geometrical mean of three replicates and error bars the S.D.



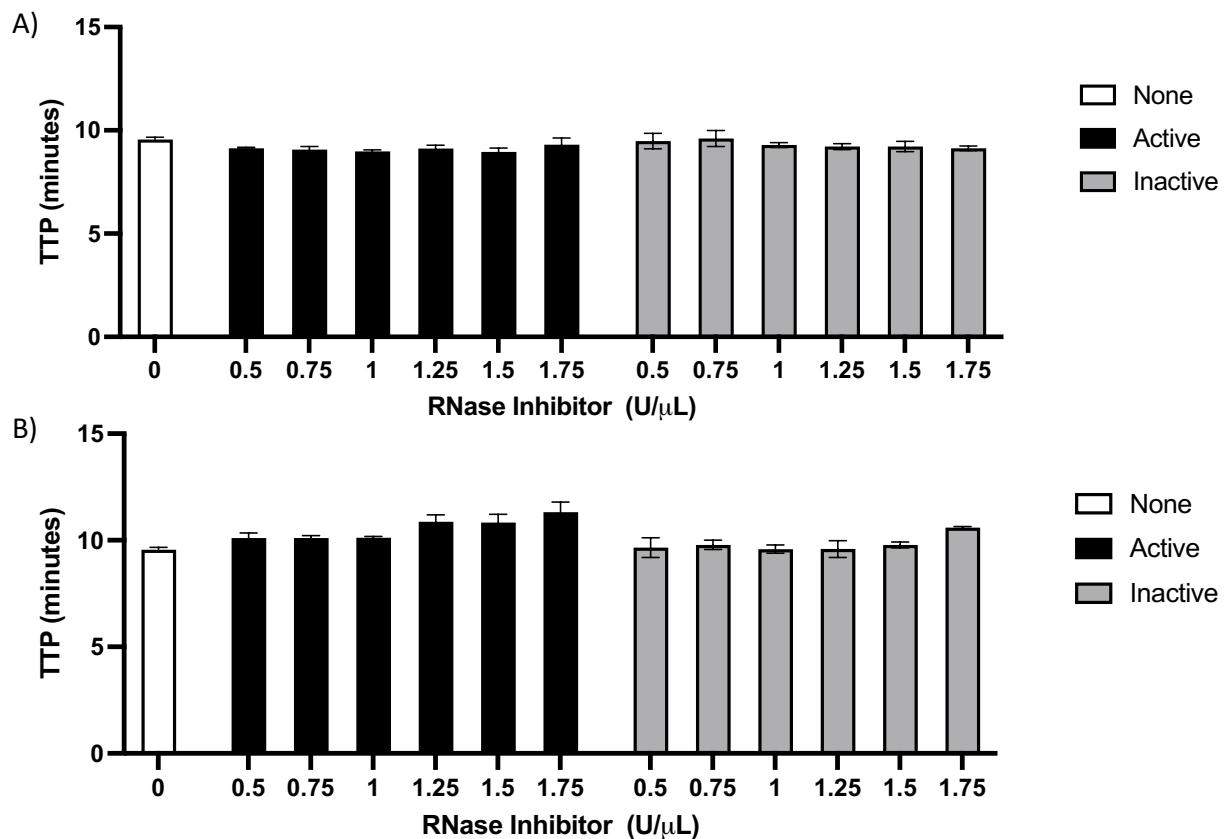
Supplementary Figure 8. 2-step amplification of EpiScript and RapiDxFire RT. The TTP achieved in the SARS-CoV-2 LAMP assay using EpiScript and RapiDxFire in two-step LAMP where the transcription took place in a pre-incubation step prior to amplification in LAMP, compared to one-step RT-LAMP using RTx. Columns represent the geometric mean of three replicates and error bars the S.D.



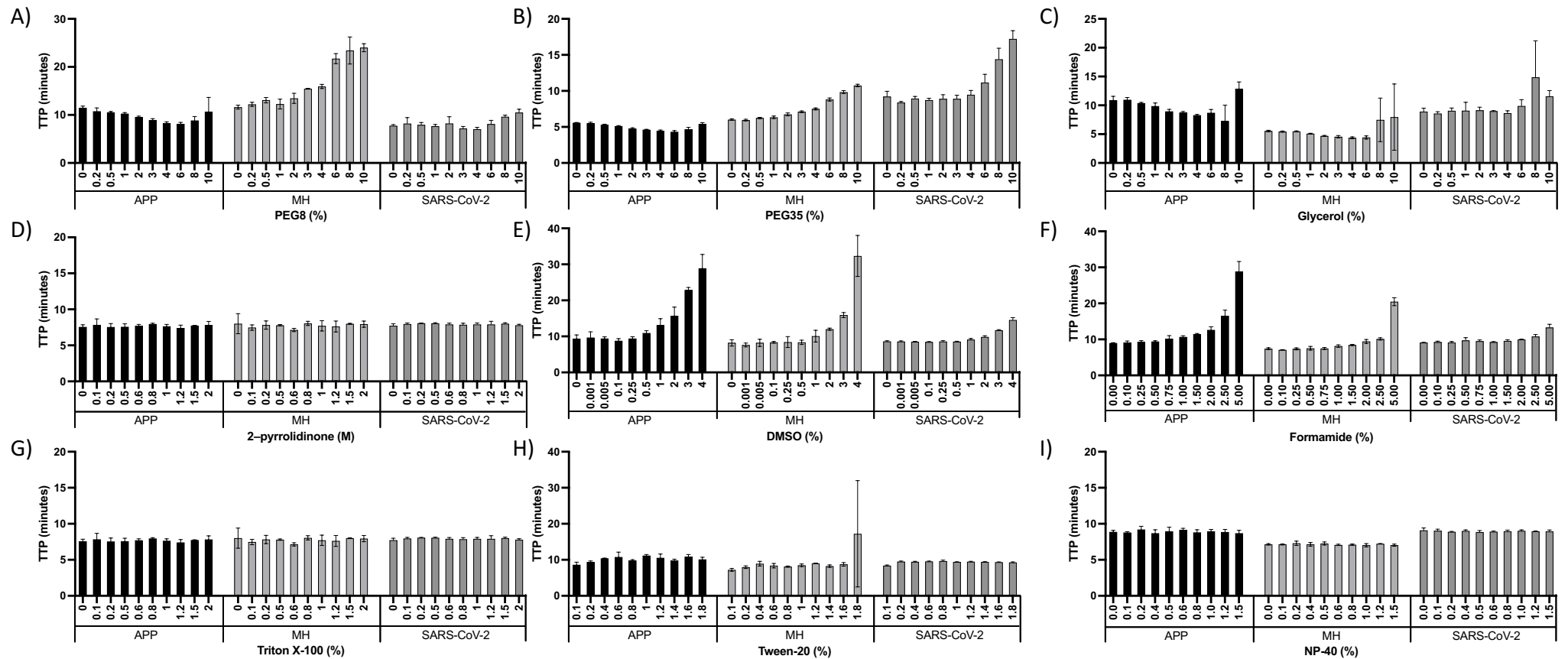
Supplementary Figure 9. Two-step RapiDxFire RT in LAMP across a range of temperatures and concentrations. The TTP achieved by the SARS-CoV-2 assay when 8,000 copies/μL was incubated across a range of temperatures in the RapiDxFire RT reaction, and subsequently 1 μL of the transcription reaction amplified at different temperatures in 5 μL LAMP reactions, with one-step RT-LAMP as a control, using RTx and WS Bst 2.0 (A). 10-fold serial dilutions of SARS-CoV-2 RNA transcribed with RapiDxFire RT at different concentrations, compared to one-step LAMP with RTx at 0.45 U/μL (B). Columns represent the geometric mean of three replicates and error bars the S.D.



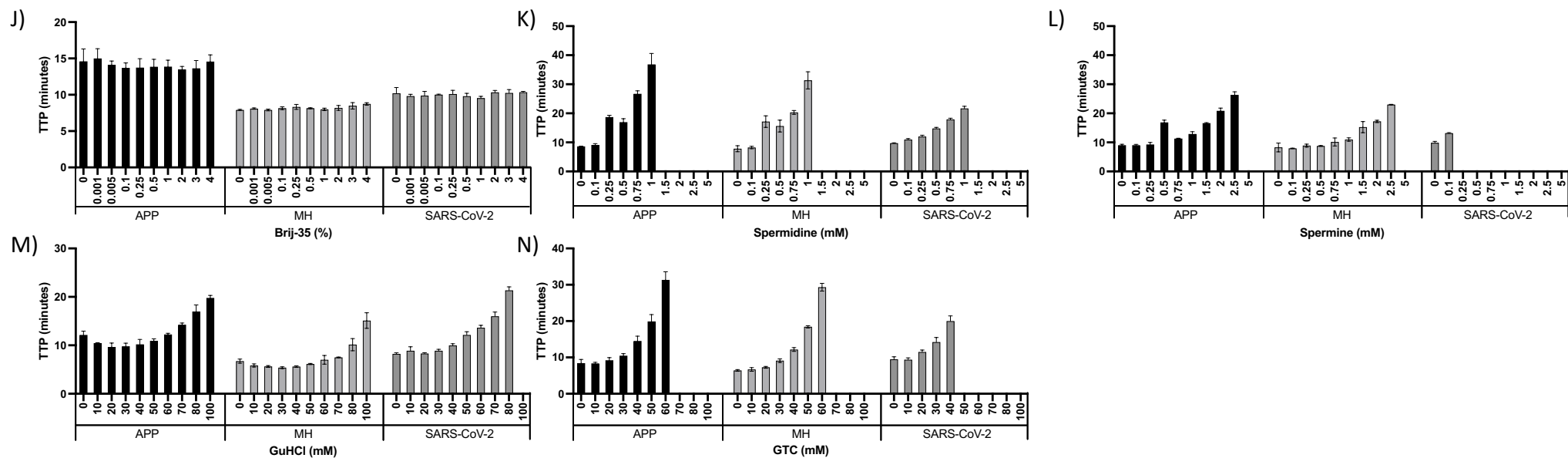
Supplementary Figure 10. LavaLAMP amplification of three LAMP assays. The TTP achieved when LavaLAMP was used to amplify two RT-LAMP assays (FluA and FluB) and one LAMP assay (PM8), in the presence of 200 copies/ μ L of each target. The optimised protocol used WS *Bst* 2.0 and RTx as a comparator. Columns represent the geometric mean of three replicates and error bars the S.D.



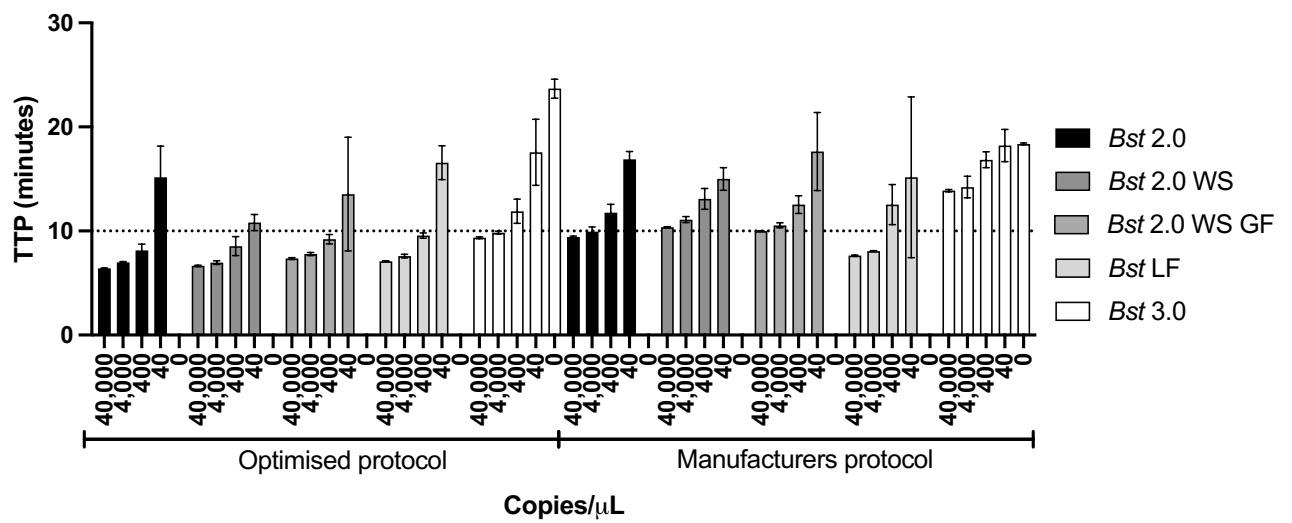
Supplementary Figure 11. The use of RNase inhibitors in RT-LAMP reactions. The addition of either active or heat-inactivated Riboguard (A) and RiboLock (B) in RT-LAMP reactions detecting SARS-CoV-2 RNA. Columns represent the geometric mean of three replicates and error bars the S.D.



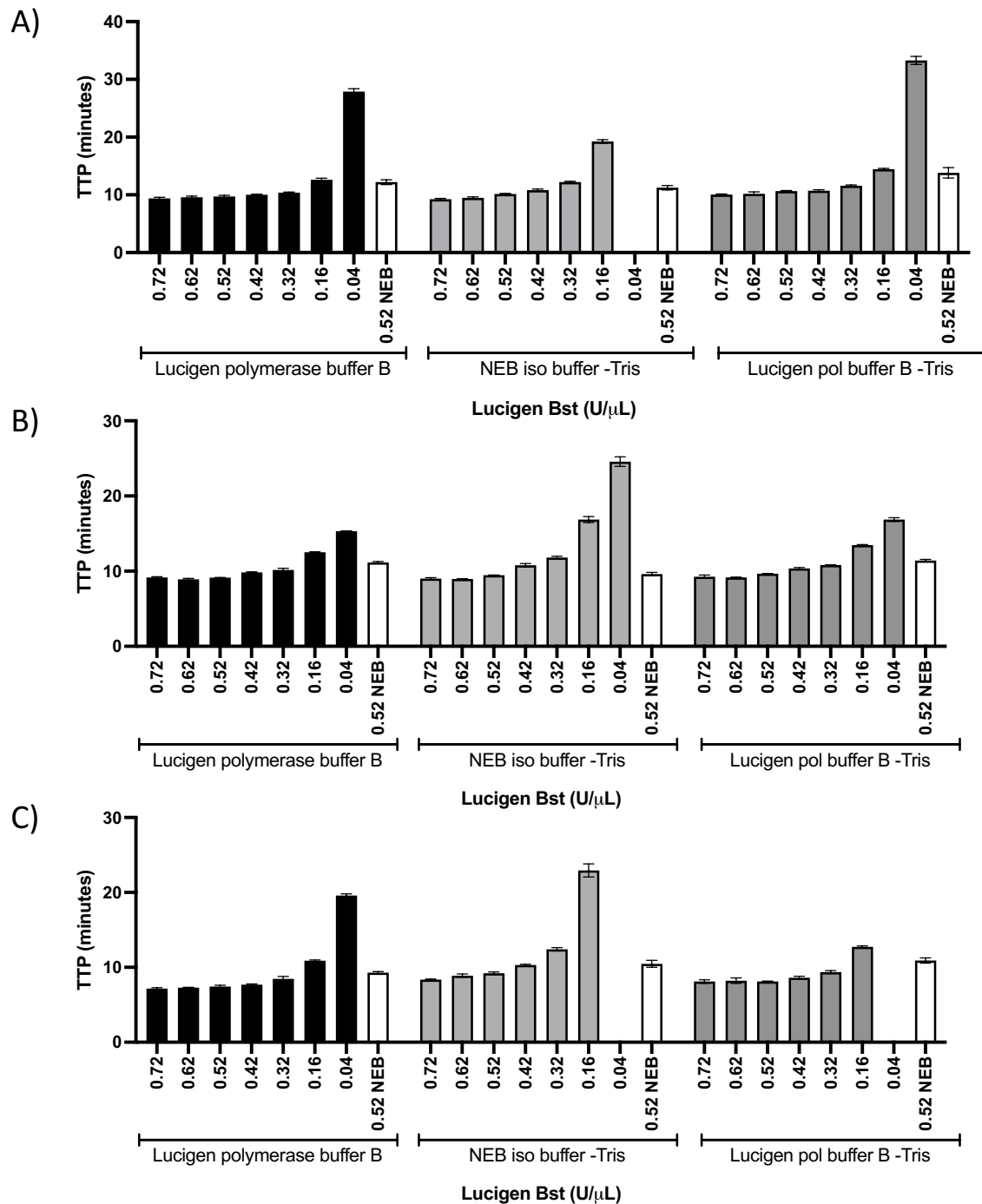
Supplementary Figure 12. Addition of compounds in LAMP. Additives were titrated into two LAMP reactions, APP and MH and one RT-LAMP reaction, SARS-CoV-2 three crowding agents PEG8 (A), PEG35 (B) and glycerol (C). Three solvents, 2-pyrrolidinone (D), DMSO (E) and formamide (F). Three surfactants, Triton X-100 (G), Tween-20 (H) and NP-40 (I). Columns represent the geometric mean of three replicates, and error bars the S.D. Continued on next page.



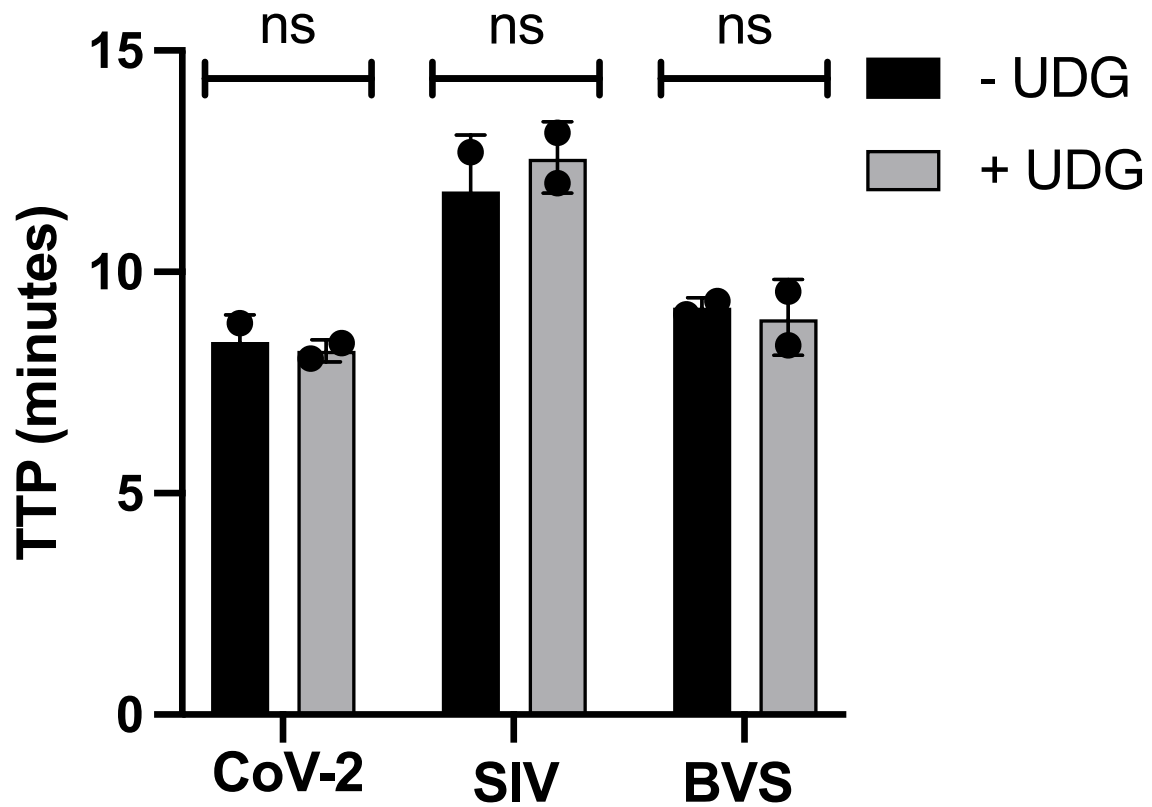
Supplementary Figure 12. Addition of compounds in LAMP (continued). Additives were titrated into two LAMP reactions, APP and MH and one RT-LAMP reaction, SARS-CoV-2 three crowding agents One detergent, Brij-35 (**J**). Two polyamines, spermidine (**K**) and spermine (**L**). Two chaotropic salts, GuHCl (**M**) and GTC (**N**). Columns represent the geometric mean of three replicates, and error bars the S.D.



Supplementary Figure 13. Optimised and manufactures recommended protocol in LAMP. 10-fold serial dilutions of SARS-CoV-2 synthetic DNA were compared using five *Bst* polymerases supplied by NEB. Columns represent the geometric mean of three replicates and error bars the S.D.



Supplementary Figure 14. Lucigen *Bst* titration in RT-LAMP reactions. Serial dilution of Lucigen *Bst* in SIV (A), BVS (B) and SARS-CoV-2 (C) using three different buffers, the Lucigen polymerase B buffer, which was supplied by the manufacture (black columns) with NEB isothermal (iso) buffer -Tris (light grey columns) required for a pH change to be observed using NEB *Bst* and a version of Lucigen's polymerase (pol) buffer B that is devoid of Tris (dark grey columns). For each condition, control of 0.52 U/μL of NEB WS *Bst* 2.0 DNA polymerase was used (white columns). Columns represent the geometric mean of three replicates, and error bars the S.D.

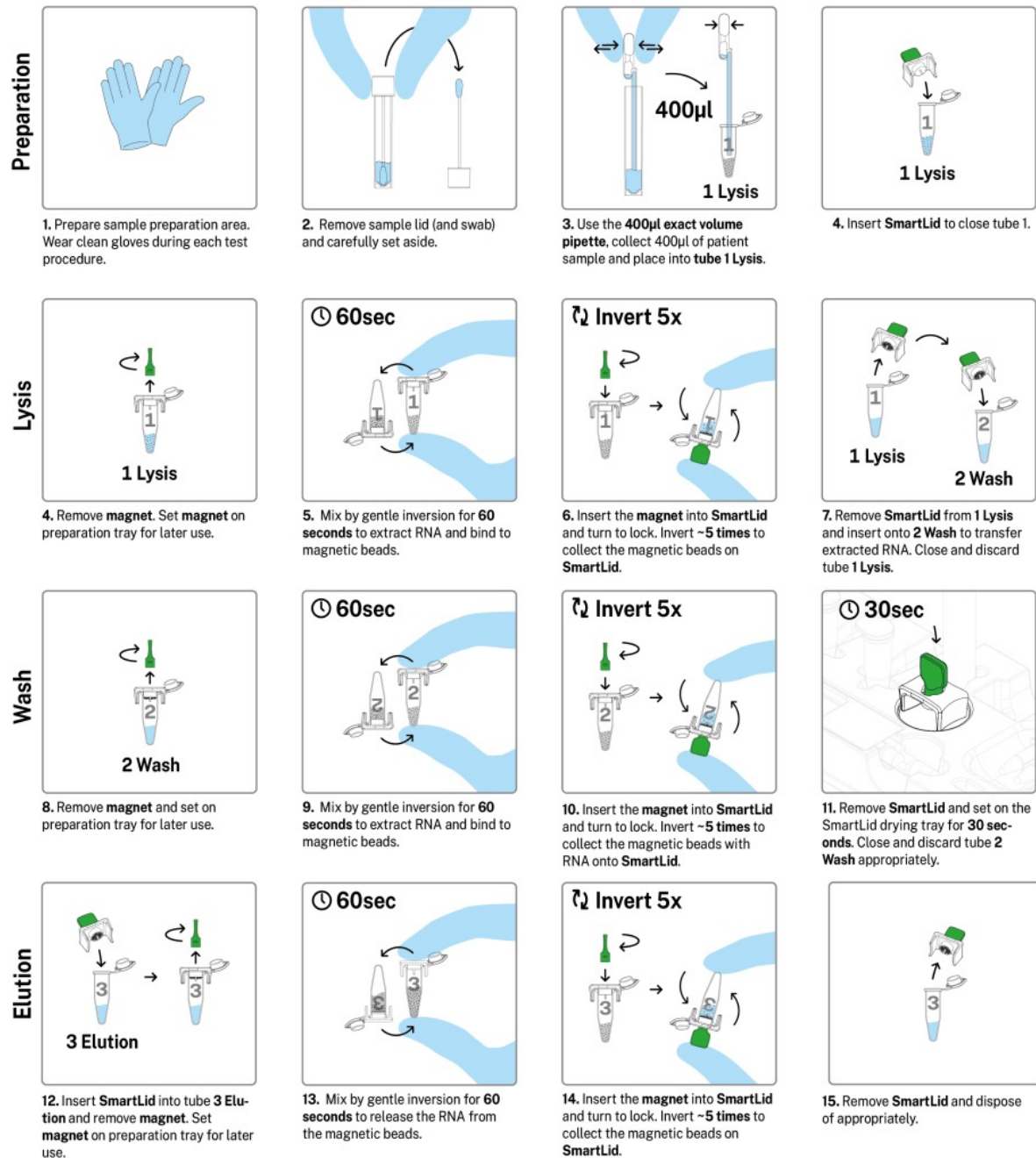


Supplementary Figure 15. Addition of UDG into three RT-LAMP reactions. The TTP of RT-LAMP reactions targeting SARS-CoV-2, SIV and BVS in the presence of absence of UDG, performed in duplicate at 250, 1,000 and 2,000 RNA copies/ μ L, respectively. Columns represent the geometric mean of three replicates, datapoints the individual replicates and error bars the S.D.

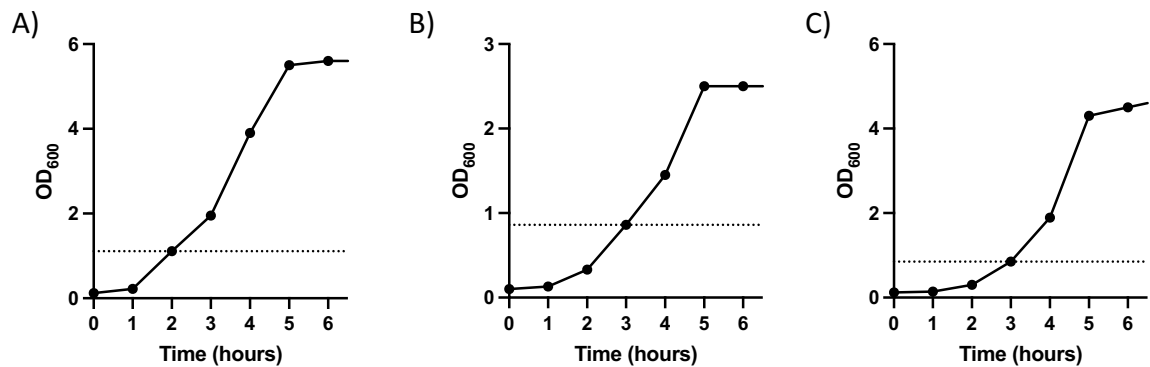
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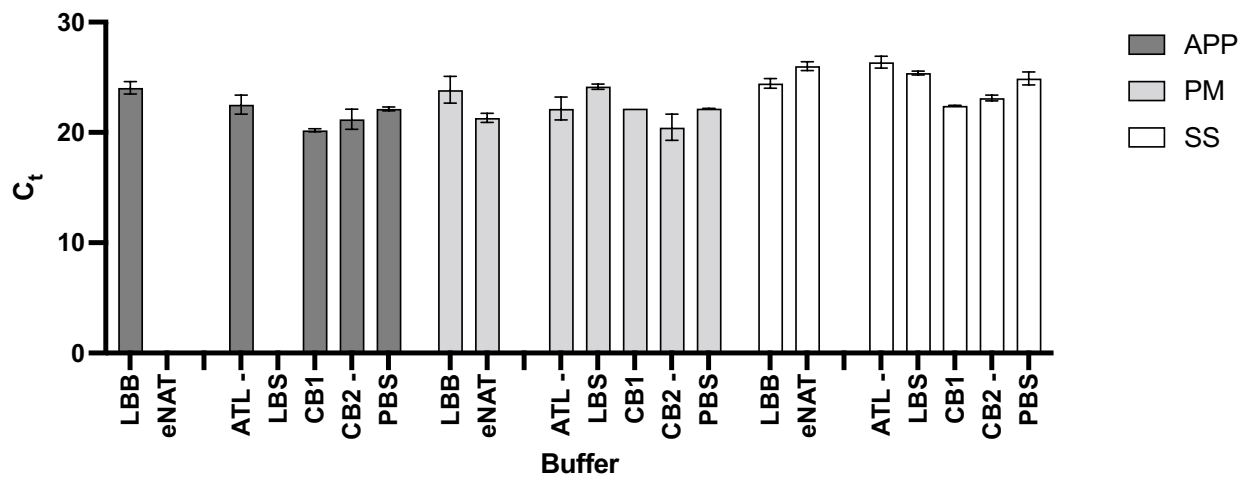
protondx



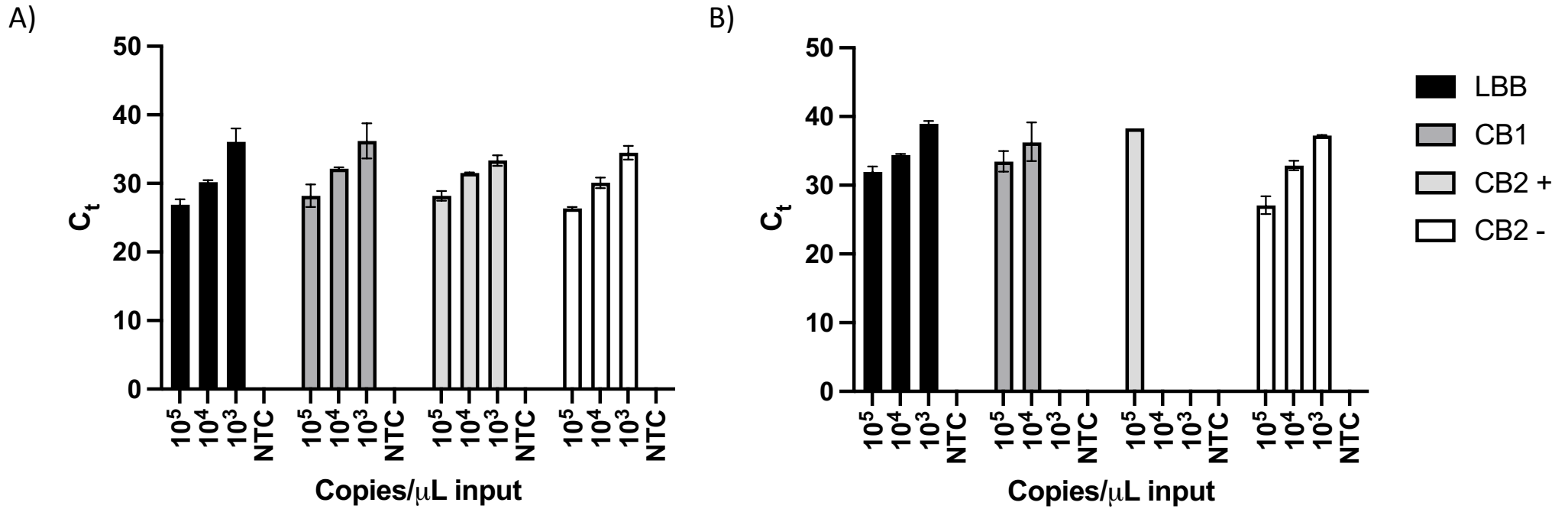
Supplementary Figure 16. SmartLid SARS-CoV-2 extraction process. The IFU user guide for the extraction of viral RNA from nose and throat swabs, developed by ProtonDx. Reproduced with permission from the Copyright holder, ProtonDx Ltd.



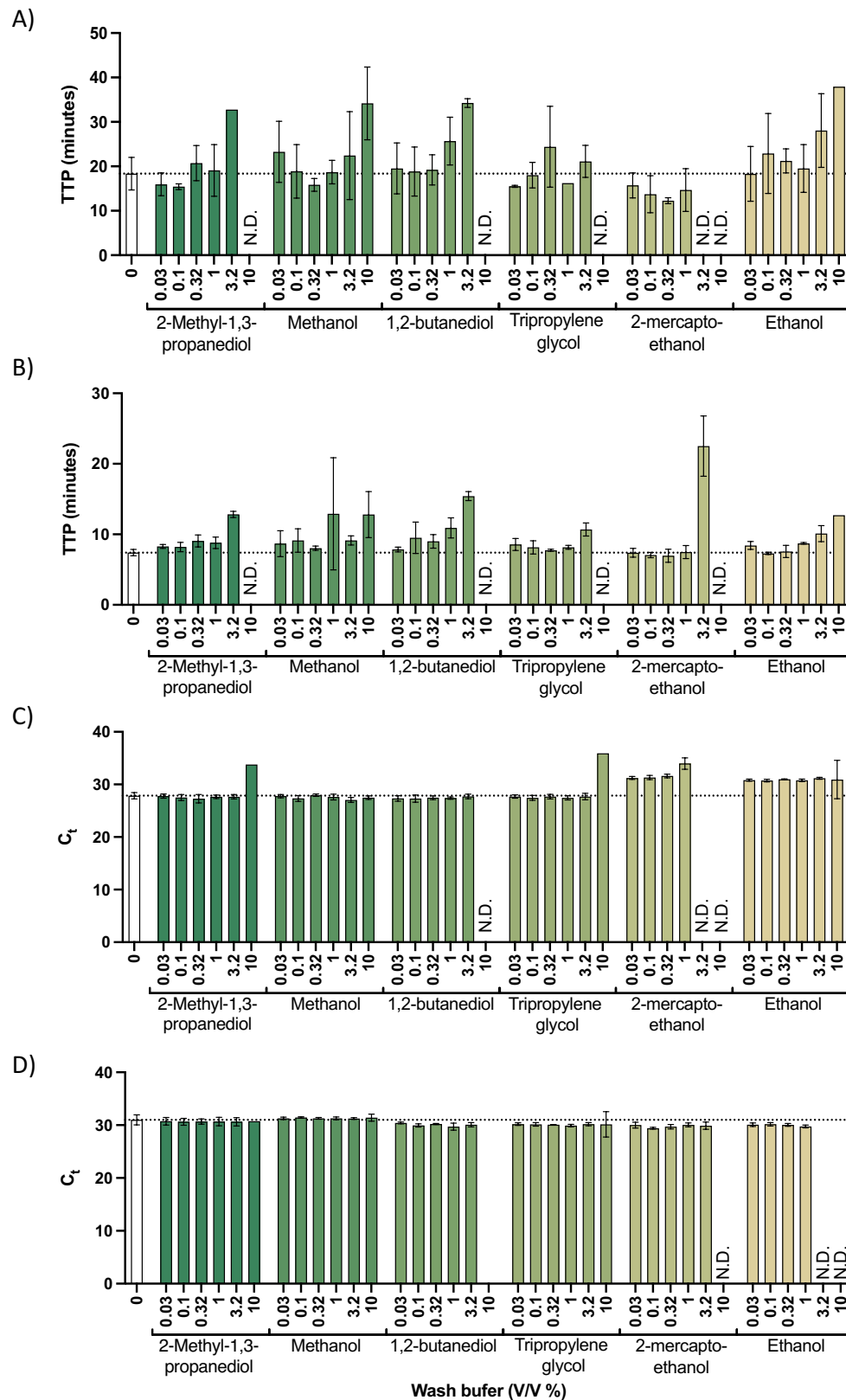
Supplementary Figure 17. Bacterial growth curves. Growth curves produced from the bacterial culturing of APP isolate, MIDG 2331 (A), PM isolate, MIDG 2214 (B) and the SS isolate, P1/7 (C). OD₆₀₀ readings were taken every hour over the course of 6 hours. The dotted lines depict the OD₆₀₀ reading at which the respective isolate was in early growth phase and used in bacterial lysis buffer experiments.



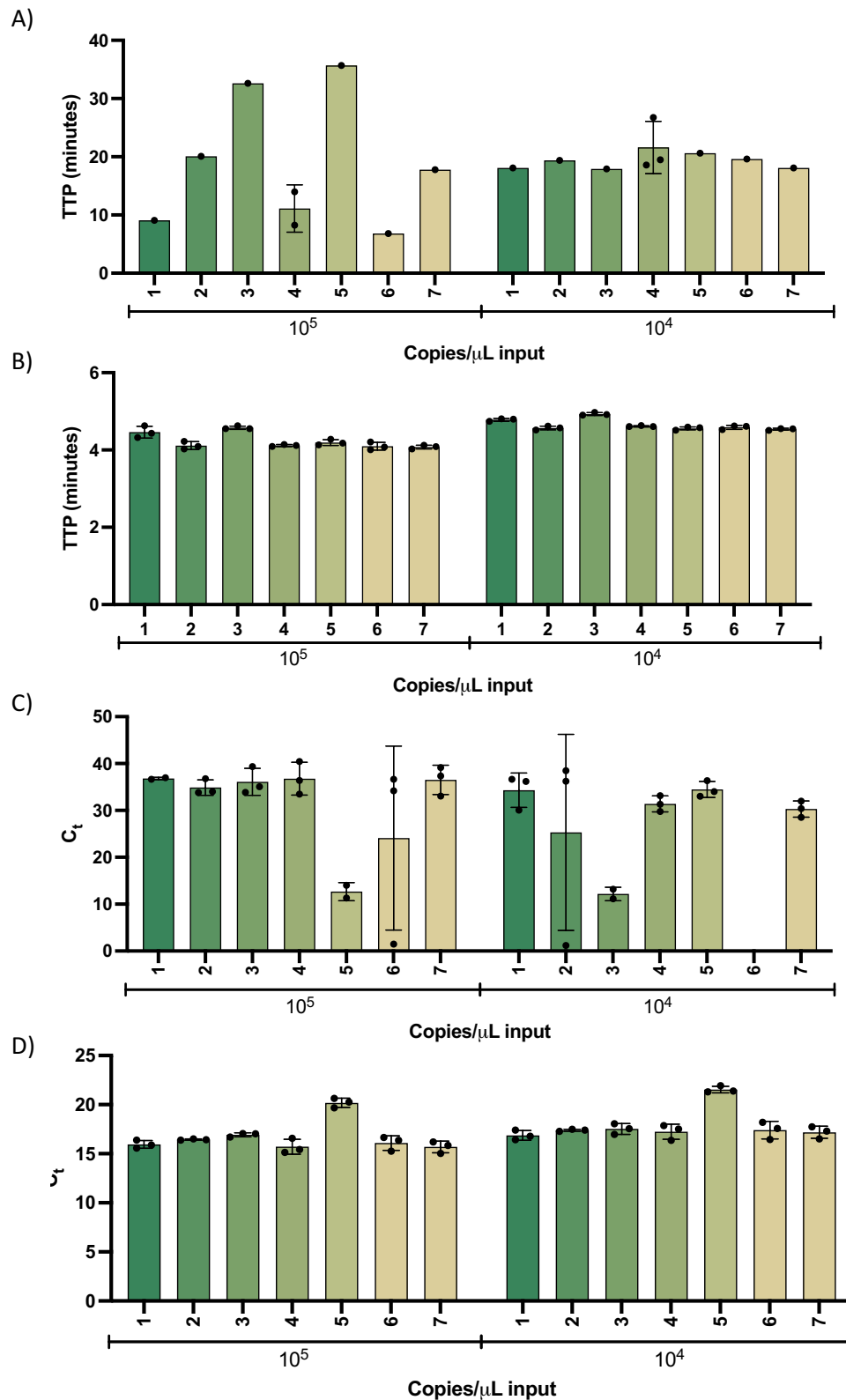
Supplementary Figure 18. Amplification of free gDNA of bacterial pathogens in the presence of lysis buffer. The chemical lysis of three bacterial pathogens using different lysis buffers. Including lysis and binding buffer (LBB), eNAT buffer, buffer ATL without proteinase K (-), Lysis and binding solution (LBS), Custom buffer 1 (CB1), custom buffer 2 (CB2) and PBS as a control. The C_t value achieved with qPCR for the detection of APP (dark grey columns), PM (light grey columns) and SS (white columns) using the diluted supernatant (1:20) of bacteria treated with the panel of lysis buffers. qPCR reactions were performed in triplicate, with columns displaying the geometric mean and error bars the S.D. The absence of a C_t value represents non-detectable amplification.



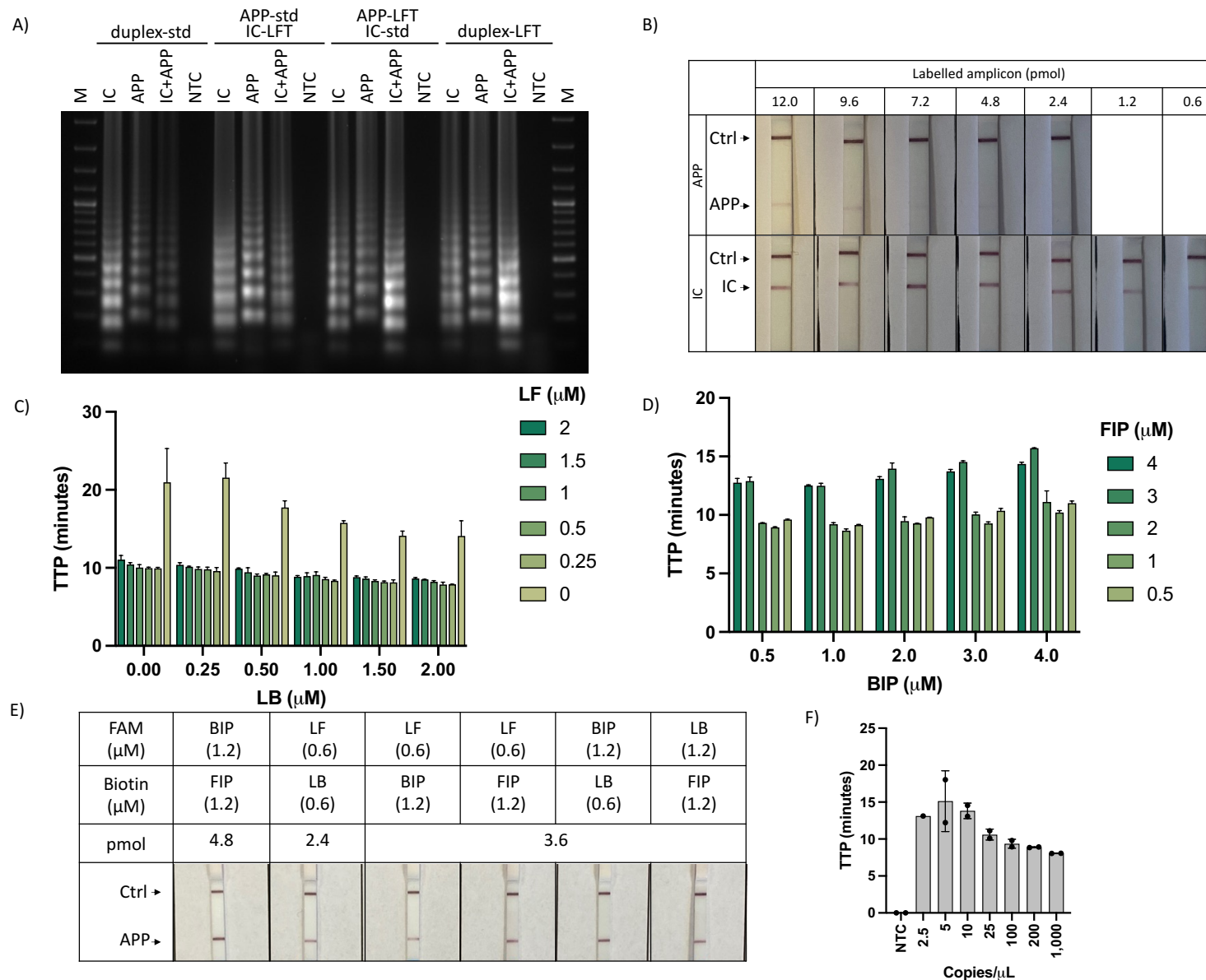
Supplementary Figure 19. Limit of detection using different lysis buffers. The qPCR amplification of negative lung samples spiked with different concentrations of APP serovar 8 gDNA into four different buffers (A) or into a pathogen-negative lung sample homogenised into four different lysis buffers (B). The lysis buffers included: lysis and binding buffer (LBB), custom buffer 1 (CB1), custom buffer 2 with and without proteinase K (CB2+ and CB2-, respectively) Amplification was performed in duplicate with columns representing the mean and error bars the S.D.



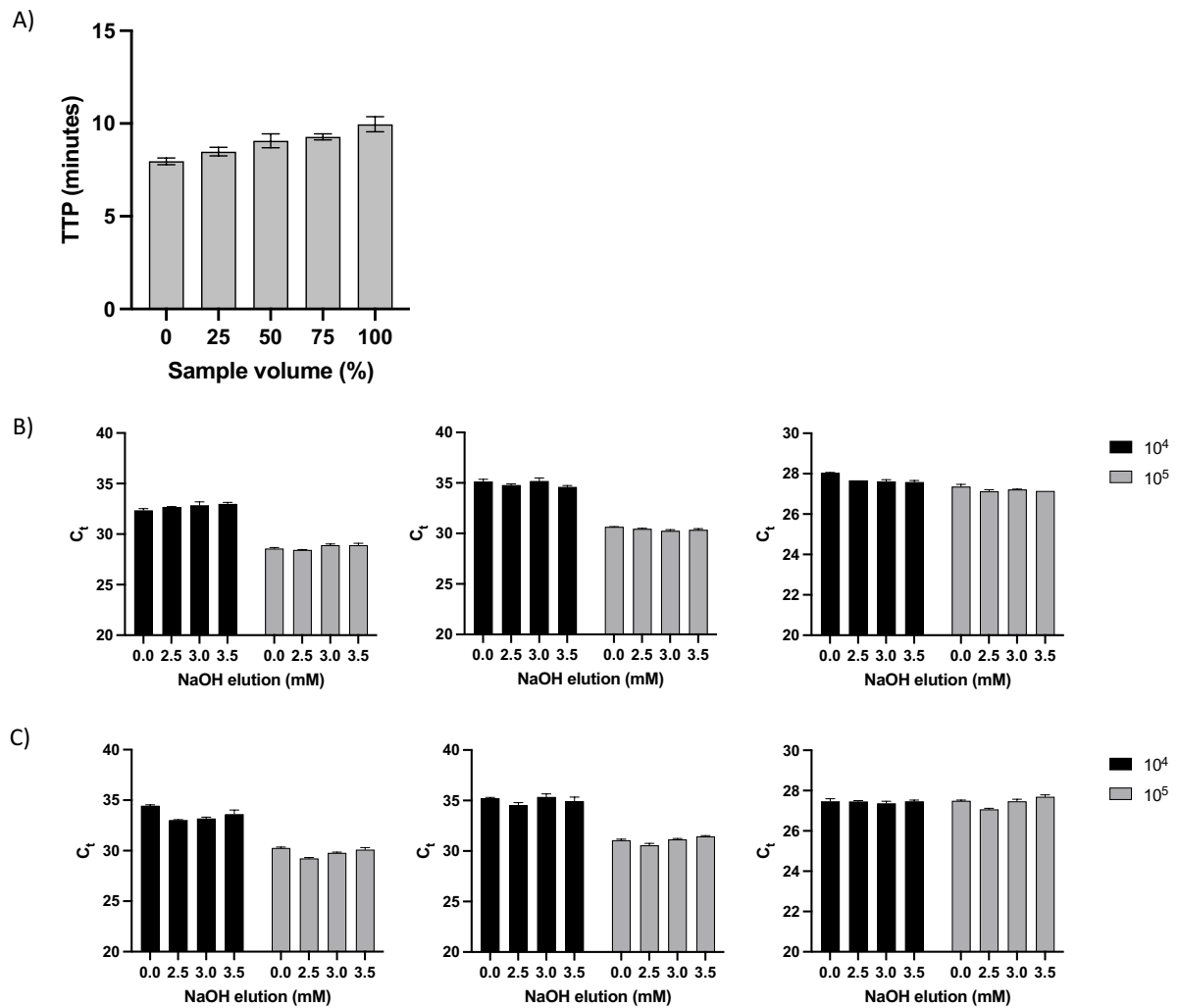
Supplementary Figure 20. Wash buffer inhibition of amplification chemistries. Titration of wash buffers into LAMP reactions detecting APP (A) or MH (B) and qPCR reactions detecting APP (C) or the IC assay - *cytB* (D). Concentrations of bacterial gDNA (A, B and C) or the IC synthetic DNA kept constant, and buffers titrated into the amplification reaction. A concentration of zero (white) represents the no-buffer control and dotted lines signify the TTP or C_t achieved with the no-buffer control. The columns indicate the geometric mean of triplicate reactions and error bars the S.D.



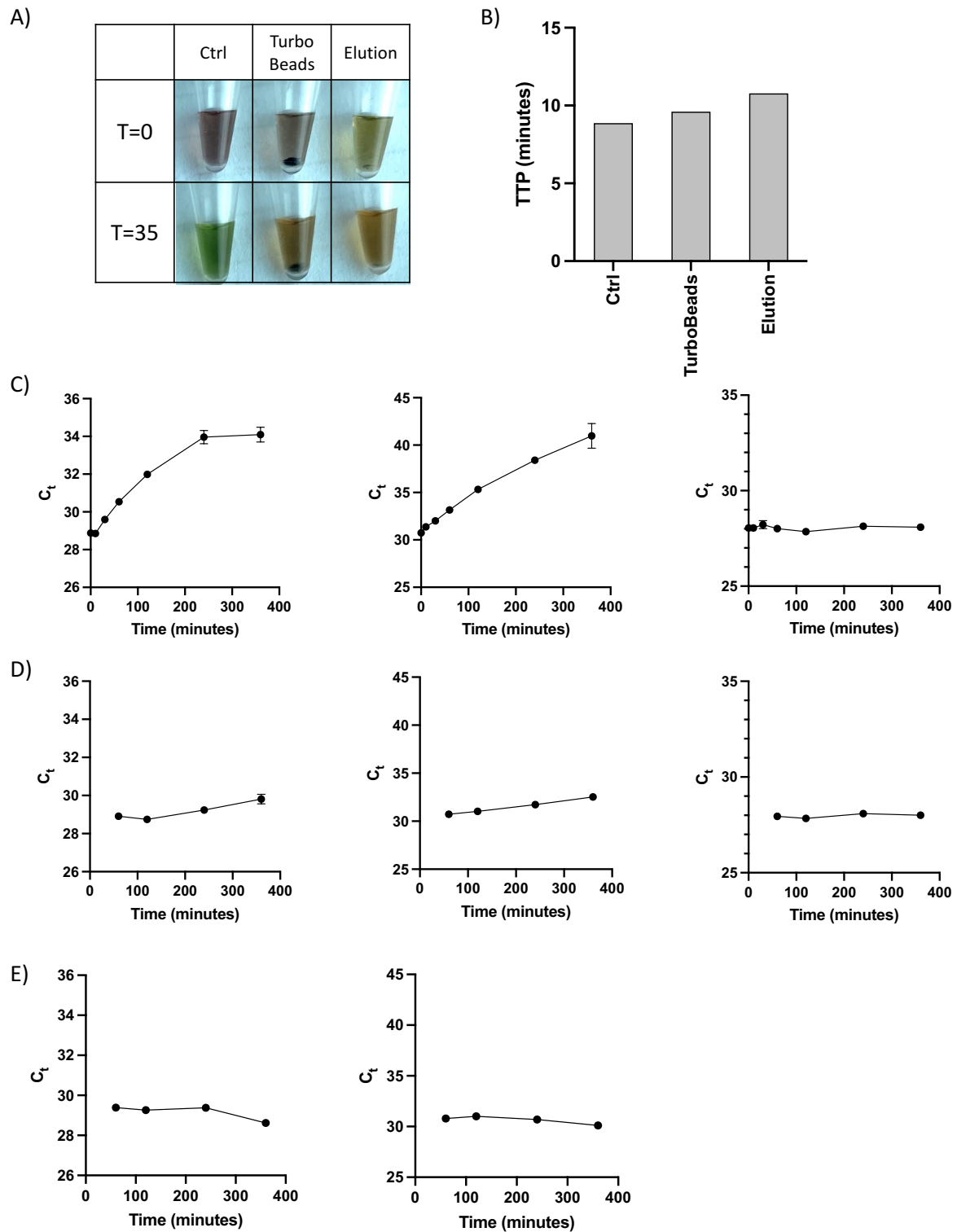
Supplementary Figure 21. Lysis buffer compatibility with SmartLid. The amplification of lung samples homogenised in by vortex and spiked with 10⁵ or 10⁴ copies/μL of APP serovar 8 gDNA. The lung homogenate was extracted with SmartLid, using a panel of seven wash buffers, 70% v/v 2-methyl-1,3-propanediol (1), methanol (2), 1,2-butanediol (3), tripropylene glycol (4), 2-mercaptoethanol (5), ethanol (6) and 80% v/v ethanol (7). The sample eluent was assessed in LAMP assays detecting APP (**A**), *cytB* (**B**) and in qPCR assays detecting APP (**C**) and *cytB* (**D**). Columns represent the geometrical mean of amplification performed in triplicate, data points the individual replicates, and error bars the S.D.



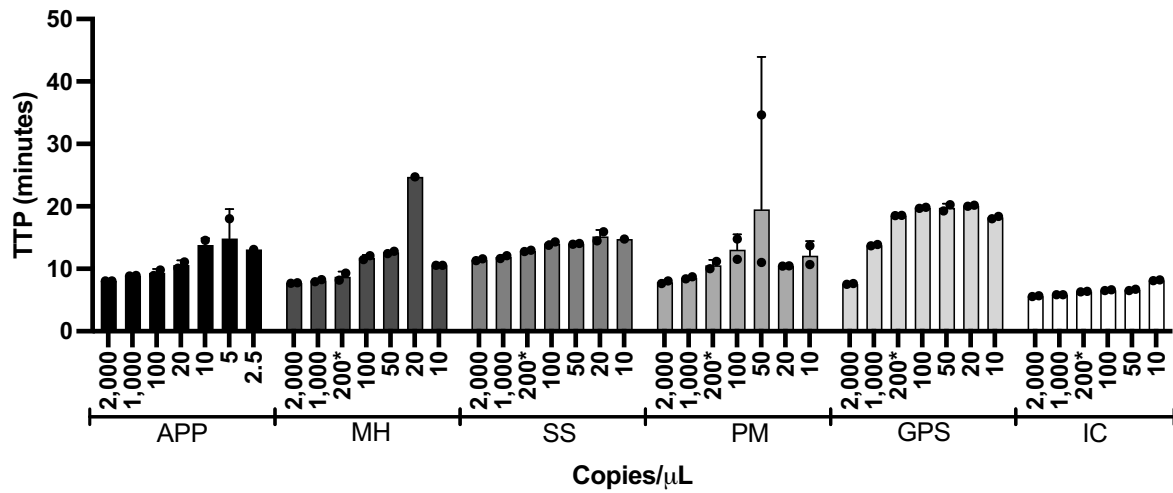
Supplementary Figure 22. The optimisation of LFT strips. The inclusion of LFT primers for the detection of APP and IC, to form a duplex LFT-LAMP detection (**A**). The singleplex amplification of APP or IC with different total amounts of labelled product run up the later flow strip (**B**). The titration of the APP loop primers LF and LB (**C**) and FIP and BIP (**D**) to RT-LAMP reactions. The addition of LAMP produce containing different concentrations of labelled LFT APP-primers labelled with either FAM or Biotin to the LFT strip (**E**). The LoD in real-time LAMP using a serial dilution of APP gDNA (**F**). M denotes the 100 bp Plus DNA Ladder (Thermo Fisher Scientific) molecular marker.



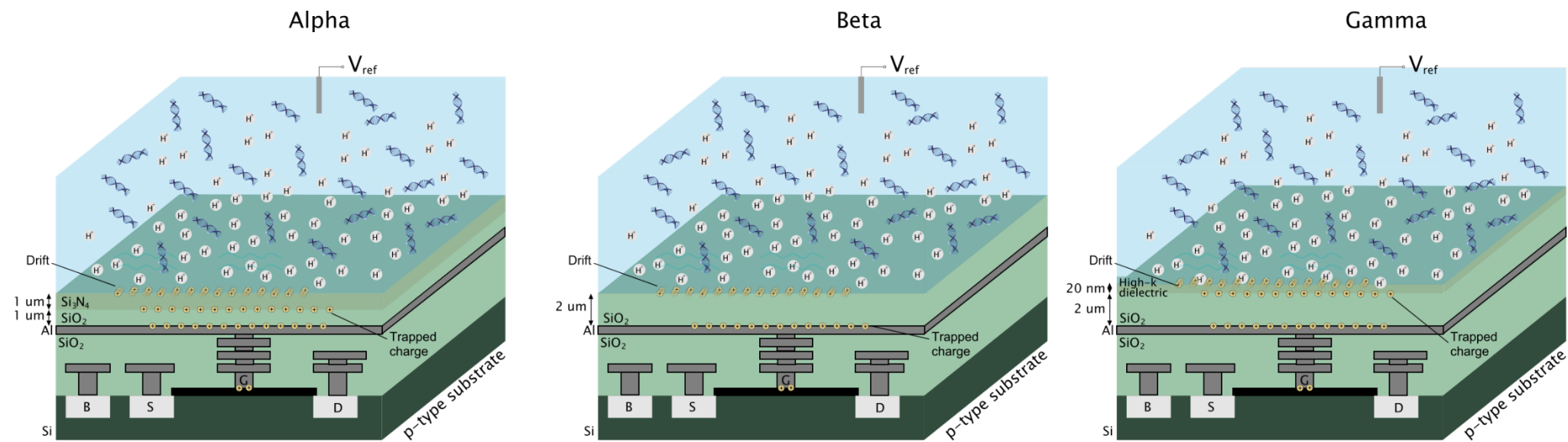
Supplementary Figure 23. Rehydration of lyophilised colourimetric RT-LAMP reactions. The rehydration of SARS-CoV-2 lyophilised colourimetric RT-LAMP reactions containing EBT and TB dye using different amounts of sample volume and spiked with 200 copies/ μ L of SARS-CoV-2 RNA into the LAMP reaction **(A)**. The increase in NaOH in the elution solution for Qiagen extraction **(B)** and SmartLid extractions **(C)**, containing spiked SARS-CoV-2 negative nose and throat swabs. Detection took place with qPCR for SARS-CoV-2 using CDC primer sets n1 **(left)**, n2 **(centre)** and human RNaseP **(right)**.



Supplementary Figure 24. SmartLid compatibility with lyophilised colourimetric RT-LAMP reactions. The colour change (**A**) or real-time detection (**B**) achieved with lyophilised colourimetric RT-LAMP reaction containing EBT and TB dye amplified in the presence of 200 copies/ μ L alone (ctrl), with TurboBeads or with SmartLid Eluent (Elution). The stability of SARS-CoV-2 RNA and human gDNA in SmartLid 4 mM SmartLid eluent stored at room temperature (**C**), 4°C (**D**) or -80°C (**E**). Detection took place with qPCR for SARS-CoV-2 using CDC primer sets n1 (**left**), n2 (**centre**) and human RNaseP (**right**). Data points represent the average of triplicates and error bars, the S.D.



Supplementary Figure 25. Real-time limit of detection using LAMP. Serial dilutions made of gDNA for APP, MH, SS, PM and GPS reference isolates and the IC synthetic DNA control. Columns represent the average of duplicate reactions (data points), and error bars, the S.D.



Supplementary Figure 26. CMOS-ISFET surface types. Alpha surface type classically used in the CMOS-ISFET architecture consisting of a 1 μm Si_3N_4 and 1 μm SiO_2 layers (**left**). Beta surface type consisting of just 2 μm of SiO_2 (**centre**). Gamma surface type with a 2 μm SiO_2 layer and a thin 100 nm proprietary high-k dielectric layer (**right**). Image used with the permission of Dr Nick Moser (ProtonDx).

Appendix III

Figure no.	Type of work:	Name of work	Source of work	Copyright holder	Permission date	Permission Granted	Permission note
1.3	Image	Acute pleuropneumonia. Taken on farm in Cyprus.	Personal communication	Credit: Andrew Rycroft Royal Veterinary College	06.11.2022	Yes	Written permission
1.4	Image	How to further improve <i>M. hyo</i> vaccine efficacy?	Pig progress (Website) https://www.pigprogress.net/health-nutrition/how-to-further-improve-m-hyo-vaccine-efficacy/	CEVA Animal Health	06.11.2022	Yes	Written permission
1.5	Figure	Fig.3. Left lung, pig 129 (G2) inoculated with 107 CFU of <i>Pasteurella multocida</i> type A (strain #11246). Diffuse fibrinous pleuritis	<i>Pasteurella multocida</i> type A as the primary agent of pneumonia and septicemia in pigs Oliveira Filho et al. The Journal Revista Pesquisa Veterinária Brasileira. Vol 38, Issue 8, pp. 716-724 (2015)	The Journal Revista Pesquisa Veterinária Brasileira does not oppose the use of the images as long as they are accepted by the authors of the article	06.11.2022	Yes	Written permission
1.5	Figure	Fig.4. Pericardium sac and heart, pig 140 (G3) inoculated with 106 CFU of <i>Pasteurella multocida</i> type A (strain #11246). Diffuse fibrinous pericarditis.					
1.7	Figure	Fig 1. Establishment of SS2 meningoencephalitis and non-meningoencephalitis pig models.	Differential protein profiling of cerebrospinal fluid in piglets with severe meningoencephalitis caused by <i>Streptococcus suis</i> type 2 compared to controls Lui et al. Frontiers in Cellular and Infection Microbiology. Vol 8, 35 (2018)	Copyright © 2018 Liu, Jia, Guo, Sun, Zhu, Li, Qu, Jiang, Wang, Gu, Sun, Feng, Han and Lei.	N/A	Not required	Distributed under the terms of the Creative Commons Attribution License (CC BY)
1.8	Figure	Fig 2. (A) Symptoms of piglets infected with <i>G. parasuis</i> ... (D) pericardial thickening, typical “fluff heart”; (F) pulmonary congestion, bleeding, necrosis.	Comparison of the <i>Glaesserella parasuis</i> virulence in mice and piglets Qi et al. Frontiers in Veterinary Science. Vol 8, 659244 (2021)	Copyright © 2021 Qi, Li, Chen, Ding, Xue, Wang, Wang, Ding and Zhao.	N/A	Not required	Distributed under the terms of the Creative Commons Attribution License (CC BY)
4.7B	Image	N/A	Collaborative research	Biofortuna Ltd.	01.12.2022	Yes	Written permission
5.1	Image	N/A	Funakoshi promotional material https://www.funakoshi.co.jp/	Copyright © Nippi Inc.	06.11.2022	Yes	Written permission
5.3	Image	N/A	Personal communication	Copyright © ProtonDx Ltd	18.01.2023	Yes	Written permission
6.11	Figure	N/A	Personal communication	Copyright © ProtonDx Ltd	18.01.2023	Yes	Written permission
Supplementary 18	Figure	N/A	Personal communication	Copyright © ProtonDx Ltd	18.01.2023	Yes	Written permission
Supplementary 26	Figure	N/A	Personal communication	Dr Nick Moser	19.01.2023	Yes	Written permission

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Organisation: Royal Veterinary College

Job title: Emeritus Professor of Veterinary Microbiology

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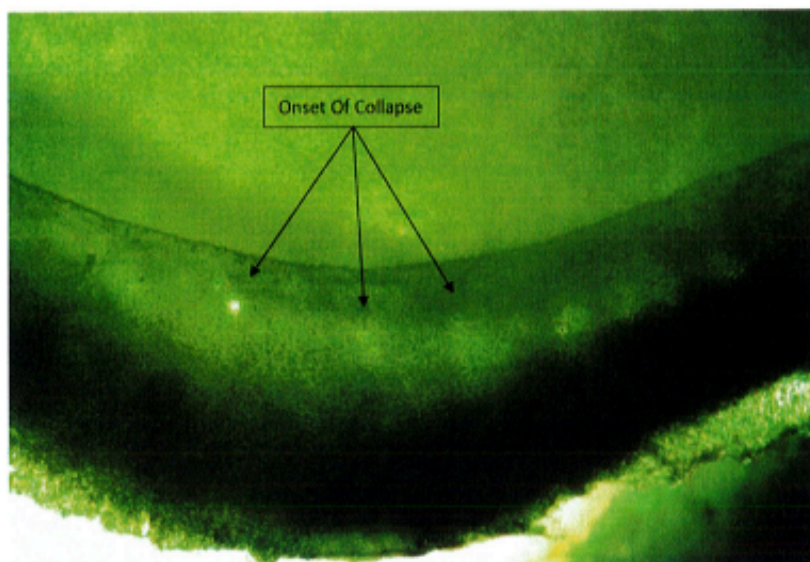
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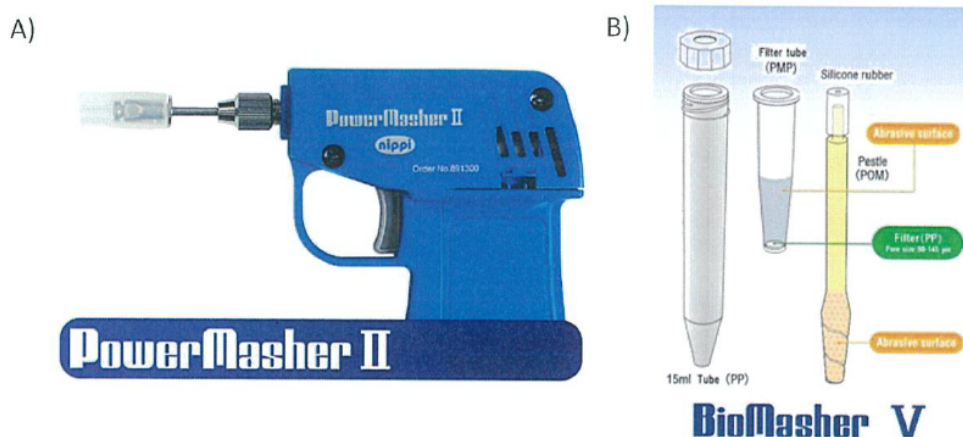
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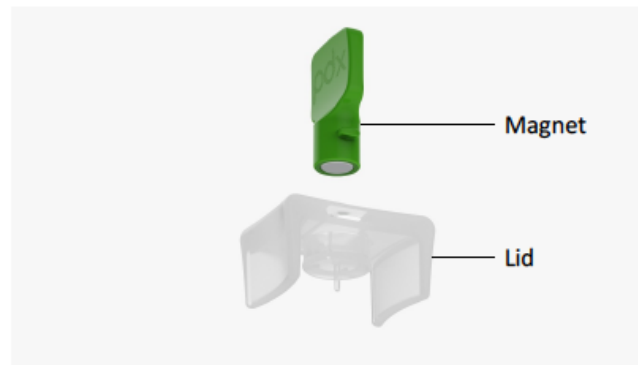
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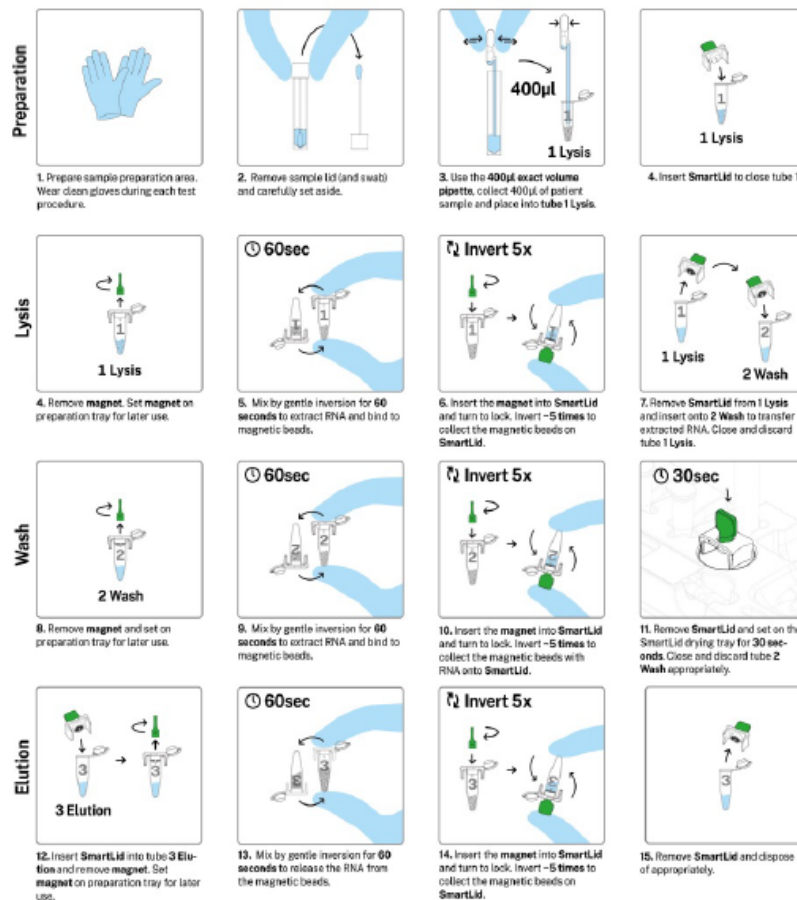
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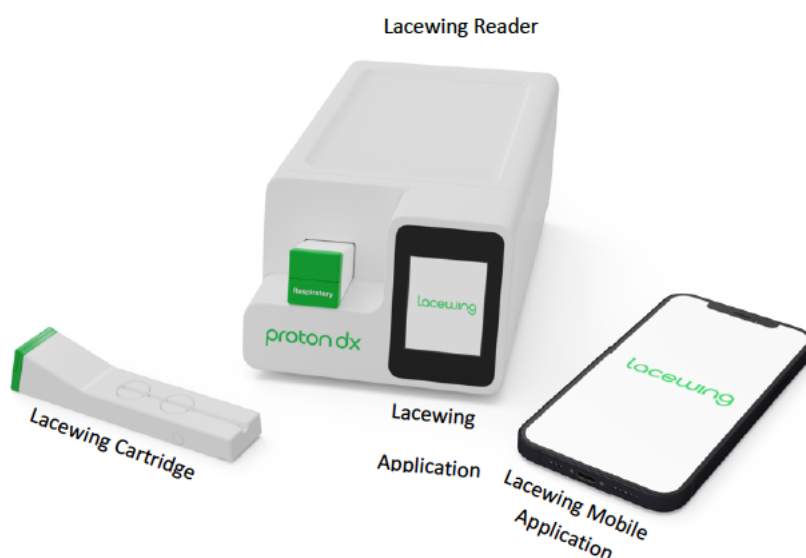
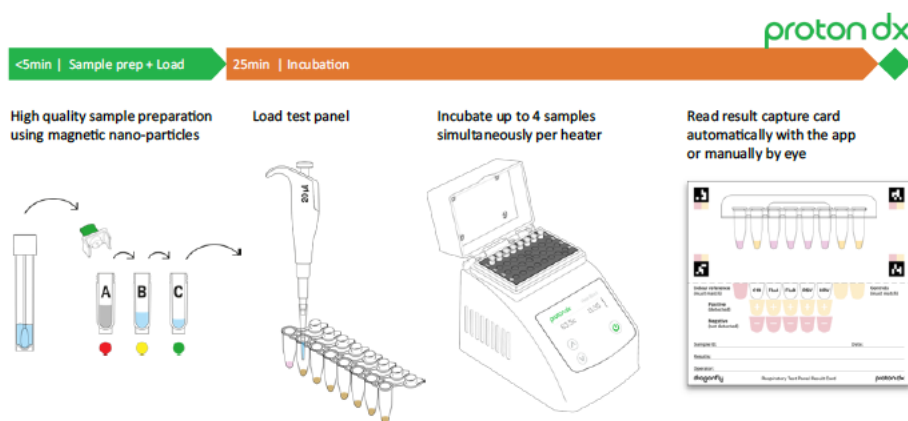


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Organisation: ProtonDx

Job title: Head of Engineering and Product Development

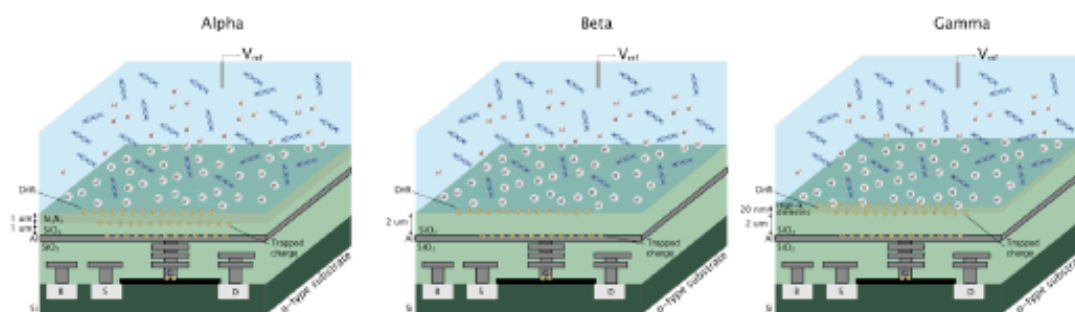
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	Alpha	Beta					Gamma				
	1	1	2	3	4	5	1	2	3	4	5
Droplet setup with DI water											
Gain [V/V]		0.42	0.42	0.43	-	0.37	0.43	0.41	0.46	-	0.41
Vref [V]		-4.73	-4.73	-4.22	-4.95	-4.76	-3.61	-3.95	-3.71	-4	-3.95
Pixels in range [%]		84.74	70.1	63.4	0	68.8	26.3	24.9	34.6	0	41.3
Offset std in range [mV]		584.3	559	603.2	-	561.6	486.1	555.6	493.8	-	601.1
Droplet setup with DI water after 4 hours of UV											
Gain [V/V]		0.43	0.42	0.43	0.43	0.42	0.4	0.4	0.33	0.3	0.4
Vref [V]		-3.29	-0.7	0.13	0.15	-0.36	1.13	2.62	3.01	2.67	1.35
Pixels in range [%]		89.3	62.3	65.6	88.1	61.6	61.6	80	74.3	77.7	48.7
Offset std in range [mV]		558.5	592.3	620.4	649.8	443.9	563.6	566	663.3	680.6	621.1
Manifold with pH buffers											
Gain [V/V]	0.41	0.42	0.43	0.43	0.43	0.41	0.4	0.42	0.41	-	0.39
Vref [V]		0.79	-4.5	3.55	5.7	4.06	1.42	-0.85	2.6	-	-0.12
Pixels in range [%]	58.8	43.5	51.3	48.1	43.7	42.8	23.5	38.5	35.1	-	40.8
Offset std in range [mV]		783	681.2	692.7	613.2	318.1	538.1	564.2	640.3	-	384.3
Output drift rate [mV/min]	8.54	-2.2	-2.3	-1.4	-1.2	-1.4	-1.6	-2.1	-1.4	-	-1.6
Output pH sensitivity [mV/pH]	5	5.2	5.1	5.3	6.8	7.5	19.9	18.8	14.5	-	17.5
Input pH sensitivity [mV/pH]		12.4	11.9	12.2	15.8	18.2	49.7	45.2	35.2	-	44.9



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Organisation: Imperial College London

Job title: Research Associate