



CRANFIELD UNIVERSITY

KARINA CAETANO SOUZA

**A PCR-BASED METHOD FOR SARS-COV-2 VARIANT DETECTION IN
WASTEWATER**

School of Water, Energy and Environment

Masters by Research

Academic Year: 2021 - 2022

Supervisor: Dr Zhugen Yang

Associate Supervisor: Professor Ewan McAdam

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ABSTRACT

The COVID-19 outbreak, caused by the SARS-CoV-2 virus, rapidly evolved into a worldwide pandemic, as declared by World Health Organization on 11th March 2020. The continued spread since early 2020 has resulted in many variants of this virus. Most mutations found along its genome are known as single nucleotide polymorphisms (SNPs) where only one base pair is mutated. Current real-time qualitative polymerase chain reaction (RT-qPCR) protocols, so called gold-standard method, are used to confirm if a person is positive or negative for the virus. There is a lack of available technology for rapid identification of variants. In order to identify the presence of a viral variant it is necessary to perform sequencing. Sequencing is expensive and may take hours to days to complete. Due to its cost, sequencing is widely unavailable in most countries. Even in countries where sequencing is available, like the UK, the number of samples sequenced are less than 10% of total cases due to extremely high cost.

Wastewater-based epidemiology (WBE) is a novel approach that would help monitor and possibly revert the current health crisis. It has been reported the presence of SARS-CoV-2 RNA in faeces of infected individuals. This makes it possible to detect and monitor SARS-CoV-2 in wastewater samples, providing a health status report of the population within the catchment. To this context, WBE also enables to monitor the dissemination of variants for early warning of the outbreak within the defined population.

In this project, a RT-qPCR method was developed targeting unique SNPs of SARS-CoV-2. This assay uses two probes, both targeting the same sequence, one with the SNP and the other non-SNP. These mutations are found in the N-gene of SARS-CoV-2 viral RNA, a conserved region that contains unique SNPs specific to each variant for differentiation. By using two probes, binding competition occurs, and the differentiation is done by observing an earlier detection with the SNP probe (~6 cycles). In addition to this, this method can be coupled with a melt curve analysis for further confirmation.

Currently, there is a lack of available technology for rapid identification of variants of concern within the community. This assay can be implemented for routine WBE. By developing a SNP-PCR assay to detect specific variants of concern using WBE, it would be possible to accurately detect variants. This information provides a comprehensive health report on the population that could possibly help revert the current health crisis.

Keywords: Wastewater Based Epidemiology, SARS-CoV-2, Real-Time Polymerase Chain Reaction, Single Nucleotide Polymorphism, Variant detection, Nucleocapsid Gene, Delta Variant, Omicron Variant.

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LIST OF ABBREVIATIONS

ACE2	Angiotensin converting enzyme 2
AIDS	Acquired Immunodeficiency Syndrome
Bp	Base pairs
°C	Celsius
CDC	Centre for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
COVID-19	Coronavirus Disease 2019
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
ECDP	European Centre for Disease Prevention
EtOH	Ethanol
FDA	Food and Drug Administration
g	Grams
HCL	Hydrochloric acid
LAMP	Loop-mediated isothermal amplification
M protein	Membrane proteins
MERS	Middle East respiratory syndrome
mL	Millilitre
N protein	Nucleocapsid protein
NaCL	Sodium chloride
PCR	Polymerase chain reaction

qPCR	Quantitative polymerase chain reaction
RCF	Relative Centrifugal Force
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RPM	Revolutions per minute
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription - Polymerase chain reaction
S protein	Spike glycoprotein
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus (1)
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
SDS	Safety Data Sheet
SGTF	S gene target failure
SNP	Single-nucleotide polymorphism
TAE	Tris-acetate-EDTA
T _m	Melting temperature
μL	Microliter
V	Volts
VOC	Variant of concern
VOHC	Variant of high consequence
VOI	Variant of interest
VUI	Variant under investigation
WBE	Wastewater-based epidemiology
WGS	Whole genome sequencing
WHO	World Health Organization

WT	Wild Type
WWTP	Wastewater treatment plant
xg	G-Force

1 INTRODUCTION

1.1 The COVID-19 Pandemic

Since the early 2000's humans have suffered with several viral outbreaks such as SARS virus (Zhong et al., 2003; Navas-Martin and Weiss, 2004), Ebola virus (Omaswa et al., 2015 ; Dixon and Schafer, 2014), Zika virus (Chang et al., 2016), and currently the COVID-19 pandemic caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) virus. COVID-19, the diseases caused by SARS-CoV-2, currently has resulted in the death of 6.31 million people (Worldometer Info, 2022) [Accessed 10/06/2022]. The World Health Organization (WHO) was informed of a cluster of cases caused by this novel virus at the end of December 2019, and a draft genome of the novel virus was rapidly identified and available on GenBank. The COVID-19 outbreak rapidly evolved into a worldwide pandemic, as declared by WHO on 11th March 2020 (WHO, 2020d). The pandemic continues to spread and kill on a global scale.

The primary mode of transmission of this disease is via droplets produced while breathing, coughing, sneezing, etc (Karia et al., 2020). In addition to the primary mode of transmission, reports have shown that SARS-CoV-2 has been detected in urine and faeces of humans which suggests a possible faecal transmission (Chen et al., 2020). Transmission is at its highest during incubation period which is suspected to be between 2-14 days. Many COVID-19 patients are asymptomatic making the spread of this disease harder to contain. It is believed that approximately 35% of infections never develop clinical symptoms, and thus are asymptomatic (Sah et al., 2021). In the attempt to contain the spread, most countries have invested in mass testing schemes and opted in blanket isolation, which involves lock-down policies (including international travel restrictions) and people staying at home.

The SARS-CoV-2 positive-sense RNA genome is approximately 30kb in size that encodes for 29 proteins. This is exceptionally long for an RNA virus as most RNA viruses such as AIDS and Ebola viruses genome size (10kb and 19kb, respectively) do not surpass 20kb. SARS-CoV-2 is an enveloped β -coronavirus (beta-coronavirus) that is genetically similar to SARS-CoV (SARS) (Kaur et al., 2021). The viral genome is surrounded by a lipid bilayer coated with a nucleocapsid protein (N). On the envelope three other types of proteins are found: spike (S) glycoprotein, envelope (E), and membrane (M) proteins. Each of these proteins play a vital role in the viral life cycle. The M protein incorporates essential viral

components into virions during morphogenesis. The N protein later connects with the M protein and the viral genome to initiate genome packaging for new viral particles. Then, the E protein forms ion channels on the viral membrane and also assists in viral assembly. As for the S protein, it is critical for the viral life as it is required for viral entry into cells. The S protein is formed by two domains, S1 and S2. The S1 sub-unit of the S protein, where the receptor binding domain is located, binds to the peptidase domain of angiotensin-converting enzyme 2 (ACE2) present on certain human cells. The S2 domain mediates further membrane fusion and allows the virus to enter the cell. It is known that both SARS-CoV-2 (COVID-19) and SARS-CoV (SARS) use ACE2 as their receptor but studies show that SARS-CoV-2 has a 10 to 20-fold greater affinity to the ACE2 enzyme than its predecessor SARS-CoV (Zheng, 2020) also making this virus more transmissible than its predecessor. The ACE2 is a peptidase that is present on the cells surface and is usually expressed in cells present in the epithelia of the lungs and small intestines. Once inside the host's cell, the virus will hijack and reform intracellular membranes to replicate its genome. The viral genome can be targeted by specific diagnostic tests to confirm the presence and quantify the amount virus in a patient. Tests such as PCR can determine the viral load, which is a numerical expression of the quantity of virus present in the fluid being tested (i.e., blood, spit, etc).

1.2 The SARS-CoV-2 Variants of Concern

The first report of a SARS-CoV-2 variant of interest was in December 2020. This variant was identified in the United Kingdom September 2020 (Chen et al., 2021), and later named B.1.1.7 due to its lineage (CDC, 2021b). Also in December 2020, another variant was reported in South Africa, named B.1.351, was first identified May 2020 (CDC, 2021b). The scientific names (i.e., B.1.1.7, B.1.351, etc.) are given after investigation of the origin of the variant and the lineage it derived from is identified. Since the appearance of B.1.1.7 and B.1.351 in December 2020, many countries began reporting variants. In Brazil, January 2021, a variant was identified and named P.1. Also in 2021, India reported another viral variant circulating, named B.1.617. Many others have been reported, and this reinforced the need for a better categorization system.

A variant categorization system was proposed by the WHO early 2021 (WHO, 2021). Variant of Interest (VOI) is a variant that present mutations that are predicted or known to affect virus characteristics (i.e., transmission, immunity, infection severity, etc.). VOI's are known to cause cluster of cases and increased prevalence. Variant of Concern (VOC) is a

viral variant that meet the criteria for VOI and now present evidence of global public health significance. VOCs are known to affect virus characteristics such as increase transmissibility, immune escape (including vaccine induced immunity), or cause more severe disease. In addition to VOI and VOC, two other categories have been created: variant under investigation (VUI) and variant of high consequence (VOHC). A VUI has been reported several times and is being monitored for characteristics of VOI. A VOHC has evidence that prevention measures have been significantly reduced (i.e., vaccines) and presents a major threat to global health. Currently, there are no VOHC reported.

Table 1: Current SARS-CoV-2 Variants of Concern.

WHO Label	Pango Lineage	GISAID Clade	Location/Date Reported
Alpha	B.1.1.7	GRY	United Kingdom – September 2020
Beta	B.1.351	GH/501Y.V2	South Africa – May 2020
Gamma	P.1	GR/501Y.V3	Brazil – November 2020
Delta	B.1.617.2	G/478K.V1	India – October 2020
Omicron	B.1.1.529	GR/484A	South Africa – November 2021

Alpha B.1.1.7

The alpha variant contains 17 mutations in its genome compared to the original SARS-CoV-2 strain (Rambaut et al., 2020). In total, 8 mutations are in the spike gene, which is essential for viral life. Due to specific mutations the virus is able to bind tightly to cell receptors and result in certain characteristics. Alpha is known to be 30 to 50% more infectious when compared to the original SARS-CoV-2 strain (J. Zhang et al., 2021). Alpha is also associated with a higher viral load in nasopharyngeal tests (Ellison, 2021). The viral load is thought to be an indicator on how infectious the person is and may be associated with increased disease severity and mortality (Fajnzybl et al., 2020).

The mutations are labelled based on the amino acid change/deletion and its location. The N501Y mutation, received this name due to an asparagine (N) changing to a tyrosine (Y) at the position 501 of the spike gene. This mutation is what allows this variant to bind more tightly to cell receptors consequently enhancing viral transmission (Liu et al., 2021). The H69-V70 and Y114-145 deletions in the spike gene result in an altered shape of the spike protein

and may assist this variant from escaping antibodies (B et al., 2021). In addition to this, the H69-V70 deletion is also responsible for a target failure in RT-PCR diagnostic tests. This is due to the primer/probes over lapping where this deletion took place and results in the primers/probes not binding properly during the assay. This failure termed 'S gene target failure' (SGTF) resulted in laboratories opting for a multiplexed RT-PCR diagnostic assay, targeting several genes in order to reduce chances of a false negative. Currently, these two (N501Y and H69-V70) are some of the defining mutations for this variant, although the other mutations may give certain advantages, there is currently no evidence they do.

Beta B.1.351

The Beta variant has caused increased worry as some studies have shown its ability to escape our vaccines (D. Zhou et al., 2021). This variant is characterized by 12 mutations in its genome, 8 are in the spike gene (Ramanathan et al., 2021). It shares the N501Y mutation with the Alpha variant which is associated with higher transmissibility (Planas et al., 2021). Beta also presents the mutation K417N in the spike gene which is also linked to higher transmissibility (Wibmer et al., 2021) The mutation that is causing the unexpected vaccine escape is E484K, also located in the spike gene. The E484K mutation is affects the receptor binding domain of the spike protein. This particular mutation reduces antibody neutralization and has shown to decrease the efficacy of the vaccines made available through emergency use (Wibmer et al., 2021).

Gamma P.1

The Gamma variant was first reported in Japan and was linked to travelers from Brazil. This variant quickly became dominant in South America shortly after its first report. Gamma is a close relative to Beta, and they share many mutations. This variant is characterized by 17 mutations in its genome, 10 of which can be found in the spike gene (Voloch et al., 2020). Sharing major mutations with other variants such as N501Y, E484K and others, makes Gamma highly transmissible and the able to escape the immune response (P. Wang et al., 2021). It also presents similar mutations such as K417T, which is similar to the K417N mutation seen in Beta. This variant has quickly become dominant in most of South America.

Delta B.1.617.2

The variant B.1.617 first appeared in India early 2021. This variant has been called a double-mutant and triple-mutant due to its many versions, currently 4 versions. The version

B.1.617.2 has been named Delta. Delta is highly transmissible, studies suggest it is roughly 60% more transmissible than Alpha (Callaway, 2021), which was 30 to 50% more transmissible than the original SARS-CoV-2. Within 3 months, Delta became dominant in most of Europe and currently is responsible for approximately 95% of current cases in the UK (Public Health England, 2021). Delta presents an increased resistance to vaccines available and poses a serious threat to those who have not taken the jab or have not taken the second dose yet (Bernal et al., 2021). This variant presents many mutations, most of which have never been seen in other variants. The mutation in the spike gene L452R allows the spike protein to bind with higher affinity to the ACE2 receptor (Tchesnokova et al., 2021). The mutation D614G, which is shared with other variants of concern, alters the fitness of the virus making it more transmissible and resistant (Plante et al., 2020). Also in the spike gene, P681R seems to give the virus a boost at infecting cells (Haseltine, 2021), this mutation is unique to the B.1.617 lineages.

Omicron B.1.1.529

The Omicron variant (B.1.1.529) was first identified in South Africa in November 2021. This variant presents a large number of mutations (50+). In the spike gene alone, there are over 30 mutations. This VOC presents characteristics of being highly contagious, more than its predecessors. Similar to Alpha, it presents the S-Gene target Failure (SGTF). It also presents the K417N mutation present in several VOC, which is known to help immune escape. By January 2022, Omicron was responsible for the vast majority of cases around the world including the UK (Public Health England, 2022). Omicron has also come with new mutations sparking interest and worry including R346K, L452X, and F486V all in the spike gene which can affect how contagious it is and how it escapes our immune system.

1.3 Wastewater-Based Epidemiology

Wastewater-based epidemiology (WBE) is a technique used to provide a comprehensive report of health status within a community. Wastewater analysis is rapidly evolving and provides the opportunity to monitor real-time data for a geographical area. This technique was theorized in 2001 (Daughton, 2001) and first implemented in 2005 (Zuccato et al., 2005). When first implemented, WBE was used to trace illicit drug use (Zuccato et al., 2008). A study conducted in Belgium showed how the implementation of a real-time approach can be successful and accurate (Van Nuijs et al., 2011). The consumption of illicit drugs such as cocaine, amphetamine and heroin were evaluated by testing daily 24-hour composite

influent wastewater samples. The number of inhabitants living in the catchment area was calculated to provide a more accurate report, this was the first time in Belgium that this was done and served as an example for future studies. The detection of cocaine and amphetamine was significantly higher during the weekend whilst the detection of heroin didn't show any significant daily variation. These results were in agreement with official statistics. WBE provided consistent and logical results which could be used by local governments to act during peak times in the hopes of reducing the populations drug usage. In addition to testing the general population, WBE also allows us to target specific places such as in a penal complex (Postigo et al., 2011).

Other targets for WBE include chemicals (i.e., drug residues and their metabolites), pathogens (i.e., Polio virus, antibiotic resistant bacteria, etc.) or biomarkers for disease excreted by humans in a given area. These can be pooled at urban wastewater treatment plants and analyzed for trends. WBE tests the presence of an analyte influent concentration per capita mass load, taking into consideration the daily flow and the population in that region. WBE presents many advantages over traditional diagnosis techniques. One advantage of WBE, particularly for the COVID-19 pandemic, is that this technique identifies the presence of SARS-CoV-2 in wastewater which corresponds to cases in a certain region. It is estimated that around 80% of transmission cases are missed because they are asymptomatic (Li et al., 2020). Though the analysis of sewage, not only would the asymptomatic/pre-symptomatic cases be considered but, it has the potential to monitor the presence of variants of concern (Department of Health and Social Care, 2021).

A recent review showed that 71% of all WBE related publications occurred since the beginning of the COVID-19 pandemic (Barcellos et al., 2023). This demonstrates how WBE has been widely used throughout the pandemic all over the world. Many countries such as Netherlands, Australia, Italy, and Germany have implemented WBE programs (Ahmed et al., 2020; Baldovin et al., 2021; National Institute for Public Health and the Environment, 2020; Westhaus et al., 2021). In the United Kingdom there is routine surveillance for viruses in certain cities like London. Poliovirus, Rotavirus, Norovirus, and Hepatitis A and B are some of the viruses under surveillance via WBE in the UK. In 2020, the UK began to monitor the SARS-CoV-2 virus in wastewater as well (Hillary et al., 2021). One of the first times a coronavirus was identified in wastewater occurred in 2005 (Wong et al., 2005).

Some aspects of WBE require more attention to detail such as how samples are collected, sample transportation, sample handling, sample preparation, concentration, and normalization (Wade et al., 2020). Samples may be collected in two ways 1) a grab sample usually taken at peak flow and 2) autosampler. A grab sample will provide information (i.e., viral load) at a single point in time whilst an autosampler will collect samples regularly over a set period (i.e., 12 hours). After collected, these samples must be kept cold (approx. 4°C) until analyzed to reduce the chances of RNA degradation. Normalization is necessary to estimate the number of people contributing to the wastewater. This is to compare the RNA to fecal load.

Some drawbacks of WBE of SARS-CoV-2 specifically, is that we are limited in understanding how the virus and its RNA remain in sewers, whether its viable or not. Infectious SARS-CoV-2 in wastewater is difficult to recover and only results in a very low concentration, despite several attempts. Another limitation is that in rural areas there are fewer converging sewer networks therefore may be more difficult to gather a comprehensive report on the community health, other alternatives in this case are needed. There have also been reports that some viruses, SARS-CoV-2 included, have still been identified in sewage even after dichlorination and UV treatment (Kallem et al., 2023). This shows that there is room to improve our current wastewater treatment plants and how we treat the sewage.

WBE provides important health information about the community. This method is practical and very cost-effective for assessing and controlling outbreaks. Specifically for viral detection, there are many ways to modify and optimize the WBE approach, for example, where qRT-PCR isn't an option (due to cost, or location limitations) LAMP may be used, as discussed in section 1.4 in more detail. This means that WBE can be used in almost any country, even in the most rural areas. As it is an objective approach, governments may use the data found by WBE to make crucial decisions on lockdown policies. WBE is one of the most promising tools to monitor the spread of COVID-19 and can serve an early warning surveillance system (Hui et al., 2020).

1.4 Current Methods for SARS-CoV-2 Detection in Wastewater

It has been reported the presence of SARS-CoV-2 RNA in feces of infected individuals (Amirian, 2020). The virus can be detected in feces even weeks after a negative nasopharyngeal test (Wu et al., 2020). Viral RNA can be detected in symptomatic, pre-symptomatic and even asymptomatic people. The presence of microbes and genetic material

in feces is not uncommon and provides a possible novel way of monitoring the spread of a disease in the community. The role of fecal shedding of SARS-CoV-2 transmission still has not been fully understood. According to the WHO, currently there are no reported cases of oral-fecal transmission of SARS-CoV-2 (WHO, 2020c). Despite the lack of evidence of transmission via the oral-fecal route, there are studies that have demonstrated viable virus present either in urine or feces (Meng et al., 2021; Xiao et al., 2020). Both SARS and MERS, both which have many similarities to SARS-CoV-2, presented some evidence of oral-fecal transmission (Yeo et al., 2020). Coronaviruses are very adaptable and can remain viable at various temperatures, including on the lower side of the scale, and in humid conditions as well, this also supports the possibility of SARS-CoV-2 being viable in wastewater. Currently, viral RNA in wastewater is confirmed via real-time polymerase chain reaction but, novel methods are being proposed that may help in monitoring the pandemic situation. RT-qPCR is the gold standard for SARS-CoV-2 detection (WHO, 2020a). There are other diagnostic tests being used as well such as, whole genome sequencing (Levy et al., 2022 ; Berggreen et al., 2023), loop-mediated isothermal amplification (Ramirez-Chavarria et al., 2022), and microfluidic devices (Cao et al., 2022).

The RT-qPCR technique uses expensive laboratory equipment and requires specifically trained professionals to execute and analyses results. This method is being used to confirm diagnosis from collected nasopharyngeal samples. RT-PCR is highly specific and relatively rapid (~1 hour) after RNA extraction is completed. As most diagnosis techniques, there are some challenges to overcome such as dealing with contaminants and collecting samples of good quality. In addition to these drawbacks, if the sample is collected too early in the incubation period it may result in a false negative, although rare (Higgins et al., 2020). This method relies on a thermocycler that involves an initial step of reverse transcription of the RNA into complementary DNA (cDNA). The thermocycler will then increase in temperature allowing the DNA strands to separate. Following a temperature decrease primers/probes will bind, and annealing will take place. The various temperature changes trigger specific chemical reactions that create, new, identical copies of the desired target (International Atomic Energy Agency, 2020). These steps are repeated over 40 cycles and results analyzed once completed. PCR primers target and bind to specific parts of the genome, TaqMan probes may also added to the mix but work differently. On the 3' end of the oligonucleotide, there is a quencher while on the 5' end there is a covalently attached fluorophore. These hydrolysis probes rely on the activity of the Taq polymerase. The dual

labelled probe will be cleaved by the Taq polymerase during hybridization to the complementary target sequence. This allows for fluorophore-based detection. The fluorescence signals are measured and result in the ability to measure the quantity of accumulation of the PCR product. In addition to this, the probe increases specificity of the assay itself. RT-qPCR, also known as real time quantitative PCR, measures the fluorescence in real time and eliminates the post-amplification confirmation analysis. The fluorescence is measured after each cycle and the intensity is indicated the quantity of RNA/DNA in the sample at that time. This PCR methods is highly sensitive and is commonly used for testing wastewater samples for nucleic acid of various pathogens including bacteria, virus, and fungi (West et al., 2023; Sharkey et al., 2023; Zhan et al., 2023). With this method it is also possible to multiplex, testing different targets simultaneously (Hayes et al., 2023).

Alternatively, loop-mediated isothermal amplification (LAMP) has gained some attention recently for SARS-CoV-2 detection (Ramirez-Chavarria et al., 2022). For RNA detection, reverse transcription loop-mediated isothermal amplification (RT-LAMP) is used. This method is unique as it allows the amplification to occur without the need of expensive laboratory equipment and is less time consuming. RT-LAMP has been widely studied during the COVID-19 pandemic as it can be an implemented in distant locations with no testing facilities (Cao et al., 2022). Amplification is done at a constant temperature and can even be implemented to test complex matrixes like wastewater. Additional benefits include visually confirmed results (colorimetric) and the ability to measure quantitatively. As most methods, some limitations are present. One limitation is that RT-LAMP is less versatile than RT-PCR. This technique cannot be used for cloning and required 4 to 6 primers usually. The primers for RT-LAMP also tend to be harder to design then primers for traditional RT-PCR.

The use of Whole Genome Sequencing (WGS) has also been highly recommended and used throughout the pandemic (Schmiede et al., 2023). This comprehensive method allows analysis of the entire genome. This is especially important for monitoring the appearance and transmission of SARS-CoV-2 variants (European Centre for Disease Prevention and Control, 2021). WGS is done by using a sequencer, a machine that is highly specific and quite complex. The sequencer identifies each nucleotide bases and provides them in order in a report format. The data is then analyzed and can take anywhere from several hours to days to be completed. As this method allows for single nucleotide polymorphism (SNP) detection, it is possible to identify which clade the sequenced virus belongs to and observe trends in mutation. WGS in combination with epidemiological data

can assist governments to make informed decisions on how to best control the outbreak (i.e., implementing more restrictions on travel, easing lockdown, etc.). Limitations of this method include data that is difficult to interpret if not properly trained, expensive equipment, and very time consuming.

Biosensors are analytical devices that are, in most cases, sensitive and specific. These powerful tools are used in our daily life (i.e., to detect insulin levels, pregnancy tests, etc.) and are now being used for detection of SARS-CoV-2, even in wastewater (Cao et al., 2022). These devices have changed the world of diagnostics and are essential now, during the COVID-19 pandemic. Lateral flow point-of-care diagnostic devices are being used as fast (10-30 min) tests for IGG/IGM antibody presence and antigen presence during the COVID-19 pandemic. A Cochrane review showed that the antigen detecting point-of-care devices were accurate 72% of cases (Dinnes et al., 2021). These devices are great to use in low resource areas and also to pool symptomatic cases for further analysis (i.e., RT-PCR, WGS).

With the oversaturation of health care units and increased demand for diagnostic testing, supply-chain shortages are affecting the capacity of testing in many places. Although these assays are specific and allows us to rapidly detect/identify positive cases, the fact that many infected individuals are asymptomatic results in part of the cases being missed and continuation of spread. There is not only a need for more testing due to increase in cases worldwide, but also a need for a multiplexed test due to the rapid emergence of variants. Ideally a test that follows the ASSURED criteria that can differentiate the variants that is more accessible than WGS

1.5 Aims and Objectives

This project aims to develop a real – time polymerase chain reaction (RT-PCR) based method to detect SNP mutations in variants of concern. Individual objectives are stated below, and this thesis also contributed to a book chapter on WBE for educational outreach. To address the overall aim the following objectives were put in place:

- (1) Bioinformatic analysis of variants of concern to determine unique SNPs. Design SNP-specific primers and probes.
- (2) RT-qPCR assay development for specific SNP detection, optimising the assay condition for a promising sensitivity for SNP detection (Delta and Omicron); and

exploring the possibility of a multiplexed RT-qPCR for the detection of multiple variants (i.e., Delta + Omicron)

- (3) Variant detection in wastewater samples (SNP-PCR assay), including validation of the developed SNP RT-PCR assay by testing raw wastewater samples (spiked and real samples); and validation of the testing with other techniques (e.g., sequencing.)

1.6 Thesis Structure

This thesis has been structured in the journal paper format with one technical chapter (Chapter 3) addressing the established aim and objectives. Chapter one is a literature review where current technologies and gaps of knowledge are address. Chapter two consists of an education and public outreach book chapter submitted to The International Microbiology Literacy Initiative. In this chapter, topics around WBE and biosensors are discussed. Chapter three titled: *Development of a PCR-Based Method for Single Nucleotide Polymorphism Detection*, is focused on the design and development of a PCR assay for variant detection in wastewater. Chapter four is the overall discussion of this thesis.

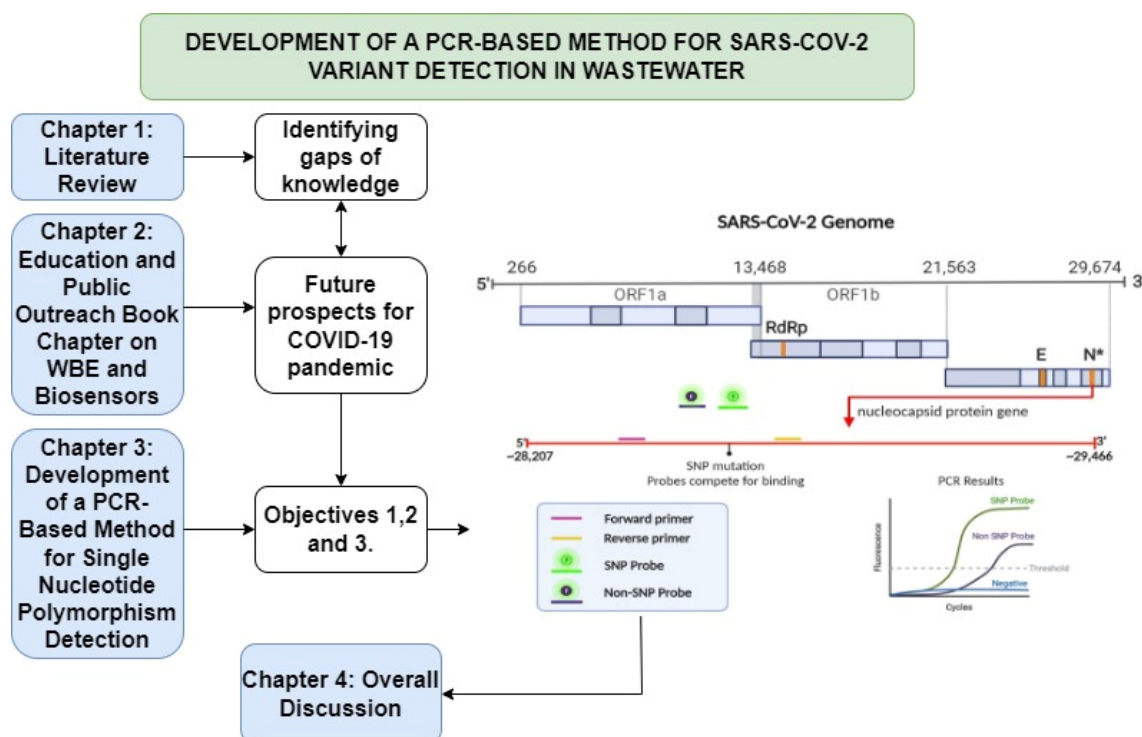


Figure 1-1: Thesis structure

1.7 References

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2 EDUCATION AND PUBLIC OUTREACH BOOK CHAPTER

2.1 Abstract

The COVID-19 pandemic has resulted in many challenges for scientists. When a microorganism spreads quickly like SARS-CoV-2 it can become hard to control. Wastewater-Based Epidemiology is a growing scientific topic that looks at testing sewage for the presence of disease-causing microorganisms. WBE provides us with a comprehensive health report on the population as we all contribute to sewage and shed microorganisms in our stool. Current testing methods like polymerase chain reaction (PCR) present some disadvantages like the long processing time. This would result in a delay in response and the virus would continue to spread. The scientific field of biosensor research and development is changing the way we test. Biosensors have been around for a long time, and we even use them on our day-to-day life (i.e., glucometer, pH strips, lateral flow tests, etc). Here we discuss the importance of WBE and Biosensor research and development. The use of biosensors for wastewater analysis could serve as an early warning system for the COVID-19 pandemic and for future pandemics.

2.2 Wastewater-Based Epidemiology (WBE)

Wastewater-based epidemiology (WBE), or wastewater surveillance, is a growing scientific topic that can help us understand disease prevalence in the population and establishes early warning system for future outbreaks. WBE involves taking a sample of untreated sewage and testing for specific targets, such as chemicals (e.g., metabolites of illicit drugs), nucleic acid (DNA/RNA) of microorganisms, so-called biomarkers, which are shed from the human. It provides a non-invasive and near real-time method to monitor the population health and disease prevalence in the community.

By analysing wastewater within the defined local treatment plant, we can see in real-time trends in health, for example, if a virus is spreading in that community. This technique was theorized in 2001 (Daughton, 2001) but first tested in 2005 (Zuccato et al., 2005). At first, WBE was used to trace illicit drug use (Zuccato et al, 2008). Drug use can be estimated by analysing untreated wastewater, this technique is now used globally. Before the use of WBE the most common methods of surveillance included population surveys, police seizures and interviews with known consumers. These methods had many limitations such as limited population data, self-report bias and being laborious. WBE was a promising and innovative

tool governments could use. Quickly this approach expanded to other targets such as pathogens (e.g., polio virus, antibiotic resistant bacteria), biomarkers of disease (e.g., for diabetes), and even human hormones (e.g., from contraception, hormone therapies) excreted in a given area. WBE has also been used to see the impact of certain products on the environment, such as liquid household waste.

There are typically five steps for wastewater analysis. First, raw wastewater is collected. Following this, samples are prepared (e.g., concentration methods) and analysed (e.g., by polymerase chain reaction, sequencing). Next, the concentration number of the target is multiplied by the daily flow rates of wastewater. The flow rate is how many litres of wastewater are processed per day. This calculation will estimate the 'daily load of the target'. This number may not be an accurate representation as the number of people using the toilet in a specific area per day may vary, and even rainfall can alter this. To correct this and obtain the estimated total daily load, a correction factor is then applied. We now know the presence of that target within the community on a specific day. In order to know how much an individual person may contribute to this total daily load we divide it by the rough number of people that serve that treatment plant. This results in the per capita value of loads within the local population (e.g., illicit drug consumption, viral load, etc). This value can be compared to other cities to see trends

Ethical considerations for any type of study must be taken into consideration by researchers, governments and institutions, WBE is not an exception to this. Data from WBE is not specific to an individual, for this reason WBE approaches are generally seen as low ethical risks. Any possible risks arising from general population studies or of site-specific studies (e.g., specific school, workplace, hospital) must be assessed and mitigations put in place. For this reason, ethical guidelines were developed by researchers from Europe and Australia and are internationally recognized (National Health and Medical Research Council, 2007). These guidelines have been revised, and amended, if necessary, on a regular basis. Mitigations suggested for WBE studies include anonymising data (i.e., omitting locations of site-specific studies), plans for effective communication to media and seeking approval from ethical committees (SCORE, 2016). Ultimately, public health monitoring by WBE benefits the general population, this is clear after the success of the Global Polio Eradication Initiative (GPEI).

In 1988, the Global Polio Eradication Initiative (GPEI) was created. This is currently known as the largest international public health initiative in history (Tebbens et al., 2010). This allowed wastewater monitoring to gain much attention and funding. Environmental surveillance of polio virus allowed scientists to identify the “silent” circulation of poliomyelitis, the disease caused by this virus. Governments used this information to adjust prevention measures such as vaccination campaigns. Since the implementation of the GPEI, the number of reported polio cases has decreased from ~350k cases in 1988 (125 countries) to 1000 cases in 2001 (10 countries) (Centre for Disease Control and Prevention, 2001).

After SARS-CoV-2 RNA was found in Dutch wastewater samples in 2020, many countries turned to WBE to help manage the situation. Success stories like the implementation of GPEI, and how it significantly reduced the number of Polio cases, are used as basis for developing wastewater surveillance in many places.

2.3 WBE as a new public health tool for the COVID-19 pandemic

The COVID-19 pandemic caused by the SARS-CoV-2 virus came with many challenges. One of the biggest challenges was that some individuals don't show symptoms, also known as asymptomatic spreaders. For others, there may be a period when they are positive but not yet showing symptoms, these people are known as pre-symptomatic. Infected individuals shed SARS-CoV-2 RNA in faeces (Amirian, 2020). In some cases, the RNA can be detected in faeces even weeks after a negative test (Wu et al., 2020). Viral RNA is detected in symptomatic, pre-symptomatic and even asymptomatic people. This makes WBE an attractive approach for the COVID-19 pandemic. For those who don't show symptoms, the individual may not see the need to be tested. This results in more cases in the region as they continue to spread the virus unaware. This is particularly worrying now that some of the variants are known to be more contagious. Consequently, we have seen a speedy increase in the number of cases in the UK and all around the world. Though the analysis of sewage, not only would the asymptomatic/pre-symptomatic cases be considered but, it has the potential to monitor the presence of variants of concern (Department of Health and Social Care, 2021).

Many countries such as Netherlands, Australia, Italy, and Germany have implemented WBE programs (Ahmed et al., 2020; Baldovin et al., 2021; National Institute for Public Health and the Environment, 2020; Westhaus et al., 2021). In the United Kingdom there is routine surveillance for viruses in certain cities like London. Poliovirus, Rotavirus, Norovirus, and

Hepatitis A and B are some of the viruses under surveillance via WBE in the UK. In 2020, the UK began to monitor the SARS-CoV-2 virus in wastewater as well (Hillary et al., 2021). Governments may use the data found by WBE to make crucial decisions on lockdown policies. Many universities around the world also began testing wastewater samples from student accommodations. One of the first studies done was at the University of Arizona, in the United States (Betancourt et al., 2020). They concluded that by using WBE and frequent testing (i.e., PCR), the transmission of COVID-19 was lower than expected as intervention measures were put in place. Sewage analysis resulted in health reports highlighting areas where action was needed. Without this information, it's likely the virus would have spread resulting in higher number of positive cases. WBE is one of the most promising tools to monitor the spread of COVID-19 and can serve an early warning surveillance system (Hui et al., 2020).

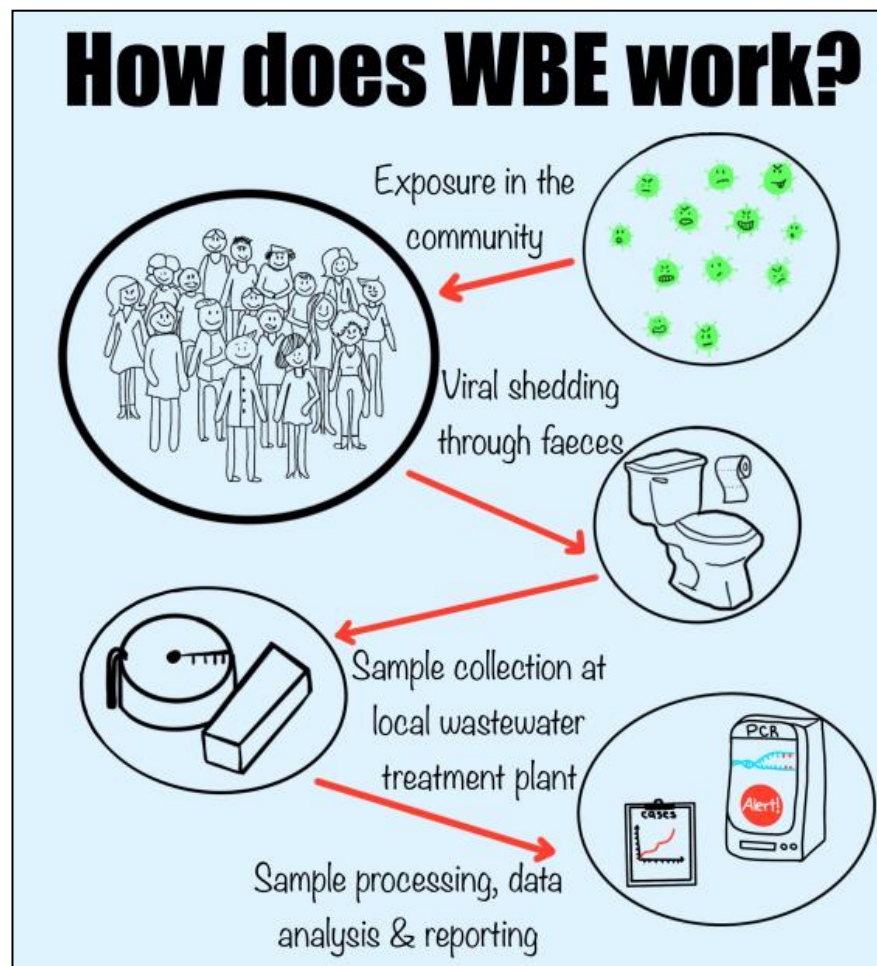


Figure 2-1: How does WBE work?

Once there is an exposure within a community, the members of that community will excrete the target of interest via faeces. This target of interest ends up in local wastewater treatment plants where samples are collected. This sample is then processed in the laboratory (i.e., concentration, PCR, etc) where a comprehensive report of that target is provided. This number is then corrected to the population number that correspond to that wastewater treatment plant. The per capita value of that target of interest can then be reported to governments for timely decisions to be made (i.e., lockdown policies).

Finding a solution to a pandemic is much more than finding an effective vaccine and treatments for the disease. It is necessary to think about how we can prevent the spread of the microorganism, which would also lower the chances of new variants appearing. In addition to this, it is important we implement new approaches that predict and prevent future pandemics. WBE is an attractive approach for the current COVID-19 pandemic and any future pandemics.

Looking at our current problem, there are many steps that are being taken to help mitigate. Polymerase chain reaction (PCR) tests are being done to confirm if a suspected individual is positive for the virus. PCR uses expensive laboratory equipment (e.g., thermocycler) and requires specifically trained professionals to execute and analyse results. It is highly specific, meaning it will be able to detect SARS-CoV-2 RNA even in a mixed sample. It is also relatively rapid taking about 1-2 hours including sample preparation. Besides the limitations mentioned above (e.g., expensive, need of trained professionals), there are some others that we should address. Current protocols being used in diagnostic laboratories don't look at identifying specific variants, meaning they produce only a negative or positive result for SARS-CoV-2 viral RNA. This information is crucial, so we understand which variants are more prevalent in the population. It is also a known fact that individuals may test positive for up to 90 days even after recovering. They aren't infectious but their bodies continue to shed viral particles (proteins and nucleic acid) that can result in a positive PCR test.

The gold-standard method for identifying variants is called Whole Genome Sequencing (WGS) or sequencing for short (European Centre for Disease Prevention and Control, 2021). It is a comprehensive method that allows scientist to see the entire genetic makeup of the virus. This is done using a sequencer, an expensive equipment that is highly specific and quite complex. It works by identifying each nucleotide base in order and provides a report in the end. Once done, the report data can be analysed to identify mutations that are characteristic of specific variants. The whole process from sample preparation to data processing can take days to be completed. Not only is the equipment expensive but it requires

highly trained professionals and hours of work. Long turnaround time means little number of samples that can be processed unfortunately.

Lateral flow tests, or rapid tests, for COVID-19 have been widely used all around the world. These tests provide results in 15-30 minutes and can be done at home. These sensors can be used to confirm if a person is positive or negative for the SARS-CoV-2 virus, similar to PCR. For this reason, they also present a similar limitation; they cannot detect variants. The sensitivity and specificity of lateral flow tests are lower than other methods mentioned, but still provide valuable information to the population. Another limitation is that there is no control on reporting results. Individuals may use these tests in the comfort of their homes and may not report their result to the government. These tests unfortunately cannot be used for WBE. Samples tested are either nasopharyngeal swabs or saliva. Wastewater is a complex matrix and could potentially interfere with the results. There are, however, biosensors that can be designed for WBE. Biosensors are used in our everyday life, including the detection of SARS-CoV-2 (e.g., lateral flow test). Biosensors usually follow the ASSURED criteria meaning quality-Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Deliverable. There are many types of biosensors which we will discuss in the next section, but how can a biosensor be a solution to the COVID-19 pandemic?

Paper-based sensors have been used for decades (e.g., pH strips) and present a promising prospect for WBE. They present many advantages for example, a paper-based sensor is cost-effective and user-friendly and can be used on site without the need of expensive equipment or trained professionals to interpret results. The make-up of these sensors tends to be very simple (e.g., paper and wax) meaning it is cheap to fabricate and easy to store. Materials used for biosensor development can be environmentally friendly and sustainable. Plastics and polymers can be detrimental to the environment, for this reason many scientists are opting for other materials. Recently there has been a growing interest in using textile from plant sources in sensing technology (Libanori et al., 2022).

With a short detection time (approximately 30 min), these sensors can assist in monitoring the pandemic as traditional detection methods are not suitable for onsite rapid detection. By using alternatives to PCR such as Loop-Mediated Isothermal Amplification (LAMP) it's possible to keep the specificity without the need of laboratory equipment. LAMP is very similar to PCR but has some advantages such as not requiring expensive equipment for its reaction to occur. LAMP also can be easily modified to target specific variants of concern as well. The

detection of variants of concern is important for pandemic surveillance. If a variant that results in high consequence (i.e., vaccine escape), it would be crucial that this variant is detected as early as possible. WBE is a near real-time and would be the best approach to this situation. Currently, paper-based sensor using LAMP technology is very promising.

2.4 Basics on Biosensors

As the scientific field of biosensor research and development became more popular, the definition has evolved. In 1997, the International Union of Pure and Applied Chemistry (IUPAC) agreed to the following definition:

A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with a transduction element. Because of their ability to be repeatedly calibrated, we recommend that a biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps, such as reagent addition. A device that is both disposable after one measurement, i.e., single use, and unable to monitor the analyte concentration continuously or after rapid and reproducible regeneration should be designated as a single-use biosensor.

The first biosensor ever made was used for oxygen detection. This discovery was done in 1956 by Leland C. Clark Jr., who is also known as the “father of biosensors” (Heineman & Jensen, 2006). His first sensor failed but, as most scientific research, the most important lessons are learned from failure. Leland wanted to measure the oxygenation of blood and his first sensor sadly failed. The electrode, which is responsible for electrical conduction, distorted the signal after it absorbed some blood components that weren't of interest. He then had the idea to using cellophane wrapper. This wrapper helped by allowing only low molecular weight substances, like oxygen, reaching the electrode to generate a signal. By using his sensor, it was possible to see an increase or reduction in current, indicating the oxygen concentration in blood. This sensor has been updated with better components however, it is a key tool used in medicine and environmental monitoring till this day.

This discovery open doors for more biosensor research and development. In 1975, Frieder Scheller, introduced a glucose sensor and later in 1977, a multi-functional biosensor using enzyme reactions (biology) and sensor technology (Renneberg et al., 2007). His work

showed that enzymes communicate with electrodes which sparked curiosity for developing biosensors with different detection methods. In the next section we discuss types of biosensors and how they are made.

2.5 Biosensors and how they are made

Biosensors have certain characteristics that make them attractive for clinical and industrial use. Optimising their properties can result in a high-performance sensor. Selectivity is undoubtedly one of the most important properties. The ability to detect a specific target in a complex sample containing multiple possible targets is important. As an example, a biosensor that can detect SARS-CoV-2, the virus that causes COVID-19, in a sample that contains other viruses such as Influenza A and B. Another important feature is reproducibility, which means that results are consistent. It's important that a biosensor is capable of giving the same results for the same sample, consistency allows us to confirm the result with confidence. Stability and sensitivity are also indispensable features to have. Stability allows concrete results even in ambient disturbances such as high temperatures for incubation of samples. Sensitivity is the minimum amount of the target the biosensor is able to detect, also referred to as limit of detection (LOD). This number is necessary to know, the lower the limit of detection the better. Generally speaking, the materials used to produce biosensors are of low-cost, this allows for mass-production and affordability once they reach the market. All these characteristics allow biosensors to have several applications. From drug discovery, disease detection and even environmental monitoring; the opportunities are endless. Depending on the target of interest, the mechanism of detection can be modified, creating a wide range of biosensor types.

Table 2: Types of biorecognition elements and types of transducers used in biosensors.

Biosensors	
Bioreceptors	Transducers
proteins, enzymes, nucleic acid, peptide, tissues, whole cells, organelles, phages, antibodies,	1. Optical (i.e., measure fluorescence, bioluminescence, surface plasmon resonance, optic fibre) 2. Electrochemical (i.e., can be conductometric, amperometric, potentiometric, and impedimetric) 3. Mass-sensitive (i.e., magnetoelastic, cantilever) 4. Calorimetric (measure colour change)

A biosensor is made up of two main parts: a bioreceptor and a transducer (Fatoyinbo & Hughes, 2012). The first, a bioreceptor is made of a biological component such as tissue, microorganisms, enzymes, antibodies, nucleic acid, etc. The bioreceptor targets specifically the target of interest, in other words, the analyte. The transducer is the mechanism of detection. A physiochemical component that detects the changes and turns it into a measurable signal. The two most common are optical biosensors and electrochemical biosensors.

Optical biosensors

This type of biosensor has a transducer which uses light to measure a signal. Optical biosensors are very interesting as they are easy to read. They are able to correlate various changes (concentration, mass, number of molecules) in light characteristics (Damborský et al., 2016). A great example of an optical biosensor is those that detect bacteria for medical diagnostics. Surface plasmon resonance (SPR) is a type of optical sensor that is used to detect the interaction between two molecules (Bakhtiar, 2013). SPR is commonly used in drug discovery and is of great importance.

Electrochemical biosensors

Electrochemical biosensors can be further classified into three groups: conductimetric, amperometric and potentiometric (Manjavacas & Nieto, 2016). The classification is

dependent on the electrical source. The most common targets (analytes) are nucleic acid (DNA/RNA), drugs that bind to nucleic acid, and glucose. Conductimetric sensors measure electric conductivity. This is possible as electrochemical reactions result in ions or electrons and the conductivity of the solution is altered. The downside to conductimetric sensors is that they generally have low sensitivity. Amperometric sensors are the most common electrochemical biosensors used. They have high sensitivity and target electroactive species present in biological samples. This is achieved by producing a current proportional to the concentration of the target of interest for detection (e.g., oxygen detection biosensor) (Janata, 2009). Potentiometric sensors are less commonly used as they present some challenges. The oxidation or reduction potential is calculated after an electrochemical reaction. The current flow caused by the electrochemical reaction occurs when a voltage is applied to an electrode placed in sample solution. The glucometer, which we will discuss more about in the next section, is a type of electrochemical biosensor. It is used by millions of diabetics around the world every day and its discovery were a great advancement in biosensor research and development history.

Other biosensors

Biosensors have a wide application for various targets and many new options are being introduced to the market. The possibilities of infinite due to the adaptability of these devices. Let us explore a few more different types of biosensors below.

Bioluminescence sensors use enzymes have the ability to emit photons, which are particles that carry energy (Yeh & Ai, 2019). The process of emitting photons is a result of a reaction is known as bioluminescence. Bioluminescence sensors measure the intensity of the energy release by using specific enzymes as bioreceptors which bind to target nucleic acid. Bioluminescence sensors can be used to detect a wide range of microorganisms but are of particular interest for bacterial detection.

Resonant biosensors that use an acoustic wave transducer in conjunction with antibodies. The analyte (e.g., a viral protein), is added to a membrane resulting in a change of diversification in the mass. This is measured by resonant frequency by the transducer and measured. Biosensors like this are generally used in laboratory as they are more complex.

Thermal biosensors which measure the absorption or production of heat. The change of temperature is a result of a biological reaction caused by the analyte and the bioreceptor.

The transducer then measures the change in heat and calibrates it against the concentration of the analyte (Ramanathan & Danielsson, 2001). These types of biosensors can be used for detection of pesticides and other targets like bacteria.

Nanotechnology is advancing rapidly, and nano biosensors is one of the most recent advancements in the area (Manimekala et al., 2022). Nano biosensors are powerful tools for rapid detection of genetic disorders, pathogens, drug screening and other applications. They work by measuring electrical changes caused by interactions with nanomaterials such as nanoparticles, nanotubes and quantum dots. Nanoparticle sensors usually use metallic nanomaterials and nanotube sensors generally used ferric based nanomaterials. In some cases, carbon nanomaterials may be used as well. These sensors can be used to detect various targets from microplastics to genetic material.

Wearable sensors are also an emerging type of sensors reaching the market (Sharma et al., 2021). These sensors can be present on items we use (e.g., masks) or even stick to skin. The possibilities are infinite with wearable sensors. They can be used to detect proteins, presence of illicit drugs and microorganisms via sweat or even air droplets. More advanced glucometers used by diabetics can also be wearable.

2.6 Sensors and Biosensors in our daily life

We use biosensors almost every day, they have now become a part of our daily tasks. Let's dive in further to understand how four of the most common day-to-day biosensors work: pH strips, pregnancy tests, glucose test for diabetes, and lateral flow tests for COVID-19.

Lateral flow tests for COVID-19

Lateral flow tests (LFT) for viral detection have become extremely popular since the beginning of the COVID-19 pandemic. There are many names to this type of sensor such as: rapid test, antigen test, antibody test, lateral flow test. The working principle is very similar to a pregnancy test. LFT use immunosensing technology. Immunosensing involves using coloured labels, typically antibodies, for a biological reaction and readout.

A nose swab sample is collected and mixed in a buffer solution (step 1 –Figure 2-2). The buffer solution is to assist the sample in travelling down the LFT. The buffer also ensures a suitable interaction with the materials of the test, this helps reduce any chances of skewed results. The base of a LFT is a nitrocellulose membrane where other membranes, or pads,

will be added for specific purposes. The sample-buffer solution is added to the first pad known as the sample pad (step 2 – Figure 2-2). The sample pad in some immunosensors may act as a filter and ensure accurate and controlled flow. The sample will travel to the next pad, the conjugate pad. On the conjugate pad there are mobilised antibodies that are specifically labelled with dye-coloured particles. These particles are most commonly colloidal gold and latex microspheres. If positive these antibodies will bind to the virus present in sample (step 3 –Figure 2-2). The sample will then move onto the next step, the target zone. The target zone has two main areas, the test line (T) and control line (C). On each line specific antibodies are immobilised. On the T line the antibodies target SARS-CoV-2 (the analyte), similar to mobilised antibodies found on the conjugate pad and therefore will bind to the virus in sample (step 4 – Figure 2-2). On the C line, the immobilised antibodies bind to the mobilised antibodies from the conjugate pad which will have travelled along with the sample, regardless of being positive or negative (step 5 – Figure 2-2). A control line is essential to have as it shows that the sample has been prepared correctly and proper liquid flow down the strip. The results are colorimetric as the antibodies have been tagged by coloured particles. This can be assessed by eye and the intensity of the colour indicates how much analyte present in sample. The last membrane is an absorption pad, to absorb the extra sample-buffer solution and extra mobilised antibodies that did not bind to the T or C lines (step 6 – Figure 2-2).

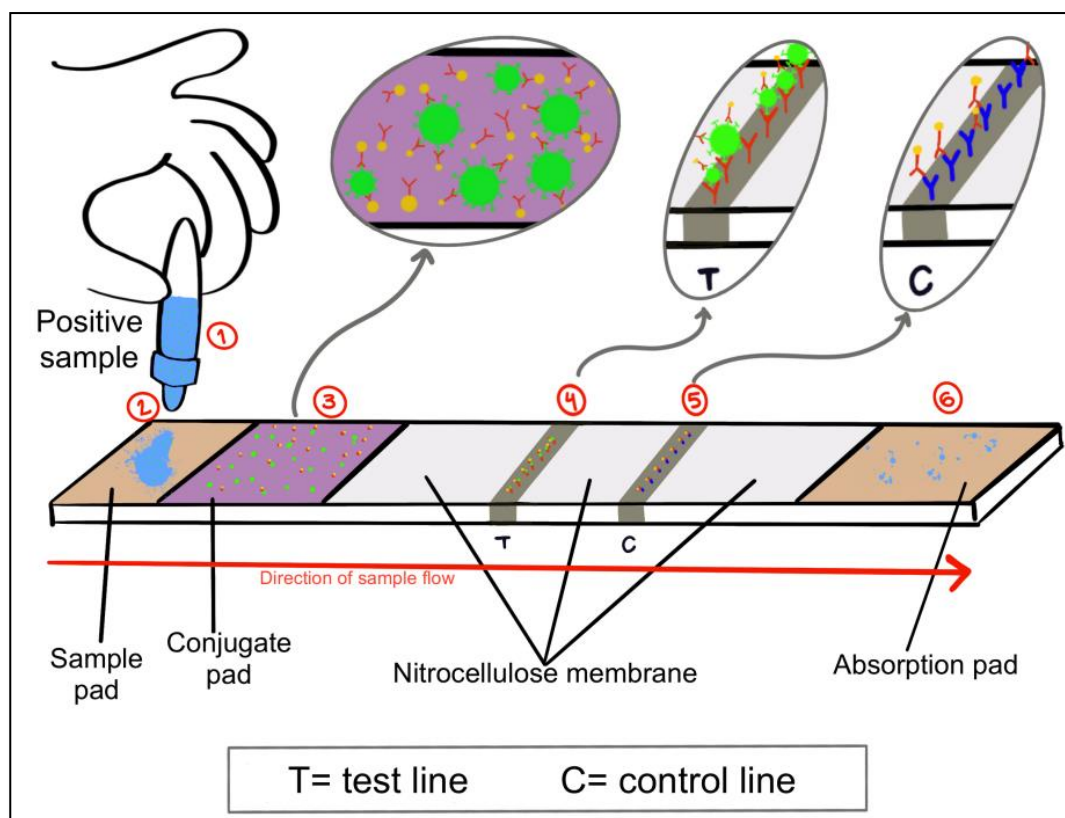


Figure 2-2: Lateral flow tests for SARS-CoV-2.

A nasopharyngeal swab sample is added to a buffer. The sample is then added to the test strip. The solution will then travel down the strip from the sample pad to the conjugate pad. Here, if positive, viral particles will bind to dye-coloured mobilised antibodies. The solution will then travel to the nitrocellulose membrane where two lines of immobilized antibodies are present. The first line (T) contains the same antibodies found on the conjugate pad (immobilized). A positive sample will result in a coloured line due to presence of virus. The second line (C) is a control to ensure the test has occurred as expected. Immobilized antibodies on the C line bind to the mobilised antibodies from the conjugate pad. Finally, any left-over sample will travel to the absorption pad.

Immunosensors like COVID-19 LFTs tend to be cheap, user-friendly and rapid. By having a long shelf life, these tests can be easily deployed and used in developing nations. There are some limitations, for example, inaccurate sample preparation results in reduced precision, limited room to optimize this test (i.e., design for specific variant detection), and limited sensitivity. Overall, LFTs have been a great tool during the pandemic and have also led to advancements in biosensor research.

pH strips

You may have seen these strips being used at your local GP to test urine, saliva and even water. These are great examples of paper-based sensor. Depending on the sample used, for example urine, when the strip is dipped in solution a colour change occurs. The strip contains pH indicators that change colour based on the sample's acidity or alkalinity. These pH indicators are immobilized weak acids and bases that will easily change colour when in contact with a different pH. A great example is methyl-red. When methyl-red comes in contact with a pH5 solution it will appear red. In contrast, in contact with a solution that has pH 6 it will become yellow. Currently, there are many different brands and types of pH strips on the market so sensitivity and range they test for may vary. For this reason, it's important to carefully select the right pH strip for the job. After the strip is dipped in solution and changes colour, a colour legend is provided so that you may compare the results. We can use pH strips to test urine and saliva is due to the changes bacteria cause to our natural pH. Normal urine pH can range between 4.6 to 8. Higher pH values (8+) for urine can indicate a urinary tract infection. In contrast, a lower pH (less than 4.6) can be an indication of kidney stones or other health problems. This information helps GP's decide if further tests are necessary.

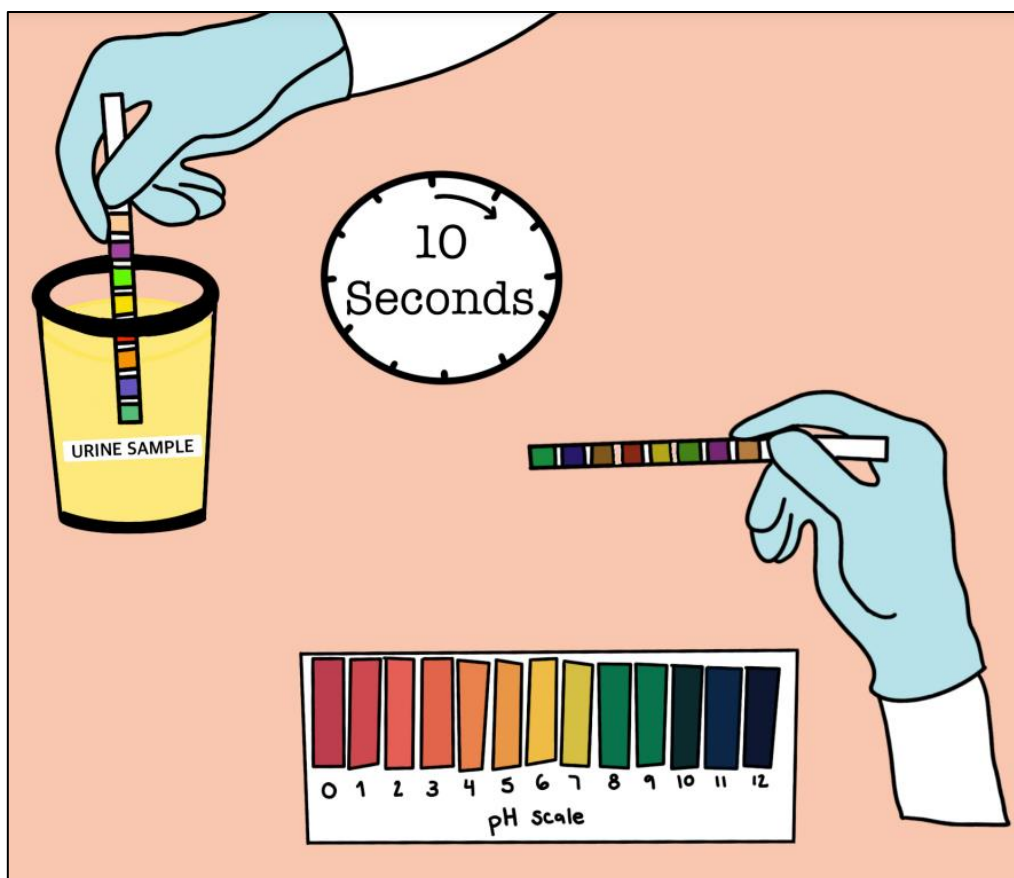


Figure 2-3: Biosensors in our daily life: pH Strips.

By using immobilized weak acids and bases, it is possible to see a colour change depending on pH of the solution. In the example image above, it's possible to see a colour change after reading a urine sample for 10 seconds. This colour change can be observed and using a reference pH scale, the results can be further interpreted.

Pregnancy tests

There are two types of pregnancy tests, urine and blood. Blood tests are done in a laboratory and urine tests are done using a biosensor at home. When a woman gets pregnant her body quickly starts preparing and this results in a lot of hormone production. Human chorionic gonadotropin (hCG) is a hormone produced primarily by cells present in the placenta. As hCG isn't usually produced when the person is not pregnant, it's a great target to indicate pregnancy. Urine pregnancy tests work by reacting to the level of hCG in urine. When the sample is added to the test there are mobilised antibodies that specifically bind to hCG. These mobilised antibodies are coloured with a dye and the colour can be seen during the readout of the test. After the antibodies bind to the hormone, they will travel down the strip and reach two lines of immobilized antibodies. One line is the test line, where if coloured indicates presence of hCG, or positive result. The second line is a control line, to indicate that the test has worked properly. The immobilized antibodies on both lines are different. On the test line you will find the same type of antibody that bind to hCG. The control line has antibodies that do not bind to hCG but bind to the mobilised antibodies. Most are optical sensors, where you will have a visual read out. This mechanism of action is very similar to the rapid tests for COVID-19. Over the years, pregnancy tests have been optimized and currently they have a high accuracy rate.

Glucose test for diabetes

Blood sugar levels, also known as blood glucose, change depending on what we are doing and eating throughout the day. For diabetics it's very important to monitor their blood glucose as their condition makes them more susceptible to having too high or too low glucose in blood. Diabetics have a hard time regulating this naturally and may even need to take insulin in some cases, a hormone that helps glucose enter cells. As this is a test that needs to be done on a daily basis, it would not be feasible to have them come in for blood tests every day. A biosensor was created to solve this problem. There are different types of glucose monitors available. The two most popular are: a continuous glucose monitor that is inserted

just under the skin, and a handheld glucose meter (glucometer for short). We will be discussing the mechanism of action of the handheld glucometer.

A small drop of blood is obtained by pricking the finger with a lancet. This drop of blood is placed on a strip that is inserted in the glucometer. The strip contains capillaries that allow the blood to travel and reach a chamber where a reaction will occur. Glucose present in the blood reacts with a bioreceptor, in this case it's the enzyme glucose oxidase (or dehydrogenase). The bioreceptor is then re-oxidized in the presence of an ion, also present in this chamber. The ion generates an electric current that can be measured. The amount of charge travelling is proportional to the amount of glucose in the blood that resulted in the reaction. There are, generally speaking, three electrodes in glucose test strips. One electrode is for the reaction current and the other two are counter/reference electrodes. In order to the glucometer to provide the correct blood glucose levels, its necessary to have references to compare with.

This reaction method is known as amperometric, where the meter measures the charge and uses an algorithm to estimate and give a number of blood sugar levels. However, the readout method (transducer) may vary. In some cases, there is a colorimetric change (optical biosensor) that can be compared with a standard. The glucometer tends to be accurate most times however, there are factors that might skew results. This includes ambient temperature, handling of the sensor, sensor not properly calibrated (amperometric), blood sample quality, etc. In general, the use of this sensor has dramatically improved the quality of life for people living with diabetes.

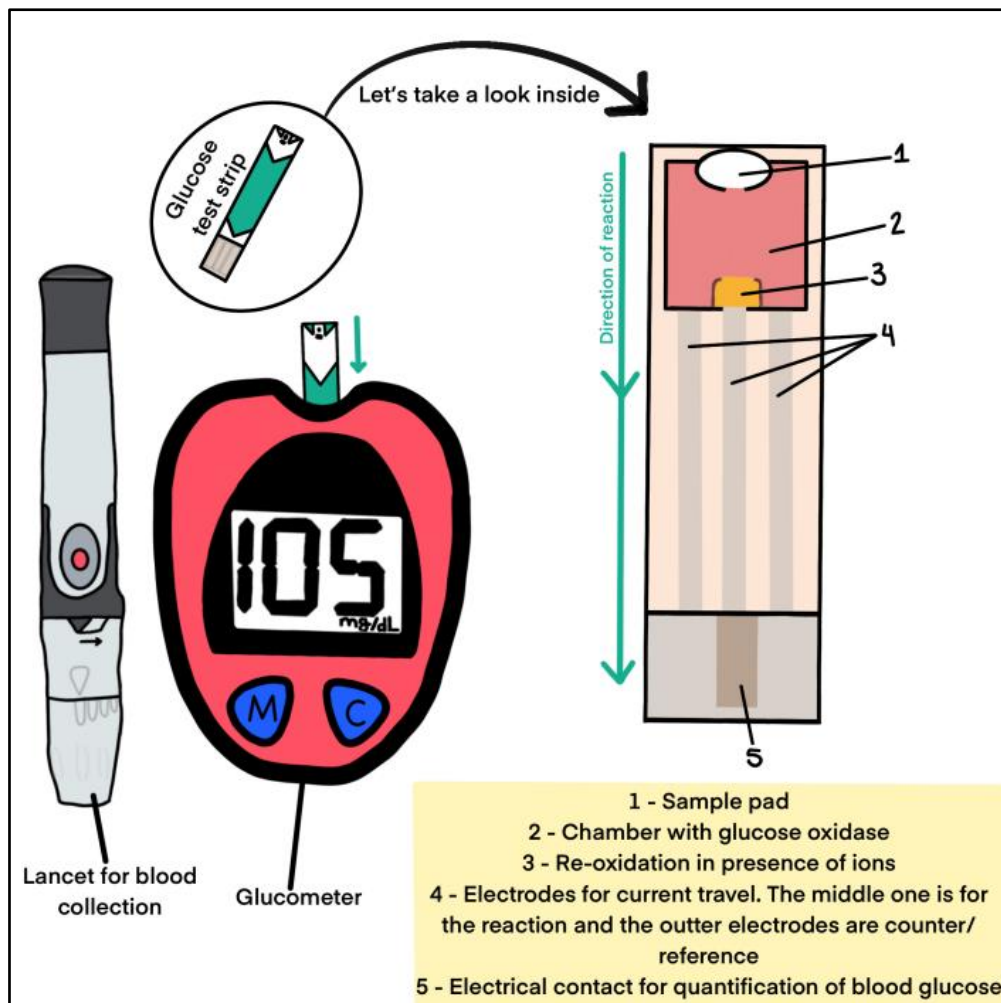


Figure 2-4: How does the glucometer work?

In the image above it is possible to see in more detail the inside of the glucose test strip where the glucose in a blood sample is captured. It is possible to see a chamber where the sample will arrive and come in contact with glucose oxidase. The sample will then interact with ions that will generate an electrical current. The current will travel down the middle electrode and finally reach the end point of the strip. At this stage the current is measured by the device and the estimate glucose in blood (mg/dL) is provided on the screen. The outer electrodes are counter/reference used for comparison.

Cranfield University, researchers work on developing biosensors for water and wastewater. Figure 2-5 below shows how a paper-based biosensor works on detecting SARS-CoV-2 in wastewater. The first step in developing a paper-based biosensor is to design it using online applications (red step 1 – Figure 2-5). Once you have found the ideal design for your sensor, it is printed using black wax instead of ink (red step 2 – Figure 2-5). The printed sensor is then placed on a heat block to allow the wax to diffuse to both sides (red step 3 – Figure 2-5). Wax is hydrophobic, meaning it doesn't mix with water. Water is the

base for all our samples. By using specific designs, we can control the sample flow in a specific orientation.

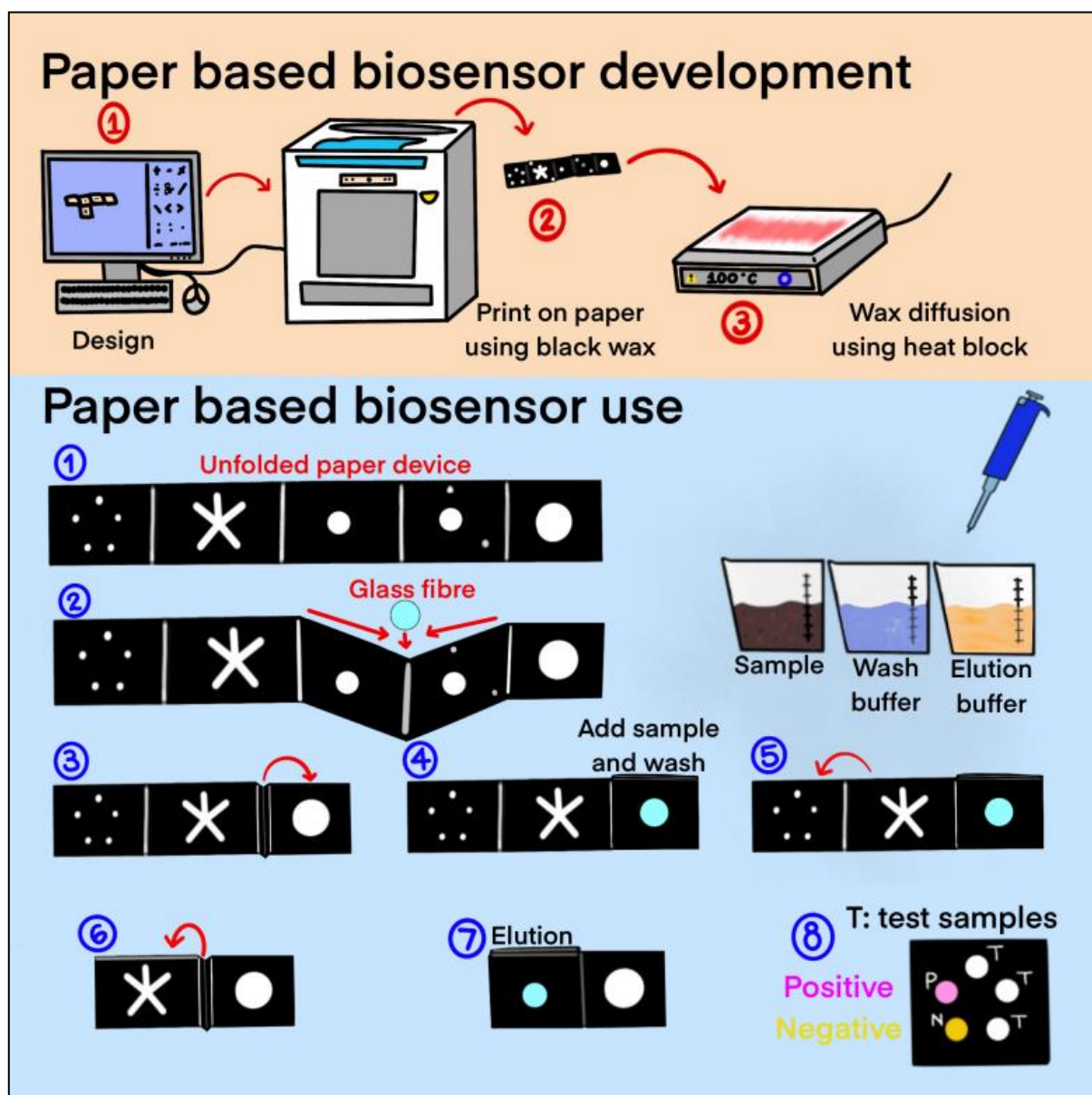


Figure 2-5: Paper-based biosensor for SARS-CoV-2 detection.

Top section showing the process of designing and developing the sensor, individual steps are shown in red numbers. Blue section below shows the use of the sensor for testing real samples (simplified version). Individual steps are labelled in blue numbers and described in text. Colorimetric readout identifying positive samples as pink and negative samples as yellow.

Now that you have a functioning biosensor, it's time to test real samples. Our design (shown below) contains 5 areas (seen in blue step 1 – Figure 2-5). A sample of wastewater is

collected and to that sample we add a lysis buffer. This buffer helps to break down everything that isn't of interest (proteins, inhibitors, cell walls, etc) but leaves the nucleic acid (RNA/DNA) intact. First step is to fold between area 3 and 4 (blue step 2 – Figure 2-5) and insert glass fibre between them. Our target is nucleic acid, which binds to glass fibre. The flap containing the glass fibre is folded to the right (blue step 3 – Figure 2-5) and this is where we add the sample that has been lysed. We need to ensure that everything that isn't of interest is washed off so to the same area we add a wash buffer (blue step 4 – Figure 2-5). Now our nucleic acid is clean and bound tightly to the glass fibre, we let this sit for a bit. Area 1 and 2 are folded together in preparation for the next steps (blue step 5 – Figure 2-5). We turn the flap containing the target of interest to the left (blue step 6 – Figure 2-5). Now it's time to unbind the nucleic acid. A strong elution buffer is added (blue step 7 – Figure 2-5), and this unbinds any nucleic acid. The nucleic acid travels down the capillaries reaching each tip of area 1. The clean nucleic acid is used to perform LAMP, as mentioned previously. If there is SARS-CoV-2 nucleic acid, a colour change will occur, and a positive result will appear pink (blue step 8 – Figure 2-5). If the sample is negative for SARS-CoV-2, the sample will appear as yellow (blue step 8 – Figure 2-5).

2.7 Biosensors for WBE

We have discussed wastewater-based epidemiology (WBE) and how it can be beneficial for managing the COVID-19 pandemic and also biosensors and how they are powerful, innovative tools. Bringing these two growing scientific areas together could change and promote how we analyse wastewater currently for public health assessment. Using biosensors that are rapid, sensitive and provide on-site detection, we can improve our public health understanding and response to outbreaks.

Metabolites for drug abuse

The drug abuse is always a problem worldwide, and how to accurately estimate the drug consumption remains a challenge due to the illegality of drugs. Currently, it is possible to monitor the community-wide drug use trends through WBE, which detects drug or drug metabolites from the wastewater (Choi *et al.*, 2018). Many biosensors have been developed for drug analysis, and a few have been also introduced to WBE.

The first biosensor to detect cocaine in sewage was designed in 2016, which was a novel electrochemical aptamer sensor (Yang *et al.*, 2016). They used this sensor to assess

changes in cocaine concentrations in a city in England over a seven-day period to understand drug consumption trends in this community. The results showed that higher cocaine concentrations could be observed on weekends compared to weekdays. There was another type of sensor developed for multiplex drug identification that can test cocaine and methamphetamine (METH) at the same time (Mao, Ma, Li and Yang, 2019). Novel Au/Ag nanoparticles targeting METH and cocaine respectively were synthesized and added to solution. When you add wastewater that contains these two illicit drugs to the solution, it will cause colour change. This colorimetric analysis may further play an increasing role of monitoring of illicit drug of abuse.

Population markers

When using WBE for quantitative or per capita assessment of health-related information, it is important to accurately estimate the number of populations in the survey area. Instead of adopting the data of real population size, concentrations of some molecules in the wastewater were used to represent the citizen level (O'Brien *et al.*, 2014). These types of biomarkers are usually virtually non-degradable and are easily detectable.

A typical approach is to estimate the population size of a wastewater treatment plant catchment area by studying biomarkers associated with human metabolism. The advantage of endogenous biomarkers, which are excreted into sewage through live urine and faeces, is that they are highly correlated with human activity. Estrogen is a common endocrine substance found in wastewater and is often considered as an endocrine disruptor (Bellet *et al.*, 2012). This is because even at very low concentrations it can cause endocrine disruption in animals and humans, with serious implications for the health of adolescents in particular. There is a biosensor for the analysis of oestrogen, based on a technique called surface-enhanced Raman spectroscopy (SERS) (Liu *et al.*, 2019). As the concentration of the sample varies, the spectrum will show peaks of different intensities, which indicate the level of oestrogen in the wastewater.

Microorganism detection

Sewage is rich in various biochemicals, thus provides ideal conditions for different types of microorganism, such as bacterial, virus and fungi. Some of them may contribute to waterborne microbial diseases. It is believed that the concentration of some pathogens could reflect the disease rate in the region, but conventional microscopic approaches for pathogens

detection are time-consuming, insensitive and lack accuracy (Ejeian *et al.*, 2018). As an alternative technique, biosensors are applied to this field for real-time identification.

A portable optical fibre-based system was developed to distinguish *Escherichia coli* (E. coli) from other pathogens in wastewater effluents (Yildirim, Long and Gu, 2014). They mixed known concentration of aptamers, a kind of short DNA sequence, with the wastewater samples that contains E. coli, and aptamers could combine the E. coli. The biosystem detects the left free aptamers, which could not hybridize with the E. coli, and generate fluorescence signal to monitor the concentration of E. coli. The higher the fluorescence intensity, the lower the concentration of E. coli. This portable analysis could be carried out within one hour on-site, with high specificity and high sensitivity.

Pandemic surveillance

The outbreak of COVID-19, caused by the highly infectious virus SARS-CoV-2, has highlighted the need for widespread monitoring of infection prevalence. Nevertheless, accurate estimation of the extent of SARS-CoV-2 infection remains challenging because of limited diagnostic testing capabilities and asymptomatic and oligosymptomatic infections. As an alternative surveillance method, WBE is proposed to measure the emergence and spread of SARS-CoV-2 in communities, because SARS-CoV-2 is demonstrated to be present in faecal and urine samples from infected populations (Jones *et al.*, 2020).

The changes in virus concentration and diversity are useful information for assessing the temporal and spatial patterns of virus infection (Mao *et al.*, 2020). Besides, detections in wastewater are likely to precede the identification of new COVID-19 cases in the community. Accordingly, the use of WBE in the early stage of virus outbreaks makes it possible to identify where the virus is starting to develop and spread, and to take early warning and intervention measures based on the analysis.

Currently, sewage analysis during the COVID-19 outbreak has primarily relied on the lab-based polymerase chain reaction (PCR) technology, which is time-consuming and expensive. Biosensors, which have already been used for point-of-diagnostics, are promising to provide a more efficient way for WBE. One has reported that the paper-based platform that could be used for the quantitative monitoring of genetic biomarkers within communities through the analysis of wastewater (Yang *et al.*, 2017). Similar technique (e.g., Lateral Flow Test) has been applied for COVID-19 test during the pandemic for nucleic acid testing.

Although there are few commercially available biosensors that could be introduced for on-site WBE monitoring at the moment, it is believed that paper-based device holds great potential for this application as relevant technologies are widely used for sewage analysis and individual test.

2.8 Conclusion and Perspectives

Although sewage is a complex matrix, this matrix contains a variety of biochemicals that can provide important information about drug consumption, chemical exposure and public health through sewage research. The rapid development in the field of biosensors makes it possible to detect biomarkers or pathogens from urine, sera, and saliva. In addition, given the fast response times, low cost, minimal sample handling, high data resolution and remote operation capabilities, these novel sensors can be used for wastewater analysis, promoting the wider application of WBE. Biosensors hold great promise as a powerful analytical tool in wastewater research, but a number of challenges remain. Since the complex matrix of sample may affect and interfere the detection, biosensors are required to have a broader detection range. In addition, it is desirable that a single biosensor is capable of multiple detection at the same time. Besides, it is necessary to evaluate biosensors with the conventional wastewater analysis methods. We believe that effluent sensors can reach the ultimate aim of on-site monitoring and give real-time data within the scope of WBE by addressing these challenges and technical restrictions. WBE is seen as ethically low-risk and highly accurate. Using WBE for outbreaks has been successful before when the Global Polio Eradication Initiative was created in 1988.

For the COVID-19 pandemic and future pandemics, biosensors in WBE will provide valuable information. Not only would the data provide a real-time analysis of public health, but it may also assist in the detection of novel variants. By using biosensors over traditional methods (i.e., PCR, sequencing) it would also reduce time and cost drastically whilst providing population level information. Biosensors can be easily stored, and its user-friendly design allows for it to be used for on-site detection. We believed that paper-based biosensors have the capability to be applied to WBE, offering real-time and continuous information. This early warning system will allow scientists and governments to respond in a timely manner. By using WBE and biosensors, the transmission of COVID-19 can be reduced as intervention measures are put in place. WBE is one of the most promising tools to monitor the spread of

COVID-19 and can serve an early warning surveillance system (Hui et al., 2020) for the government for effective intervention and management public health.

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3 DEVELOPMENT OF A PCR-BASED METHOD FOR SINGLE NUCLEOTIDE POLYMORPHISM DETECTION

3.1 Abstract

Testing methods used to detect SARS-CoV-2 currently cannot provide variant specific information. Whilst these methods are very accurate in determining a positive/negative result, they do not provide the whole picture (i.e., if in sample a viral variant is present). Wastewater-based epidemiology (WBE) has grown exponentially in the past years and is very promising as it provides a comprehensive health report of the population. Developing methods that can provide information such as if a specific variant is present in sample, in conjunction with WBE, can be essential tools during pandemics. In this chapter we discuss the development of a PCR-Based method to detect single nucleotide polymorphisms (SNPs). SNPs are mutations that occur in the viral genome and result in viral variants. In some cases, a single SNP is responsible for a new variant that can pose significant threat. By choosing unique SNPs that match to specific variants, we are able to determine their presence in a positive sample. The intended use of this PCR-based method is for WBE, where this vital information can allow scientists and governments to respond to pandemics in a timely matter (i.e., by implementing lockdowns, mass testing, vaccinations, etc). Data collected by the development of this method can also be used to develop more protocols using other techniques such as LAMP allowing testing to be done onsite or in rural areas. By developing a variant-specific method for WBE, we can provide better statistics on variant dissemination and improve our response to the pandemic.

3.2 Introduction

COVID-19 disease, caused by SARS-CoV-2 virus, evolved into a worldwide pandemic rapidly. The pandemic was officially declared by the World Health Organization (WHO) on 11th March 2020. The continued spread since 2020 has resulted in many variants of this virus (Alexiev et al., 2021). Most of the mutations found along the genome are known as single nucleotide polymorphisms (SNPs) where only one base pair is changed. Some variants can be more transmissible and possibly cause more severe disease (Ciuffreda et al., 2023). There is, however, a lack of available technology for rapid identification of variants. Wastewater-based epidemiology (WBE) is a novel approach that would help monitor and possibly revert

a health crisis like the COVID pandemic. WBE tests the presence of an analyte influent concentration per capita mass load, taking into consideration the daily flow and the population in that region. The presence of SARS-CoV-2 RNA in faeces of infected individuals was reported early on in the pandemic (Park et al., 2020). This makes it possible to detect and monitor SARS-CoV-2 in wastewater samples, providing a health report of the community. This technique has been used before for other viral diseases such as Polio and proved to be very helpful (Battistone et al., 2014).

Current approved methods to detect SARS-CoV-2 include lateral flow tests and reverse transcription polymerase chain reaction (RT-PCR) based methods. These methods, however, are used to confirm if a person is positive or negative for the virus. In order to confirm the presence of a viral variant it is necessary to perform sequencing. Sequencing requires skilled personnel, is expensive and may take hours to days to complete. Due to its cost, sequencing is widely unavailable in most countries. Even in countries where sequencing is available, like the UK, the number of samples sequenced are less than 10% of total cases (data from GISAID, 2022). For these reasons, it is much harder to monitor the dissemination of a variant.

The possibility of using RT-PCR to detect variants of concern has been explored by some groups around the world. In most cases this method is coupled with another such as mass spectrometry minisequencing (Zhao et al., 2021) high-resolution melting curve analysis (Barua et al., 2021) and even ion-pair reversed-phase high performance liquid chromatography (Sascha et al., 2021). These protocols have proven work by detecting a shared mutation among variants and therefore can confirm if samples are variant positive or negative. However, these protocols do present some limitations. The main limitation being their target of interest, a shared mutation among variants. The defining mutations are mostly found in the S gene which encodes for a vital protein known as the spike. Targeting a shared mutation will not help identifying specific variants that pose more threat, such as the Delta variant (B.1.617). The S gene due to its important role in the viruses life cycle, is prone to having many mutations. Over the months we have seen increased numbers of mutations in that gene specifically. Thus, targeting a gene that is more conserved and mutations unique to specific variants would be a much better option. Moreover, coupling with other methods may also result in increased work time and some methods may be inaccessible in diagnostic laboratories (i.e., chromatography).

There is clearly a lack of available technology for rapid identification of variants of concern within the community. By developing a SNP-LAMP assay to detect specific variants of concern using WBE, it would be possible to accurately detect variants. This information would provide a comprehensive health report on the population that could possibly help revert the current health crisis. To this end, this project proposes to develop a quantitative RT-PCR assay that targets variant-specific mutations found in the N gene of SARS-CoV-2. This method would allow us to not only detect variants but also to differentiate between them. This assay will provide a solid foundation and vital information for developing rapid identification of SNPs beyond SARS-CoV-2.

3.3 Materials and Methods

Solutions in this study are described as volume/volume (v/v) or as weight/volume (w/v). All necessary chemicals, reagents and solutions used have risk assessment and CoSHH paperwork. Safety Data Sheet (SDS) of all reagents can be accessed via Cranfield Intelex system. Individual reagents and kits used in this study are mentioned in text.

Water used in this study was obtained from a Veolia PURELAB Chorus 1 Complete. The water is ultrapure (type 1 laboratory grade), double-distilled, deionized, bacteria negative, pyrogens negative, DNase and RNase free. When unavailable, Invitrogen nuclease-free water was purchased from Thermo Fisher Scientific (UK) and used in laboratory work.

All work done in this thesis obtained ethical approval via CURES system (Reference: CURES/14139/2021)

3.3.1 Collection of Samples and Processing

Samples from Cranfield's Wastewater Treatment Plant (WWTP) were collected using the ISCO 3700 sampler. The sampler was programmed to collect 250 mL of wastewater every hour for a total of 12 hours. All tested samples were influent collected from balance tank prior to any treatment. Following 12 hours of collections, the influent samples were mixed. One sample of 500 mL was collected from the mixture. Samples were then transported on ice to the laboratory and maintained at 4°C until processed.

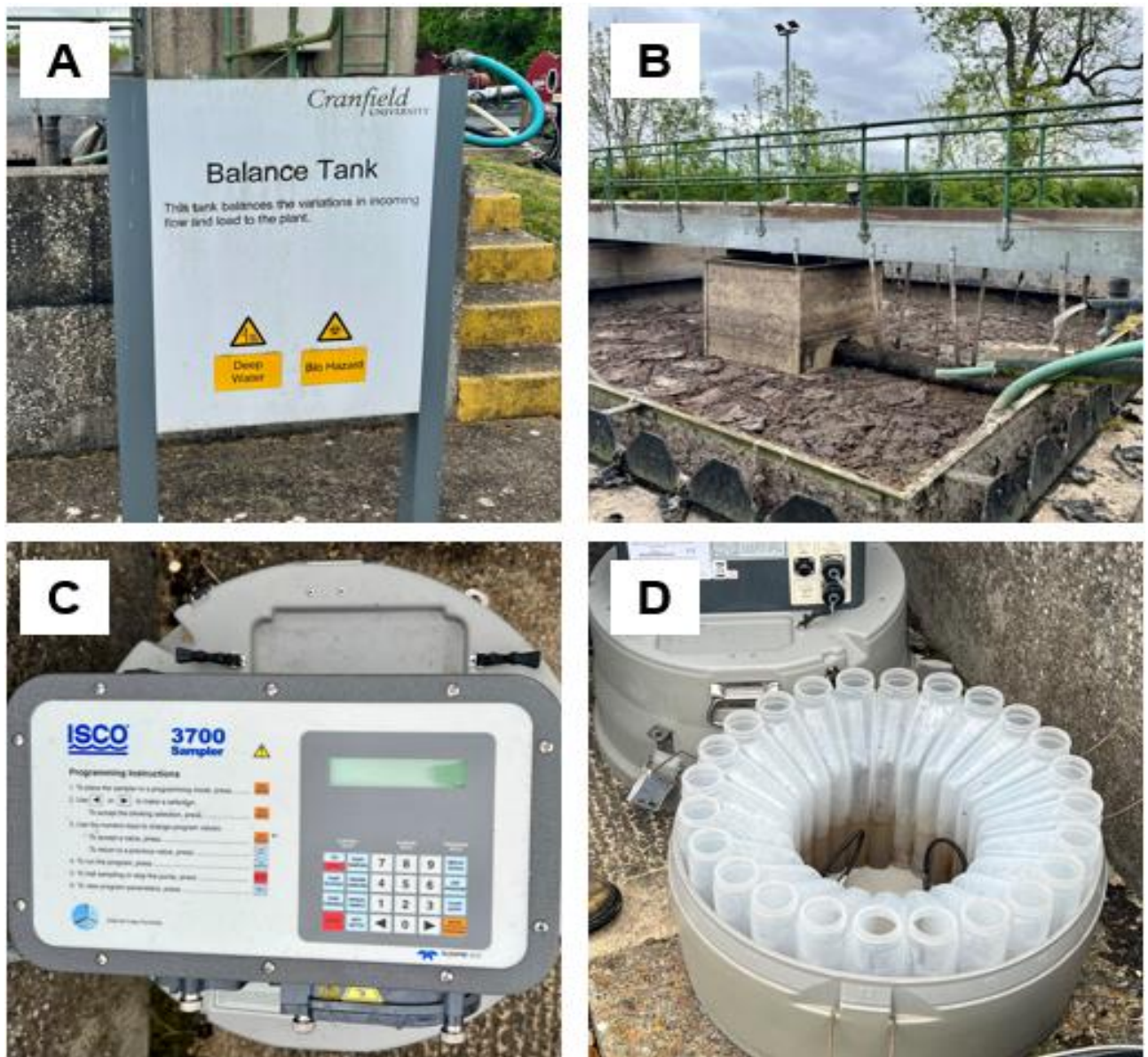


Figure 3-1: Cranfield University Wastewater Treatment Plant. (A,B) Sample collection point, the balance tank, an essential part of wastewater treatment plants. Designed to contain a large volume of wastewater relative to flow in and out. (C) ISCO 3700 sampler. (D) 250 mL collection bottles for sampler.

PEG Precipitation-based method

Samples were homogenized by inverting the bottle several times. Four aliquots of 50 mL were then centrifuged at 3,000 xg for 30 minutes in a 4°C cooled centrifuge (Thermofisher Scientific Megafuge 8R Centrifuge). Following centrifugation, 150 mL of supernatant was transferred to a 250 mL PCCO bottle without disturbing the pellet. The pH of the samples were measured and, if needed, corrected to 7-7.6 using 1M NaOH or 1M HCL. To each 150 mL sample, 50 mL of a 40% PEG 8000, 8% NaCL solution (Sigma-Aldrich) was added and inverted several times to mix. Incubation overnight (~12h) in 4°C fridge. The following day samples were centrifuged at 10,000 xg for 30 minutes in a 4°C cooled centrifuge. 1 mL of supernatant was retained, and excess supernatant was discarded into a waste container with 70% Ethanol solution (EtOH). The pellet/supernatant mix was centrifuged at 12,000 xg at 4°C for 10 minutes. Supernatant was pipetted away, and pellet retained.

The pellet was resuspended in 800 µL of TRIzol reagent (Thermofisher Scientific) followed by vortexing for 15 seconds. At this point no large particles should be visible. To the solution, 0.2 mL of chloroform (Thermofisher Scientific) was added for lysis. The samples were thoroughly mixed by shaking or vortexing and left to incubate at room temperature for 2-3 minutes. Samples were then centrifuged for 15 minutes at 12,000 xg at 4°C. The mixture will then separate into three phases: a lower red phenol-chloroform phase (proteins), an opaque interphase (DNA), and a clear upper aqueous phase (RNA), this can be seen in Figure 3-2. At this point, the upper phase was transferred to a new tube and the rest of the mixture discarded. Samples at this point were kept on ice for RNA purification or stored at -20°C until use.



Figure 3-2: Wastewater concentration by PEG-Precipitation. (A) Centrifuged sample after overnight incubation. (B) Pellet obtained prior to TRIzol resuspension. (C) Phase separation following TRIzol and Chloroform addition. Arrow pointing to the RNA layer.

Ultrafiltration-based method

Samples were homogenized by inverting the bottle several times. Two aliquots of 50 mL were then centrifuged at 3,000 xg for 30 minutes in a 4°C cooled centrifuge (Thermo Scientific Megafuge 8/8R Centrifuge). Without disrupting the pellet, the supernatant was collected. In a 10 kDA ultracentrifugal filter falcon tube (Amicon Merck), 15 mL of supernatant was added. The tubes were centrifuged for 45 minutes to 1 hour at 3,000 xg at 4°C. The flow through was discarded and 15 mL of sample was added to the top filter. Centrifugation was repeated at 3,000 xg for 45 minutes to 1 hour at 4°C. Flow through was discarded and the sample remaining above the filter was transferred to an RNase-free eppendorf. Samples at this point were kept on ice for RNA extraction/purification or stored at -20°C until use.

RNA extraction and purification

Buffers used (Table A-4) for RNA extraction and purification are supplied in the QIAamp® Viral RNA Mini Kit and prepared using manufactures protocol.

Prior to any work, the biosafety cabinet was properly sterilized using the UV light for 20 minutes and surfaces cleaned with a 70% EtOH solution. RNA Zap (Thermofisher Scientific) was used to clean the surface and, on any equipment/instruments to be used (i.e., centrifuges and pipettes). Samples concentrated using ultrafiltration methods follow the protocol from beginning. Samples concentrated using PEG-precipitation methods follow protocol after first incubation period.

To a tube containing 800 µL of AVL Buffer with carrier RNA, 200 µL of the corresponding sample was added. The mixture was then vortexed for 15 seconds followed by a quick spin in the centrifuge. The tube incubated at room temperature for 10 minutes. Following incubation, 800 µL of 100% EtOH was added to the sample and vortexed for 15 seconds. For samples concentrated using PEG; 800 µL of EtOH was added to 200 µL of sample (no lysis buffer needed). The tube is then quickly spin in the centrifuge to allow the liquid to settle at the bottom. To the QIAmp Mini column in a 2 mL collection tube, 630 µL of the mixture was added. Samples were then centrifuges for 1 minute at 8(x1000) RPM. Flow through was discarded and any remaining sample was added to the column to then be centrifuged again. Once all the sample has been added to the tube and the flow through discarded, the column is transferred to a new 2 mL collection tube.

To the column in the new collection tube, 500 µL of AW1 buffer is added. The sample is then centrifuged for 1 minute at 8(x1000) RPM. The filtrate should then be discarded, and the column transferred to a new collection tube. A second wash step is applied by adding 500 µL of AW2 buffer to the column. Samples should then be centrifuged for 3 minutes at 13.3(x1000) RPM. Again, the filtrate should be discarded, and the column transferred to a new collection tube. To elute the RNA, 60 µL of AVE elution buffer is added to the column and the samples left to incubate at room temperature for 1 minute. Following incubation, the sample should be centrifuged for 1 minute at 8(x1000) RPM. Viral RNA is then collected at the bottom of the tube, and this can be stored at -20°C until further work.

3.3.2 Design and Characterization of Single Nucleotide Discriminating Primers and Probes

Identification of SNPs in Variants of Concern

Whole genome sequences for SARS-CoV-2 were collected from platforms such as National Centre for Biotechnology Information (NCBI) and Global Initiative on Sharing Avian Influenza Data (GISAID). GISAID was originally created for rapid sharing of influenza data however, it has expanded to sharing data on SARS-CoV-2 and other viruses.

Sequences for all variants were complete genomes, high coverage and confirmed to be the variant of interest. In order to identify SNP's, using the NCBI BlastN program, sequences of interest were aligned to the original SARS-CoV-2 Wuhan Sequence (NC_045512.2, TaxID: 2697049). A comparison between 10 or more sequences of a specific

variant was done in order to confirm which SNPs were recurring and not unique to that sequence. The gene of interest for this project is the nucleocapsid (N) gene which starts at position 28274 and ends at position 29533 in the SARS-CoV-2 Wuhan sequence. For specific variants, the start/stop positions may vary due to deletions or mutations. The general size of the gene is 1200 base pairs (bp).

Primer Design

Primers used can be found in Appendices (Table A-1), all primers were ordered from Sigma-Aldrich. Bioinformatic analysis previously done allowed for several mutation candidates to be identified. Once the mutation was selected, forward and reverse primers were designed. The product size (gap between primers) aim was between 70-200 bp. Primers contained GC content between 40-60% to promote binding, increasing stability. Length of primers varied between 18-30 bp. The melting temperature (T_m) varied between 65°C and 75°C. Primers that presented secondary structure were avoided. Control primers were also used, CDC N1 and CDC N2 forward and reverse primers were used and do not overlap any mutations. All primers were checked using Integrated DNA Technologies online software OligoAnalyzer.

Primers arrive lyophilized and should be centrifuged prior to resuspension in nuclease-free dH₂O. In order to prepare a stock solution of 100 micromolar (μ M), the amount nuclease-free dH₂O needed was calculated by multiplying by 100 the number of nanomoles (nmol).

Design of SNP Discriminating Probes

Probes used can be found in Appendices (Table A-1) all probes were ordered from Sigma-Aldrich. Using bioinformatic analysis mentioned previously, several mutation candidates were found for each variant of concern (VOC). Among the several candidates, one SNP for each VOC was selected as a target. The specific SNPs selected were in ideal locations, allowing 70-200 bp between the forward and reverse primers, conserved in more than 90% of sequences searched using GISAID and NCBI and rarely found in other strains.

For each VOC, two probes were designed. One probe containing the mutated base pair and the second containing the original base pair (Wuhan reference). In order to differentiate them, different fluorophores were added. In the presence and absence of the SNP, the probes will compete for binding and the probe with highest accuracy will result in more fluorescence (Figure 3-3). The probes were design using Primer-blast, provided by the

NCBI. The location of the SNP in the probe varied, however, the aim was to maintain the SNP as close as possible to the 3' end to increase stability. Secondary structure and T_m for both probes were checked prior to ordering to ensure both could be used in the same assay. Probes were quenches to minimize noise and maximize point fluorescence. Control probes were also used, CDC N1 and CDC N2 probes were used and do not overlap any mutations. All probes were checked using Integrated DNA Technologies online software OligoAnalyzer.

Probes arrive lyophilized and should be centrifuged prior to resuspension in nuclease-free dH_2O . In order to prepare a stock solution of 100 micromolar (μM), the amount nuclease-free dH_2O needed was calculated by multiplying by 100 the number of nanomoles (nmol).

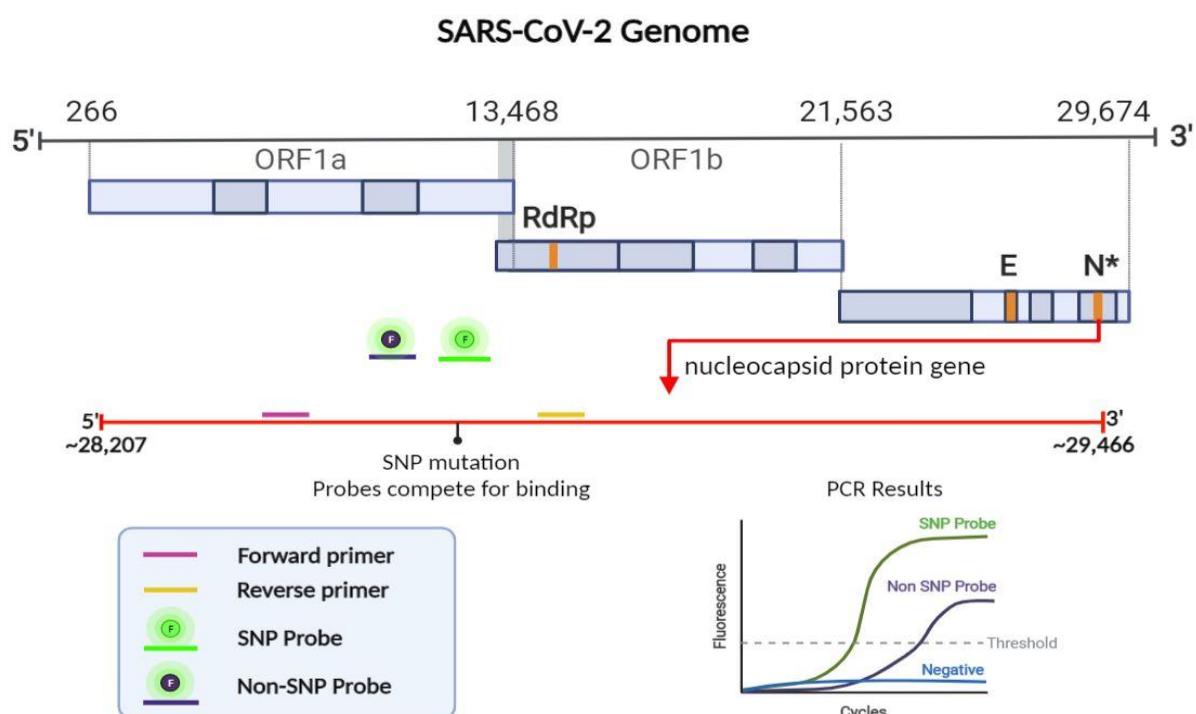


Figure 3-3: Rationale of the PCR-based SNP detection assay. Image made using Biorender.

3.3.3 Optimization of Single Nucleotide Polymorphism RT-PCR Assay

Artificial gene sequences and plasmids

Artificial gene sequences for the N-gene (Wuhan, Delta and Omicron) were purchased from Eurofins Scientific (Figure A-1, Figure A-2, Figure A-3). These gene sequences are dsDNA and arrive lyophilized. To prepare a stock solution of 1 nanogram (ng) per μL , add nuclease-free dH_2O in the same amount of concentration (i.e., 300 ng = 300 μL of nuclease-free dH_2O). Allow the solution to incubate for 1 hour at room temperature. For long term

storage the fragments can be kept at -20°C. In order to calculate the copy number per microlitre, the equation below is used. From the prepared stock solution, serial dilutions are prepared to make up 10⁶ copies/ µL, 10⁵ copies/ µL, 10⁴ copies/ µL, 10³ copies/ µL, 10² copies/ µL, 10¹ copies/ µL and 10⁰ copy/ µL.

$$\text{number of copies (molecules)} = \frac{x \text{ ng} \times 6.0221 \times 10^{23} \text{ molecules/mol}}{\left(N \times 660 \frac{\text{g}}{\text{mol}}\right) \times 1 \times 10^9 \text{ ng/g}} \quad (3-1)$$

For internal positive control, a pEX-A128 plasmid containing all SARS-CoV-2 genes (Wuhan) was purchased from Eurofins Scientific. The plasmid arrived lyophilised (1 µg) and was resuspended in nuclease-free dH₂O. (Figure A-4). From the prepared stock solution, serial dilutions are prepared to make up 10⁶ copies/ µL, 10⁵ copies/ µL, 10⁴ copies/ µL, 10³ copies/ µL, 10² copies/ µL, 10¹ copies/ µL and 10⁰ copy/ µL.

Polymerase Chain Reaction for N-gene Amplification for Transcription

The PCR reaction mix was prepared by mixing: 10 µL Brilliant III Ultra-Fast QPCR Master Mix with Low ROX (Agilent Technologies), 4 µL Betaine (Sigma-Aldrich), 1 µL SYBR Green (Thermofisher Scientific), 2 µL dH₂O, 0.5 µL forward primer (N-Gene-F-T7), 0.5 µL reverse primer (N-Gene-R-T7) and 2 µL artificial gene sequence (Wuhan, Delta or Omicron). Internal negative controls were added by replacing the artificial gene sequence with dH₂O.

The PCR was run using a QuantStudio PCR Machine (Thermofisher Scientific). PCR conditions can be seen in Table 3-3 below. Additionally, a melt curve analysis was performed at the end of the PCR. This assessed the dissociation of the PCR product and the probe by monitoring fluorescence from 65°C to 95°C with a temperature transition rate of 1°C per second.

Table 3-3: PCR conditions for N-gene Amplification prior to Transcription

N-Gene PCR for T7 Transcription	Temperature (°C)	Duration	Cycles
Initial Denaturation	98	5 minutes	1
Denaturation	98	15 seconds	40
Annealing	55	30 seconds	40

Extension	72	30 seconds	40
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Gel Electrophoresis and RNA/DNA Extraction from Gel

Information on reagents for Gel Electrophoresis can be found in appendices (Table A-5). To prepare a 2% agarose gel, 1g of agarose (Thermofisher Scientific) was weighed and mixed in 50 mL 1x TAE buffer solution (Sigma-Aldrich). When necessary, the volume of agarose was adjusted (i.e., for a 1.2% agarose gel: 0.6g agarose in 50 mL 1x TAE). The solution was microwaved for 30 seconds to 1 minute and the flask swirled until agarose fully dissolved. Once the agarose was fully dissolved, 5 μ L of Safe View Stain (Thermofisher Scientific) was added and the flask swirled.

In the casting tray with the comb already inserted, the molten agar was poured and left to sit for approximately 40 minutes. Once the gel has set, the colour will be opaque, gel was inserted in the gel tank (Bio-Rad) in the correct orientation (negative to positive). In the gel tank, 1x TAE buffer was poured, observing the max line, until the gel is submerged. This was allowed to sit for a minute and then the comb removed from the gel.

For each sample of 20 μ L, 5 μ L of loading buffer (Thermofisher Scientific) was added. To the gel, 10 μ L of the RNA or DNA ladder (Thermofisher Scientific) was added to the first well. Each sample was then loaded to individual wells. Once done, the lid was placed, and power supply connected to the corresponding electrodes. The gel was then run at 90 volts (V) for 50 minutes. Depending on product size, the voltage and duration was adjusted. Once done, the gel was visualized using the GeneSnap Software (2008) from SynGene.

Upon identification of target of interest, the gel was cut around the band using a scalpel knife. The small gel band was then placed in a microcentrifuge tube. In order to extract the target of interest, the Zymoclean Gel DNA Recovery Kit was used. Kit components can be seen in appendices (Table A-6). To the tube, 3 volumes of ADB was added (e.g., for 100 μ L of agarose gel add 300 μ L of ADB). The mixture was incubated at 55°C for 10-30 minutes. The molten agarose solution was added to the spin column in a collection tube and centrifuged for 1 minute at 10,000 – 16,000 xg in room temperature. The flowthrough was discarded and to the column, 200 μ L of Wash Buffer was added. Then, a centrifugation step at the same speed previously used for 30 seconds. At this point, the column was transferred

to a RNase-free microfuge tube and 6-20 μL of Elution Buffer was added. The sample was centrifuged at 16,000 $\times g$ for 1 minute in room temperature. Ultra-pure DNA is now extracted and ready for transcription.

RNA Transcription and Purification

In order to transcribe the artificial gene sequences (dsDNA) to RNA, the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) was used. The reaction mix was assembled by mixing 2 μL ATP (100 mM), 2 μL GTP (100 mM), 2 μL UTP (100mM), 2 μL CTP (100 mM), 2 μL T7 Polymerase mix, 2 μL $\times 10$ reaction buffer, 5 μL dH_2O , 3 μL template of interest. The solution was mixed thoroughly by vortexing. Finally, incubation at 37°C for 4 hours in order to produce RNA. Dependant on transcript size, the incubation period was adjusted for maximum yield.

The RNA was purified using the Monarch RNA Cleanup Kit (New England Biolabs). Reagents from the kit can be seen in (Table A-7). To begin, a starting sample size of 50 μL was used and adjusted using nuclease-free water if necessary. To the sample tube, 100 μL of RNA Binding Buffer was added. Immediately after, 150 μL of >95% EtOH was added. The sample was mixed by pipetting or flicking, vortexing is not recommended. The solution was then loaded onto the column and centrifuged in room temperature for 1 minute at 16,000 $\times g$. The flow through was discarded and column was transferred to a new collection tube and 500 μL of RNA Wash Buffer added. Centrifugation step was repeated using same conditions. The wash buffer step and centrifugation were repeated again. At this point the column should be transferred to a new RNase-free microfuge tube. The RNA was eluted using nuclease-free water by spinning in room temperature for 1 minute at 16,000 $\times g$. Volume of nuclease-free water varied from 6-20 μL , dependent on need. The RNA can be immediately used or stored in -20°C until use.

Measuring RNA/DNA concentration using Qubit

DNA and RNA can be quantified using the Qubit 4 Fluorometer (Thermofisher Scientific). This device includes a built-in reagent calculator to determine the amount of dye and buffer required for sample and standard preparation. Target specific assays are purchased from Thermofisher Scientific.

The High Sensitivity double-stranded DNA assay (HS dsDNA) has a quantitation range of 0.1 to 120 ng. Initially high concentration and low concentration standards are

measured. Once completed, samples can be mixed in detection buffer provided and measured for quantitation. Results can be seen on touch screen. Samples under or over the quantitation range can be measured using the Broad Range double stranded DNA assay (BR dsDNA).

The High Sensitivity RNA assay (HS RNA) is highly selective and also tolerates contaminants such as salts, free nucleotides, solvents, detergents, or proteins. The quantitation range for this assay is 5 to 1000 ng. Initially high concentration and low concentration standards are measured. Once completed, samples can be mixed in detection buffer provided and measured for quantitation. Results can be seen on touch screen. Samples under or over the quantitation range can be measured using the Broad Range RNA assay (BR RNA).

Optimizing the Concentrations of the Primers and Probes

A working stock solution of primers and probes of 10 μ M, 20 μ M and 30 μ M was prepared. Reaction mix was prepared by adding 5 μ L master mix (TaqMan® Fast Virus 1-Step Master Mix Thermofisher Scientific), 8 μ L dH₂O, 0.5 μ L forward primer, 0.5 μ L reverse primer, 0.5 μ L of probe(s), and 5 μ L artificial gene sequence. Internal negative controls were added by replacing the artificial gene sequence with dH₂O. PCR was performed using artificial gene sequences at 10⁴ copies/ μ L. Conditions of the PCR can be seen in Table .

Table 3-2: PCR conditions for testing different Primer-Probe Concentrations

PCR for Primer- Probe Concentration	Temperature (°C)	Duration	Cycles
Warm-Up	50	2 min	1
Initial Denaturation	95	15 sec	1
Denaturation	95	15 sec	40
Annealing	60	30 sec	40
Extension	75	30 sec	40

Once the concentration of primers and probes was selected, the ratio of primer-probe was evaluated. In the previous PCR, primer-probe ratio was 1:1 by adding 0.5 μ L ea. The ratio 2:1 and 1:2 was also tested using the same conditions mentioned above. Where

necessary, the reaction mix was corrected to 20 μL final volume by adjusting the amount of dH_2O added.

Optimizing the Reaction Conditions for Best Performance

Optimizing the reaction conditions can increase primer/probe binding to target and improve the assay overall. There are two main points where PCR reaction conditions can be optimized; the amplification temperature and the extension temperature. For this, the reaction mixture mentioned previously, with the identified ideal primer-probe ratio, was maintained with exception of the target which was increased to 10^5 copies/ μL . The first set of PCR reactions tested various combinations of amplification and extension temperatures. General guidance for PCR conditions can be seen in Figure 3-4.

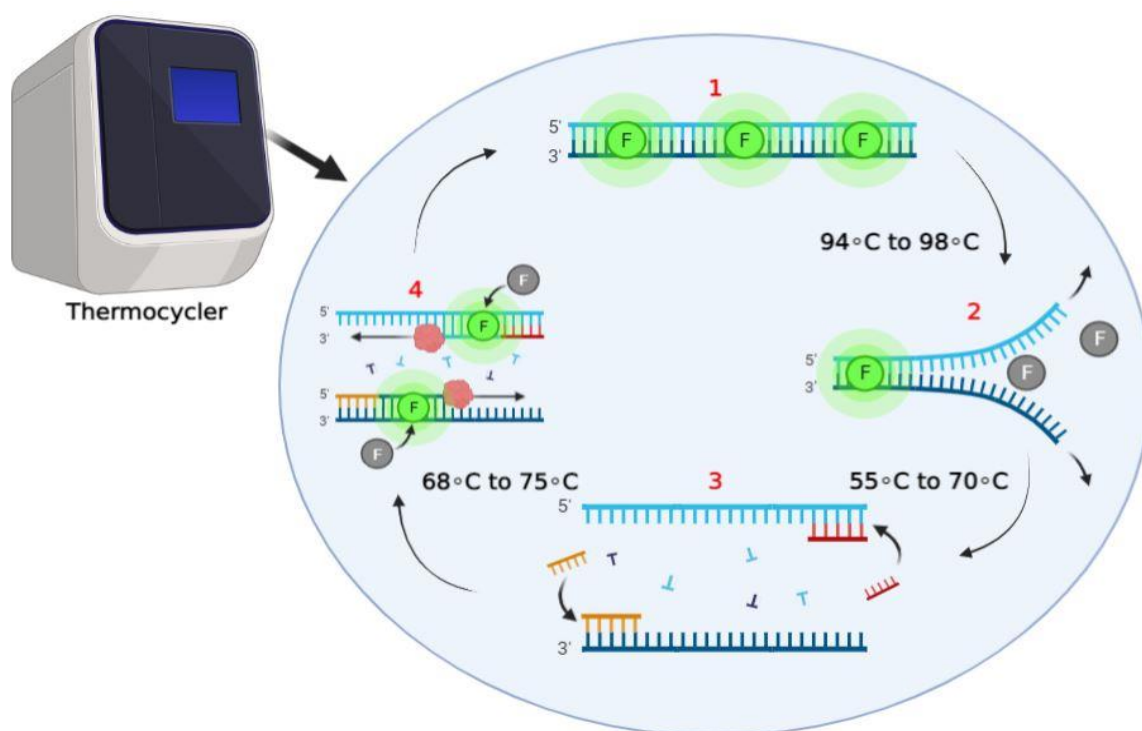


Figure 3-4: PCR conditions. (1) Hold phase, (2) Denaturation 94°C to 98°C, (3) Annealing 55°C to 70°C, (4) Extension 68°C to 75°C. Image made using Biorender.

With this in mind, initial conditions tested maintained a 7°C gap between annealing and extension steps, seen in Table .

Table 3-3: Optimization of PCR Assay by testing different annealing and extension temperatures.

PCR Test	Hold stage	Denaturation	Annealing	Extension
Test 1	95°C / 2 min	95°C / 15 sec	58°C / 30 sec	65°C / 30 sec
Test 2	95°C / 2 min	95°C / 15 sec	60°C / 30 sec	68°C / 30 sec
Test 3	95°C / 2 min	95°C / 15 sec	63°C / 30 sec	70°C / 30 sec
Test 4	95°C / 2 min	95°C / 15 sec	65°C / 30 sec	72°C / 30 sec
Test 5	95°C / 2 min	95°C / 15 sec	68°C / 30 sec	75°C / 30 sec

Once the ideal annealing and extension temperature were identified, further optimization was done by testing different extension temperatures only. This is due to the fact that probes generally have higher T_m and may need a higher extension temperature. Conditions tested can be seen in Table .

Table 3-4: Optimization of PCR Assay by testing different extension temperatures.

Hold stage	Denaturation	Annealing	Extension
95°C / 2 min	95°C / 15 sec	65°C / 30 sec	68°C / 30 sec
95°C / 2 min	95°C / 15 sec	65°C / 30 sec	72°C / 30 sec
95°C / 2 min	95°C / 15 sec	65°C / 30 sec	75°C / 30 sec

Sensitivity and Specificity

Dilutions of RNA obtained from transcription, method as described previously, were prepared at 10^6 copies/ μL , 10^5 copies/ μL , 10^4 copies/ μL , 10^3 copies/ μL , 10^2 copies/ μL , 10^1 copies/ μL and 10^0 copy/ μL . All primer-probe sets were tested against the dilutions of each target (Wuhan, Delta and Omicron) in triplicates. PCR reaction mix consisted of 5 μL master mix (TaqMan® Fast Virus 1-Step Master Mix Thermofisher Scientific), 8 μL dH₂O, 0.5 μL forward primer, 0.5 μL reverse primer, 0.5 μL of probe(s), and 5 μL artificial gene sequence. Internal negative controls were added by replacing the artificial gene sequence with dH₂O. PCR conditions used can be seen in Table . This was performed in triplicates for

each dilution. Using the average of the detection values obtained for each target, a standard curve was generated and used for quantification.

Testing Spiked Wastewater Samples

Wastewater samples from November and December 2020 were concentrated using the PEG-precipitation method with RNA purification as mentioned previously. Samples were tested using CDC N1 primers and probe. PCR reaction mix and conditions as mentioned in Table . Once confirmed negative, the raw wastewater samples with no treatment were used for spiking. The process of spiking wastewater sample is an important control measure. As wastewater is a chemically and biologically complex matrix, is important to quantitatively assess recovery rates. Wastewater can contain constituents that interfere with nucleic acid extraction.

RNA for each target was obtained from transcription, method as described previously. Using the Qubit to quantify the amount of RNA in ng/ μL , serial dilutions were prepared. A known concentration of 10^7 RNA copies/ μL was spiked into 150 mL of raw wastewater. Wastewater was then processed using PEG-precipitation and RNA extraction, methods as mentioned previously. Following this, PCR was performed using the established method and the recovery rate determined using a standard curve.

3.3.4 Assay validation

Testing Samples Collected from Quarantine Hotels June 2021

During the peak of the COVID-19 pandemic in the UK, it was mandatory that passengers quarantine in hotels situated near the airport of arrival. In June 2021, a collaboration project was established which allowed Cranfield to have access to samples of wastewater from these hotels. Due to data protection, specific locations will not be mentioned, and samples will be referred to as their sample number (Table A-2)

After collection of the raw influent wastewater, samples were transported to the laboratory. Samples were maintained in -20°C conditions. When necessary, samples were thawed in room temperature water bath and processed as mentioned previously (UF/PEG concentration, RNA Extraction). PCR was performed using the Delta primer-probe set and CDC N3 Primer probe set to determine the presence of SARS-CoV-2 variants. Established PCR reaction mix, and method mentioned previously was used. An additional step was added

to the PCR, 1 cycle at the beginning for 2 min at 50°C for reverse transcription. All samples were tested in triplicates to ensure accuracy of results.

Testing a Cohort of Samples from Cranfield's WWTP

Samples collected from Cranfield University's Wastewater Treatment Plant were collected twice a week using the ISCO 3700 sampler. Samples were transported to the laboratory in 4°C conditions and maintained in 4°C until processed later the same day. Samples were processed using methods described previously (PEG-precipitation, RNA extraction). An additional step was added to the PCR, 1 cycle at the beginning for 2 min at 50°C for reverse transcription. The PCR was then performed using primer-probe sets for Wuhan, Delta and Omicron (Table A-1). All samples were tested in triplicates to ensure accuracy of results.

Sample sequencing and Bioinformatic Analysis

Samples were sent to Sheffield University for sequencing. Sequencing results were analysed by a collaborator at Sheffield and provided for comparison to the Delta assay results. This is essential to determine method sensitivity and specificity. RNA was prepared using PEG method followed by RNA extraction using the Qiagen commercial kit. Concentration of RNA for each sample was measured (ng/ µL) using a Qubit. Samples with sufficient RNA quality were processed and sequenced on either an Illumina NovaSeq 6000 or NextSeq 500/550 flow cell. Sequences were assembled and analysed with the DRAGEN COVID Lineage (Illumina, v.3.5.3).

3.4 Results and Discussion

3.4.1 Comparison of concentration methods: PEG and Ultrafiltration

Detection of pathogens in wastewater is a complex process consisting of sampling, concentration, purification and detection methods (i.e., PCR). It is essential to identify an effective concentration method that will ensure highest nucleic acid recovery from complex wastewater samples, resulting in assay efficiency. Two methods are highly used, ultrafiltration and PEG. Several studies have conducted a comparison between various methods (Lucansky et al., 2023; Farkas et al., 2022), results vary depending on the extraction kit used, sample quality, equipment used, et cetera. Ultrafiltration consists in using a falcon tube which contains a semipermeable membrane only allowing particles under a certain size

to pass (10kDa). Samples are added to the tube and centrifuged at high speed. PEG is another method used to concentrate wastewater. PEG when combined with NaCl and added to wastewater samples results in virus precipitation due to the mixture altering the pathogen's solubility. TRIzol is then used as a lysis buffer and upon adding chloroform the separation of RNA, DNA and protein is visual.

In order to compare the two methods, a wastewater sample collected from Cranfield's WWTP was used. As toilets are flushed and wastewater produced at all moments of the day, collecting one sample at a specific time would not be an accurate representation. For this reason, samples were collected for a total of 12 hours, the busiest hours of the day for the university, and mixed to generate one sample which would be an accurate representation for that day. Both methods were done simultaneously using the same sample collected within 24 hours. Tests were run in triplicates in order to ensure accuracy.

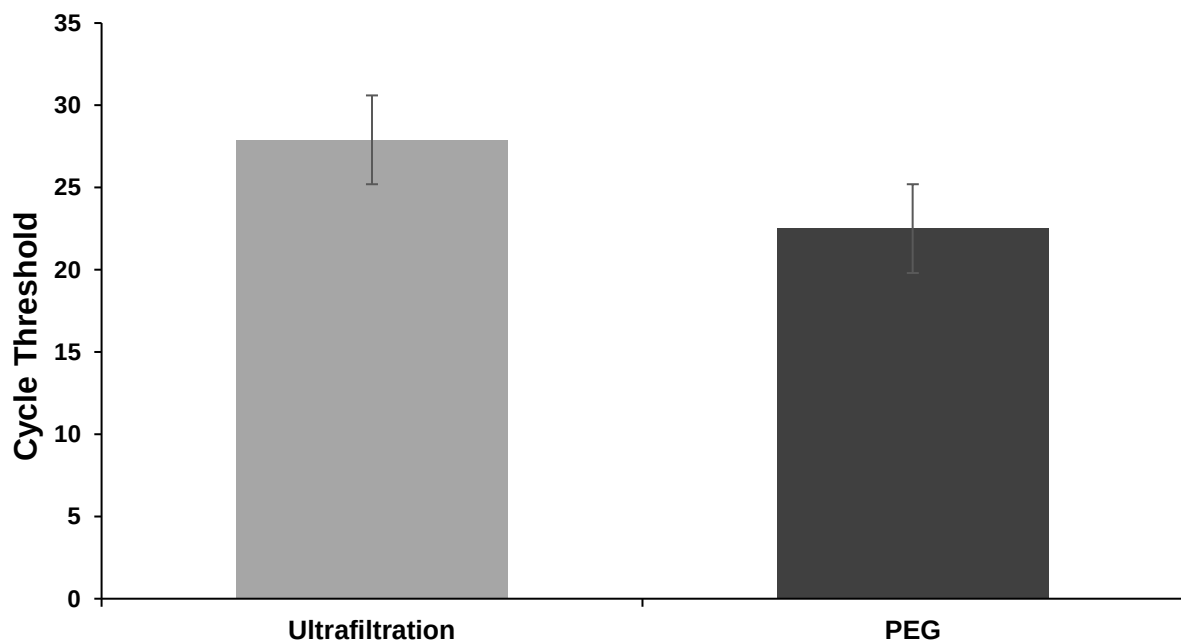


Figure 3-5: Comparison of two wastewater concentration methods: Ultrafiltration and PEG.

Figure 3-5-shows that when concentrated using PEG, the target is detected earlier compared to the ultrafiltration concentrated target. The average cycle threshold (C_t) for the PEG method is 22.5 cycles compared to the ultrafiltration method at 27.9 cycles, indicating that PEG method is more effective for enrichment in our experiment. Looking at literature, it noticeable that the results aren't the same for everyone. A study conducted in the UK, found that volume and composition of the wastewater greatly affected virus recovery rates,

regardless of what method was used (Farkas et al., 2022). By testing five commonly used methods on 115 unspiked wastewater samples, the two methods that performed the best were Amicon ultrafiltration and beef-extract-enhanced PEG precipitation. Further analysis showed that ultrafiltration worked best on lower sample volume whilst PEG worked best on samples with higher volume. This may provide an explanation as to why PEG worked better on samples tested in this thesis although, the PEG method used in this study did not contain beef-extract. In another study conducted in Slovakia showed that samples that were frozen before extracted resulted in significantly lower RNA yield (Lucansky et al., 2023). This was reassuring as university samples tested in this thesis were fresh. This study used a different extraction kit however, their comparison did show that PEG performed slightly better than ultrafiltration.

Both methods consist of an initial centrifugation to remove suspended solids. This is an important step as historical RNA/DNA can be found in solids and would interfere with the end result (Graham et al., 2021). PEG presents several advantages besides having a lower cost when compared to ultrafiltration. The TRIzol step allows for separation of proteins, DNA and RNA. Many PCR inhibitors at this stage are separated from the target of interest as they stay in the lower red-phenol layer (Figure 3-2). These inhibitors are not separated in ultrafiltration in many cases as they may be the same size of the target allowing passage through the filter. The TRIzol step also results in nucleic acid and therefore removing the need for the lysis step during RNA extraction (QIAamp kit). This shortens the RNA extraction from 1 hour to approximately 30 minutes per sample. Ultrafiltration does not result in nucleic acid and the full RNA extraction protocol is needed. In terms of practicality, ultrafiltration is a quicker and more simple method to follow. The duration of both methods is very different and should be taken into consideration. The PEG method can take 6 to 24 hours whilst ultrafiltration is 3-4 hours in duration. Taking all these factors into consideration, for our laboratory and the specific target (SARS-CoV-2), PEG did perform best and was the selected method for any further experiments done.

3.4.2 N-Gene Bioinformatic Analysis of Variants of Concern

Whole genome sequences for each variant obtained from GISAID. A BlastN (NCBI) was run to compare these sequences to the reference genome (Wuhan strain). The genome of SARS-CoV-2 offers many possible targets due to its size however, not all genes make good targets due to the viruses' natural evolution. In Vogels et al. (2020), a comparison of 9

primer-probe combinations which targeted several genes was conducted. It was seen that at low viral concentrations, not all assays were positive. This shows the importance of choosing a good target and optimizing the assay to minimize the risk of false negatives. For the Delta variant (B.1.617.2), in the N gene, a total of four SNPs of interest were identified. For the Omicron variant (B.1.1.529), in the N gene, several large mutations were found however, only one possible target SNP. In Figure 3-6 it is possible to see the SNPs identified in both variants as well as some of the other mutations (i.e., deletions and insertions).

When selecting the specific SNP of interest, it is important to ensure enough space around the target. As the amplicon size should be between 70-200 base pairs it is important to ensure there is enough room between primers for the probe to bind. For this reason, all SNPs present in delta could have been targeted as there was enough space. Omicron however, only had one possible SNP that could be targeted. Other mutations present in omicron were either not SNPs or in a location that would make it impossible for probe binding due to overlapping with the primers. SNPs that were originally or mutated to G or C base pairs, were also preferred. This is due to the fact that a higher GC content in primer/probe design results in better stability and binding. In addition to the high GC content, the SNP can be placed closer to the 3' end of the probe which also increases stability as it is the last part of the probe to bind. Other points were also further looked into (i.e., melting temperature, secondary structure, length) to ensure successful design.

	Position (bp)		Position (bp)
Wuhan	181	AAGGAAGACCTTAAATTCCTCGAGGACAAGGCGTTCCAATTAACACCAATAGCAGTCCA	240
Delta	28387	G.....	28446
Wuhan	601	AGCAGTAGGGGAACCTTCTCTGCTAGAAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCT	660
Delta	28807	T.....	28866
Wuhan	1081	AAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGGCTGATGAAACTCAA	1140
Delta	29287	T.....	29346
Wuhan	1141	GCCTTACCGCAGAGACAGAAGAAACAGCAAACCTGTGACTCTTCTCTGCTGCAGATTG	1200
Delta	29347	A.....	29406
Wuhan	28274	ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCC	28333
Omicron	1	T.....	60
Wuhan	28334	TCAGATTCAACTGGCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGT	28393
Omicron	61		111
Wuhan	28874	AGCAGTAGGGGAACCTTCTCTGCTAGAAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCT	28933
Omicron	592	AAC.....	651

Figure 3-6: Single Nucleotide Polymorphisms (SNPs) identified in the nucleocapsid gene of SARS-CoV-2 variants of concern Delta and Omicron.

Two probes were designed for each assay, a SNP probe based on variant sequence and a non-SNP probe based on the reference Wuhan sequence. As it is only one base pair mutation, binding can still occur even in the presence of a target that is not a perfect match. The likelihood of this happening is lowered when both probes are inserted in the same pot. The probes will compete for binding and the probe with highest accuracy will bind more than the other. This creates a gap of 4-6 cycles and can be used to indicate a variant (Figure 3-3).

Upon selecting the target SNP of interest and designing the probes, forward and reverse primers were designed using PrimerBlast. As mentioned previously, the amplicon size aim was 70-200 bp. Considerations taken for primer design include a high GC content, no or low secondary structure, appropriate length (18-30bp) and lower melting temperature than the probes.

3.4.3 Analytical performances

Optimizing the concentrations of the primers and probes

Protocols to be used for surveillance applications should be optimized to ensure the most accurate results. RT-PCR assays have multiple optimization opportunities such as annealing temperature, oligonucleotide concentration, ratios, et cetera; all which need to be

determined empirically (Huggett et al., 2013; Bustin et al., 2009). Primers and probes arrived lyophilized and at this stage are stored at room temperature. To prepare the stock solution tube are briefly centrifuged and resuspended in nuclease-free dH₂O. The stock solution of all primers is prepared to 100 µM. Working solutions generally are between 10 – 20 µM and the amount added to the reaction determines the final concentration. All working solutions of primers/probes were prepared to 20 µM, this allowed for less primers/probes to be added to the reaction mix whilst achieving the desired final concentration. Stock and working solutions were then stored in -20°C to preserve quality.

Generally speaking, primer-probe ratio is 2:1 for most PCR assays. As this assay used two probes instead of one, different ratios were tested. As changing the ratio does interfere with the PCR reaction mix make up, dH₂O was adjusted when necessary to ensure final reaction mix of 20 µL. A 2:1 ratio was tested by adding 0.5 µL of the working solution of primers and 0.25 µL of the working solution of the probes. Final concentrations for each were 0.5 µM and 0.25 µM, respectively. A 1:2 ratio was tested by inverting previous volumes, adding 0.25 µL primers and 0.5 µL probes. Concentrations therefor were 0.25 µM and 0.5 µM, respectively. Finally, testing the 2:2 ratio consisted of adding the same volume for both primers and probes. For this test, the volume selected was 0.5 µL resulting in final concentration of 0.5 µM.

Wuhan reactions in Figure 3-7, little difference is seen when testing 1:2 and 2:1 ratios. The 2:2 ratio did result in an earlier detection with the SNP probe however, standard deviation shows that this result is not the best option. Observing the Delta reactions, it is possible to see that there is a much wider gap in Ct between the two probes. This indicates that there is in fact a competition for binding. The lower Ct value for the SNP probe shows that the primer is more specific. The 2:2 ratio option was discarded due to having the smallest gap in Ct for Delta and due to the Wuhan reaction using this same ratio. The gap between the Ct for Delta with 1:2 and 2:1 ratios were similar. The difference was around 2 cycles, the 1:2 ratio performing a little better. Similar tests were done with the CDC primers and probes in literature and the best performing ratio was 2:1. The 2:1 ratio for the Delta assay was selected as the CDC N1 and N3 primers and probes are used in this study as references, keeping everything standardized.

Optimizing the Reaction Conditions for Best Performance

PCR conditions vary depending on the template and primers/probes used. This is because these components may have higher or lower melting temperatures (T_m). Generally, a $T_m > 60^\circ\text{C}$ can result in binding to unintended targets, in contrast a $T_m < 60^\circ\text{C}$ could possibly bind partially or not bind at all. It is necessary to adjust and optimize the PCR assay to ensure best performance. A PCR consists of three main steps: denaturation, annealing and extension. Denaturation generally occurs between $90\text{--}95^\circ\text{C}$, this is due to the kinetic energy causing the molecules to vibrate. This results in bonds being disrupted between corresponding base pairs and DNA separating into single strands. Annealing and extension steps are more specific to primers and probes used. Annealing temperature is related to the T_m of the primers, the temperature has to be sufficient to allow forward and reverse primers to bind to the template (Peters et al., 2004). A low temperature can result in undesired duplexes or intramolecular hairpins, reducing reaction efficiency. A temperature too high would not allow primers to bind to the target. Extension step is for the polymerase enzyme to bind, allowing deoxynucleotide triphosphates (dNTPs) to extend the primer to form a nascent DNA strand. The temperature and duration are dependent on the template size, and both points are important here. If the extension time is too long, it may result in smeared and unclear band in electrophoresis gels. A short extension time can fail to produce any products or even in non-specific products. Temperature can change the rate the extension takes place and also influence the outcome (Ahmed et al., 2021). Probes tend to have higher temperatures; they will bind just downstream of the forward primer. When elongation takes place, the probe is cleaved by the polymerase. This cleaving results in the quencher and reporter (fluorophore) separating and fluorescence. Extension step at the wrong temperature can decrease the fluorescence and result in erroneous results.

Given that annealing is related to the primer T_m and extension is relevant to the probes T_m , several temperature combinations were tested as seen Figure 3-7. It is possible to see that test 4, which consisted in an annealing step at $65^\circ\text{C}/30\text{sec}$ and extension step at $72^\circ\text{C}/30\text{sec}$ performed the best. Other conditions were not as successful at creating a gap between the variant target and the reference sequence. In one case, test 2, the reference sequence was detected earlier than the variant.

As the extension step is of great importance, additional tests were done modifying only its temperature. The annealing temperature was maintained at 65°C and three different

extension temperatures tested: 68°C , 72°C and 75°C (Figure 3-7). The gap of Ct for each one tested were relatively similar. It was seen that a higher extension temperature also resulted in a later detection for both Wuhan and Delta. As the template was of high concentration, a later detection is not ideal. This could result in low viral load samples not being detected. The lower extension temperature performed will however, the gap between annealing and extension was not big enough and generally low extension temperatures are used for sequences that are not GC rich, which is not this case. In the first set of tests, test 4 which used an extension temperature of 72°C has the best results. For this reason, the test conditions selected for any further testing was annealing at 65°C and extension at 72°C.

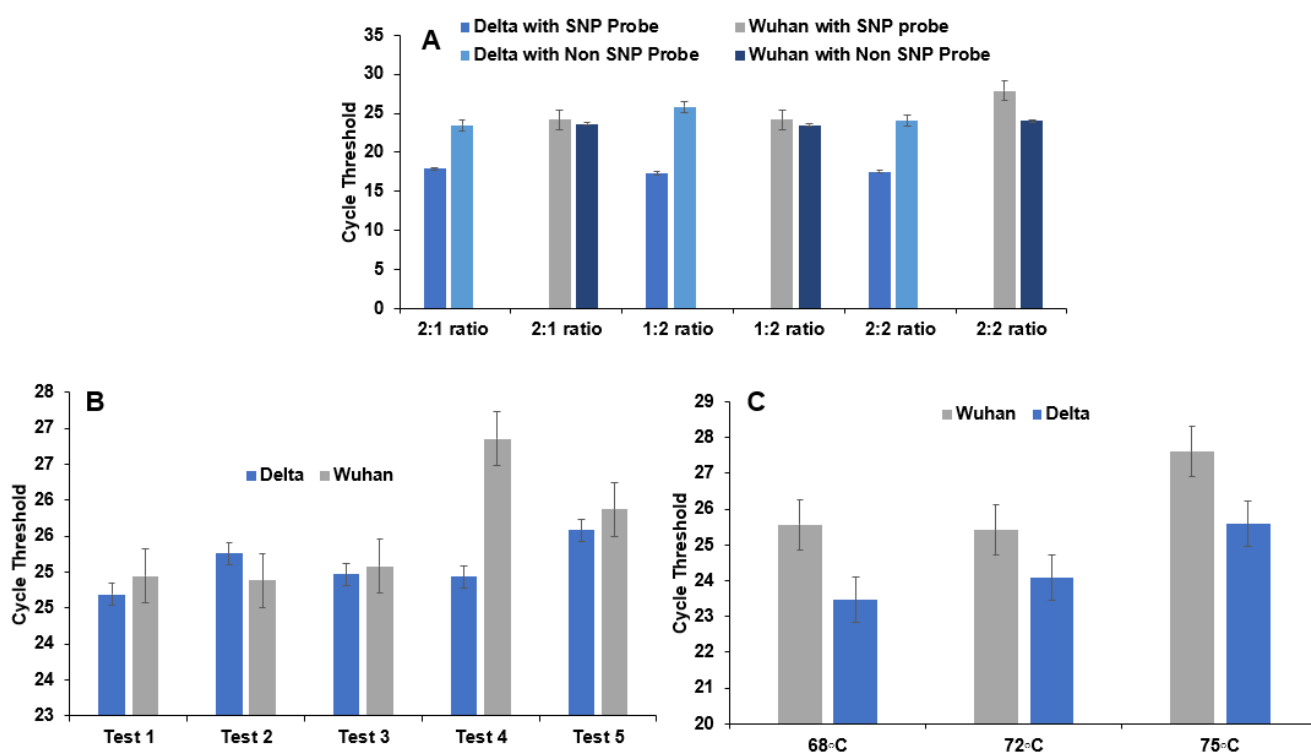


Figure 3-7: Optimizing the Delta detection assay. (A) Testing different primer-probe ratios. (B) Testing different annealing and extension temperature combinations. (C) Testing different extension temperatures.

Assay Sensitivity

Using the artificial gene sequences, dilutions of 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , 10^0 were prepared. PCR was performed in order to determine the limit of detection (LOD) of the probes and generate a standard curve for quantification. This was done for the CDC N3 set and delta assay probes. The LOD of the delta assay probes were comparable to those recommended

by the CDC. Both probes were able to detect as little as 0.25 copies/ μL . This is comparable with the CDC N1 and N3 primers and probe sets currently used in diagnostic laboratories.

PCR efficiency was calculated using the below equation. Efficiencies ranged from 98.6 to 143% (data included in caption for Figure 3-8).

$$E = -1 + 10^{(-1/\text{slope})} \quad (3-2)$$

R^2 values were calculated and for all standard curves the value was > 0.9999 . Specific R^2 values can be seen in Figure 3-8.

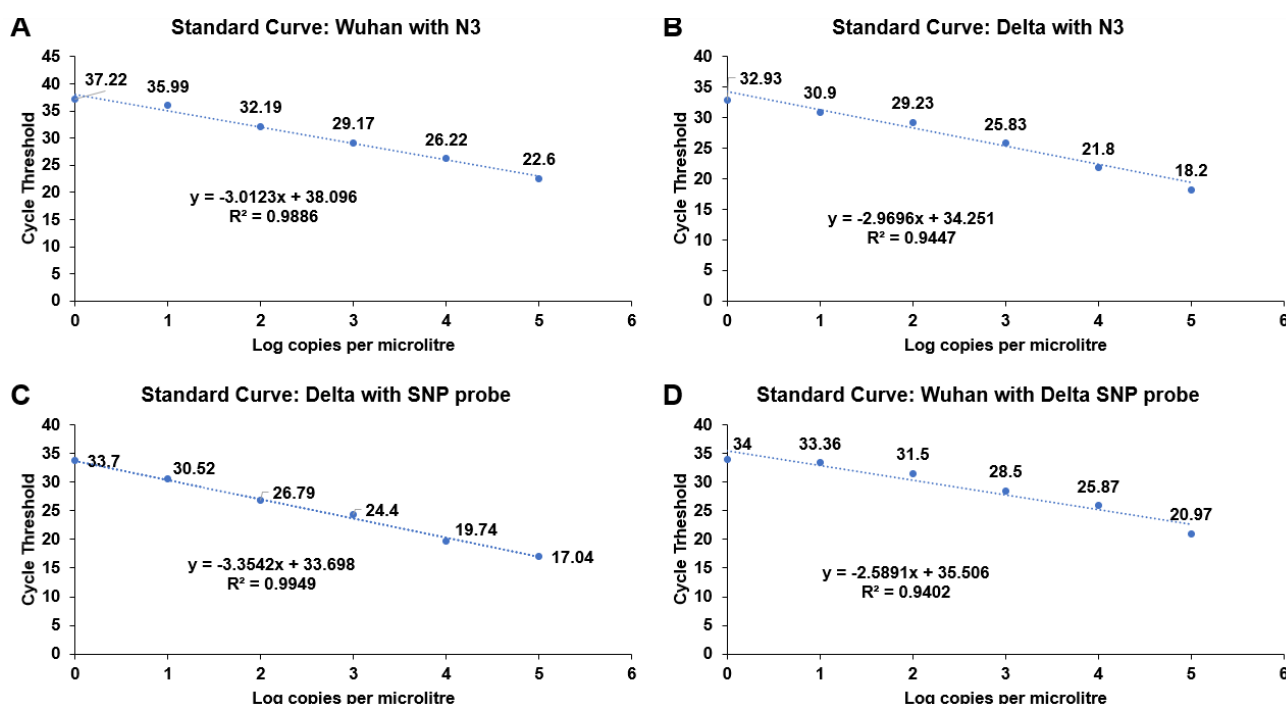


Figure 3-8: Standard Curves. (A) CDC N3 primers and probes against Wuhan (dsDNA). $E = 115\%$ $R^2 = 0.98$. (B) CDC N3 primers and probes against Delta (dsDNA). $E = 117\%$ $R^2 = 0.94$. (C) Delta (dsDNA) Sequence with SNP Probe. $E = 98.6\%$ $R^2 = 0.99$. (D) Wuhan (dsDNA) with Delta SNP Probe. $E = 143\%$ $R^2 = 0.94$

A comparison test aims to determine the difference in cycle threshold for Delta using individual probes. In separate tubes, the same reaction mix was prepared containing the same concentration of Delta target. As seen Figure 3-9, it was possible to detect Delta much earlier using the probe that contained the corresponding SNP. Delta was detected on average

5.3 cycles earlier when tested only with the SNP Probe. When inserting both probes in the reaction tube, due to the competition for binding, the difference is even more significant.

Determining ΔC_t is essential when comparing differences in C_t values for a target of interest against a reference target. A positive ΔC_t indicates that target gene is expressed at a lower level than the reference gene. A negative ΔC_t indicates that the target gene is expressed at a higher level than the reference gene. In Figure 3-9, the ΔC_t upon comparing detection for Delta (Target) and Wuhan (Reference) against a wide range of concentrations (10^5 to 10^0) can be seen. Both sequences were added at the same concentration. The negative ΔC_t indicates that when testing with the Delta SNP probe, the expression of the delta target is higher than the reference Wuhan sequence.

An additional step in order to determine if the sequence of interest has a mutated SNP or not is performing a melt curve analysis. This was done as an additional step to the N gene amplification PCR mentioned in Table 3-3. A melt curve assesses the dissociation characteristics of dsDNA. The reason why this is a great technique for SNP identification is due to SNPs giving the template a higher melting temperature than reference genomes.

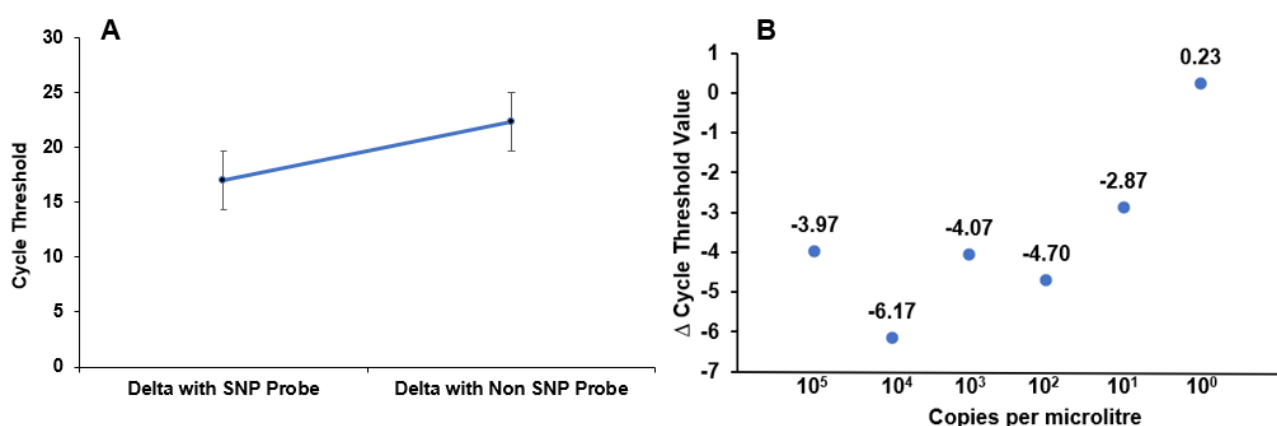


Figure 3-9: Comparing Delta assay probes. (A) Cycle threshold comparison between SNP and non-SNP probe. (B) Delta cycle threshold difference when using SNP probe against Wuhan and Delta.

As shown in Figure 3-10, the melt curve for each target indicates that Wuhan presents peak that is not high (-Rn) and more round than steep (Blue). It is easily identifiable as it is early on, seen around 75°C. Delta appears later, around 83°C and is a high steep peak (Red). Omicron presents two peaks, likely due to a rather large deletion in its N-gene. Both peaks are not high and less steep than the delta variant (Green). A melting

curve analysis can be implemented as an additional step in the process of identifying a variant of concern.

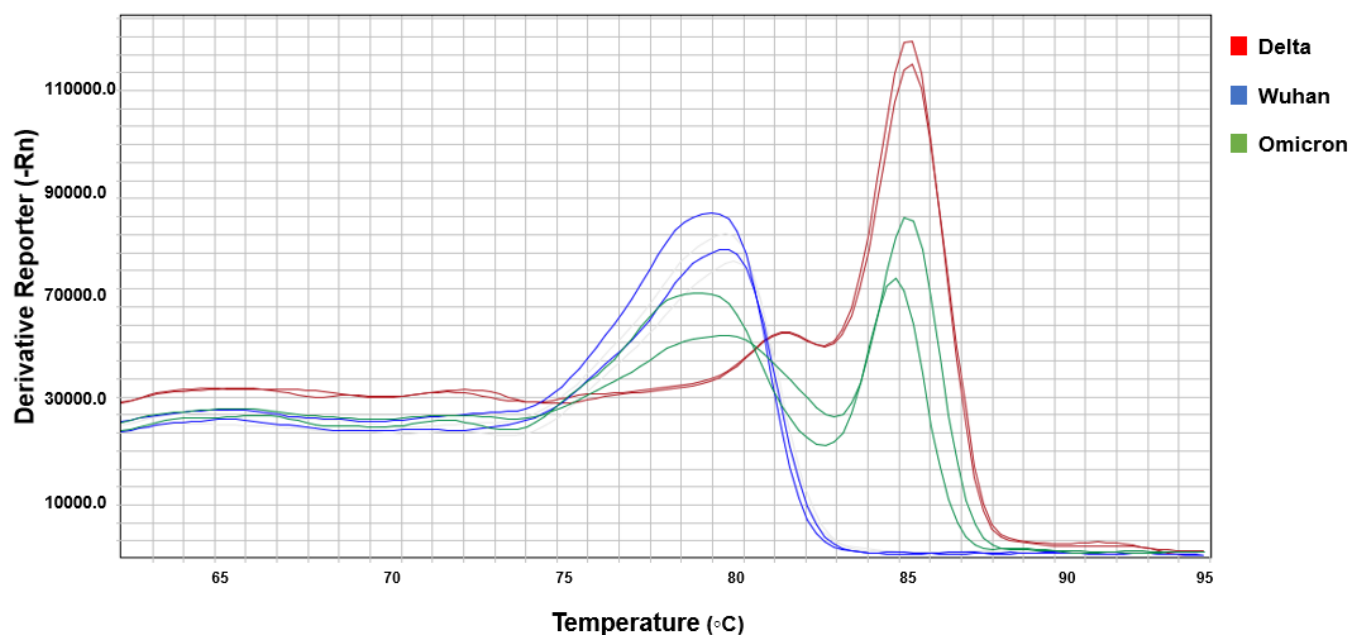


Figure 3-10: Melt curve for targets Delta, Omicron and Wuhan. Delta is seen in red; omicron is seen in green, and reference sequence (Wuhan) seen in blue.

3.4.4 Implementation of SNP Specific RT-PCR Assay for WBE

Spiked samples

For RNA preparation, PCR was done using primers with T7 promoter (3.3.3), agarose gel was used to confirm the targets had amplified (Figure 3-11). DNA was recovered from the gel using commercial kits and the transcription done. Following transcription, the RNA was purified and measured using Qubit. The concentration obtained for Delta was 19.3 ng/ μL , which corresponds to 3.798×10^{10} copies per microlitre. The concentration obtained for Omicron was 36.4 ng/ μL which corresponds to 7.163×10^{10} copies per microlitre. From this, dilutions were prepared and used for spiking negative wastewater samples for measuring recovery rate.

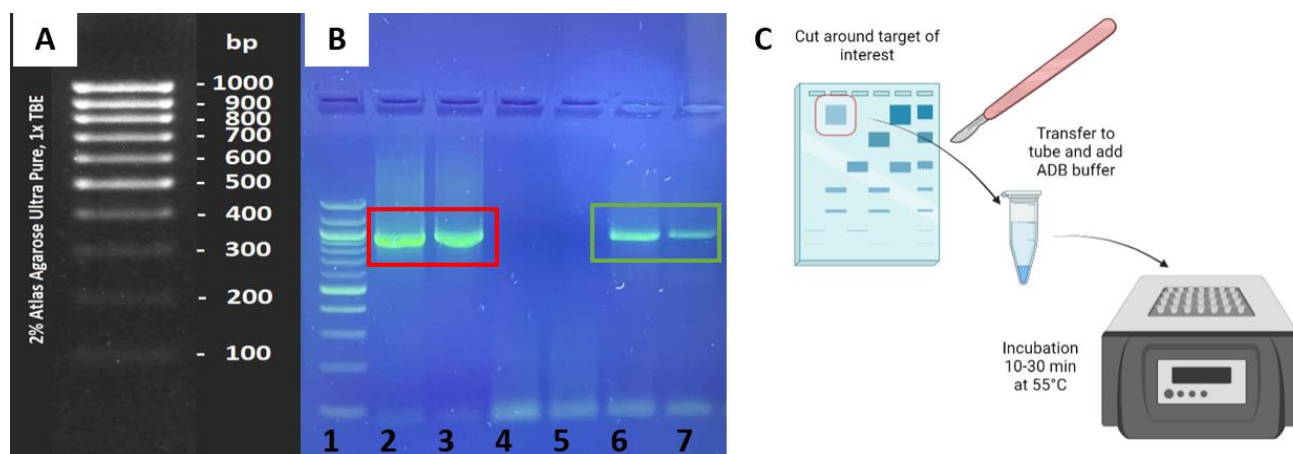


Figure 3-11: DNA Gel Recovery. (A) 100bp DNA ladder. (B) 2% Agarose Gel Electrophoresis 1: DNA ladder, 2: Delta, 3: Delta, 4: negative control, 5: negative control, 6: Omicron, 7: Omicron. (C) Cutting out target and melting gel for DNA recovery (made using Biorender).

To a negative wastewater sample (150 mL), prior to any concentration, 150 μ L of Delta was spiked at a concentration of 10^7 . Giving a final concentration of 10^5 . The sample was then processed using PEG-precipitation and Qiagen RNA Extraction procedures as mentioned previously. PCR was then performed using the established protocol with an additional step of 2 minutes at 50°C for reverse transcription at the beginning (1 cycle). Following PCR, the Ct was plotted against the correct standard curve in order to quantify the number of viral copies per microlitre and compare the amount spiked with the amount recovered.

Analysis of Samples from Quarantine Hotel

Until March 2022, it was mandatory that certain travellers arriving in the United Kingdom from “Red List Countries” needed to quarantine in hotels near the airport. Red list countries were selected based on case numbers and known spread of variants of concern.

In June 2021, raw influent wastewater samples were collected from certain quarantine hotels. Due to data protection, the name of these hotels is not mentioned here. Samples from these hotels will be referred to their specific sample number. Sample numbers and further information on tested samples can be seen in Table A-2. Samples were collected and stored in 4°C until processed. After processing samples were stored in -20°C. PEG-precipitation and Qiagen RNA extraction processes were carried out. PCR was performed using the established method testing the Delta Assay primers and probes as well as the CDC N1 primers and probe. An additional step was added to the PCR, 1 cycle at the beginning for 2

min at 50°C for reverse transcription. Any detection after cycle 35 was considered to be negative.

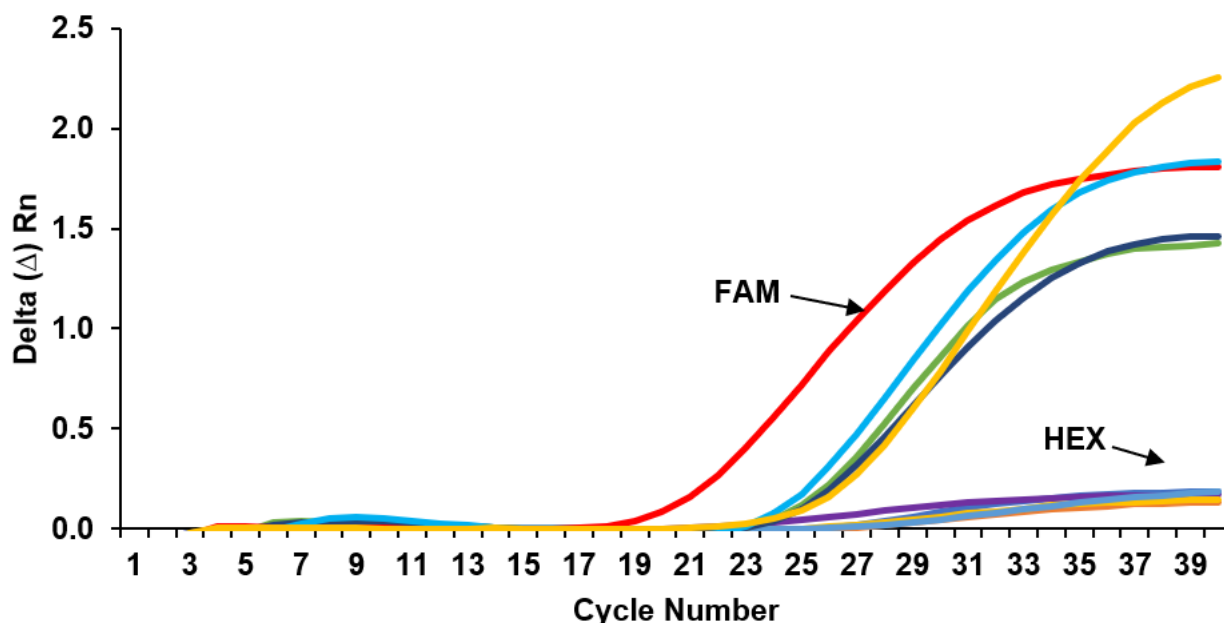


Figure 3-12: Amplification Plot of Quarantine Hotel Samples. Larger curves are samples 11-15 with SNP probe (FAM fluorophore) and smaller curves are samples 11-15 with non-SNP probe (HEX fluorophore).

In Figure 3-12 the difference in amplification curves is very clear. The non-SNP probe that uses the HEX fluorophore corresponds to the original Wuhan sequence. The SNP probe, specific to the variant Delta uses the fluorophore FAM and results in a much earlier detection. These results are strongly in line with data provided by governments at the time (June 2021). Data suggests that the Delta variant was responsible for the vast majority of cases (Figure 3-13).

The average gap of C_t between the SNP and non-SNP probes for all hotel samples is 5.55 cycles. Samples were extracted and tested with both probes simultaneously. The 5.5 cycle difference indicates that there is a high amount of Delta variant present. Due to having only one base pair change, probes can bind to sequences that do not match perfectly. This is minimized by adding both probes in the same pot, resulting in competition for binding and the probe with highest affinity would bind.

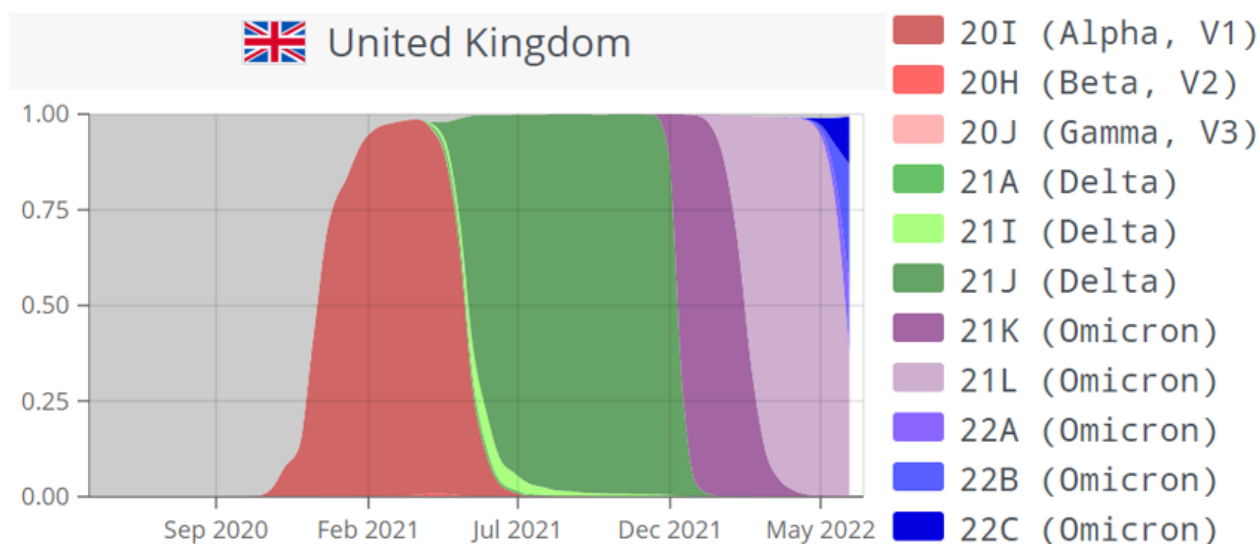


Figure 3-13: Frequency distribution of variants of concern in the United Kingdom. Grey areas are original Wuhan SARS-CoV-2. Figure obtained from CoVariants (GISAID data, 2022).

University Samples

Alpheus, a leading water and wastewater management company, closely collaborates with Cranfield University. Data from Cranfield's WWTP was kindly provided from the team at Alpheus. The table in appendices Table A-3 shows general information about the samples tested in this study. Characterization of wastewater follow APHA 1994 include testing for biological oxygen demand, pH, ammonia, nitrate, nitrogen and others. Studies such as Sweetapple et al. (2022) found that SARS-CoV-2 strongly correlates with biomarkers such as ammonium. In addition to general characteristics of wastewater, daily flow plays an important part when normalizing data. Taking into consideration the rain falls as well, as this can also influence results. In the Table A-3, daily flow rates for March to May 2022 are shown.

Since the start of the pandemic, influent wastewater samples from Cranfield's WWTP have been collected on a weekly basis. Samples tested in this study are from February to May 2022, excluding a 2-week gap in March 2022. The university also created an initiative to share COVID-19 case numbers reported between staff and students. After collected, samples were maintained at 4°C until processed. Once processed samples were stored in -20°C and thawed in room temperature water bath if further tests were required. For the purpose of testing the Delta Assay primers and probes, samples from Cranfield's WWTP from February to May 2022 were used. PEG-precipitation and Qiagen RNA extraction processes were carried out. PCR was performed using the established method testing the Delta Assay

primers and probes as well as the CDC N1 primers and probe. An additional step was added to the PCR, 1 cycle at the beginning for 2 min at 50°C for reverse transcription. Any detection after cycle 35 was considered to be negative.

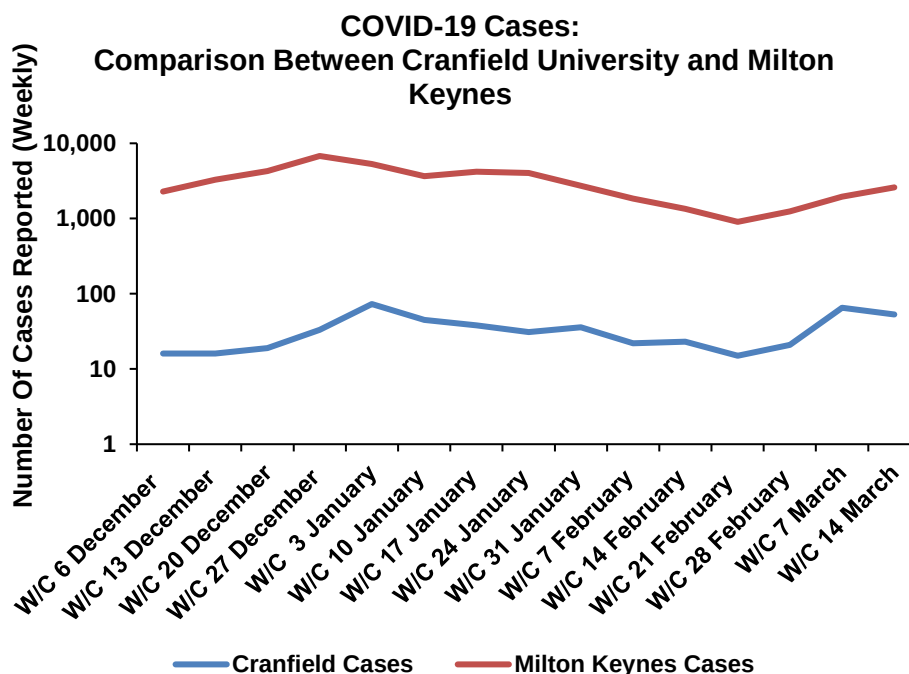


Figure 3-14: A comparison of total COVID-19 case numbers reported (weekly) at Cranfield University and Milton Keynes (Data from Public Health England Website) from December 2021 to March 2022.

Total COVID-19 cases reported in the university were shared on a weekly basis. In Figure 3-14 a comparison between case numbers from Cranfield and Milton Keynes can be seen. The date range is from December 2021 to March 2022. This initiative ended in March 2022 therefore case numbers for April onwards for Cranfield were not available. The trend for cases between both locations is very similar. Peak in cases can be seen in January and March. Cases tend to be reported less over the weekend due to less tests being conducted and lack of reporting personnel. The comparison is still valid as the overall trend of cases is similar. Samples tested after April 2022 to June 2022 presented higher cycle threshold limits (i.e., lower viral load), this is most likely due to a slight decrease in cases. This can be seen in the Milton Keynes area and is likely what occurred in Cranfield as well.

In Figure 3-15 (Part C) mean cycle threshold limits (triplicates) can be seen for both delta assay probes. The SNP probe presented an earlier detection on all samples compared to the non-SNP. This indicates that the presence of a variant, more specifically Delta, is more

likely than non-variant. There is a high possibility that other variants such as Omicron were also present in these samples due to widespread across the country (Figure 3-13) and may have been detected by the non-SNP probe. This is possible because the non-SNP probe targets the original mutation, and the SNP is specific to VOCs. A multiplexed assay testing SNP/non-SNP targets for various variants of concern would provide a better picture and possibly allow variant-specific quantification. For the samples tested, the average C_t gap was 5.6 cycles. This can be interpreted as a Delta being detected 5.6 cycles earlier than other SARS-CoV-2 variants.

Similar to the quarantine hotel samples, the wastewater samples collected from Cranfield presented a higher amplification curve and higher C_t value with the SNP probe. This can be seen in Figure 3-15 (Part A and B). This indicates the likelihood of a SARS-CoV-2 variant present in sample. For the period that these samples were collected, the variant that dominated was Omicron. As the delta assay was used and the SNP probe presented an earlier detection than the non-SNP (reference sequence), this suggests that the Delta variant is still present and spreading amongst staff and students.

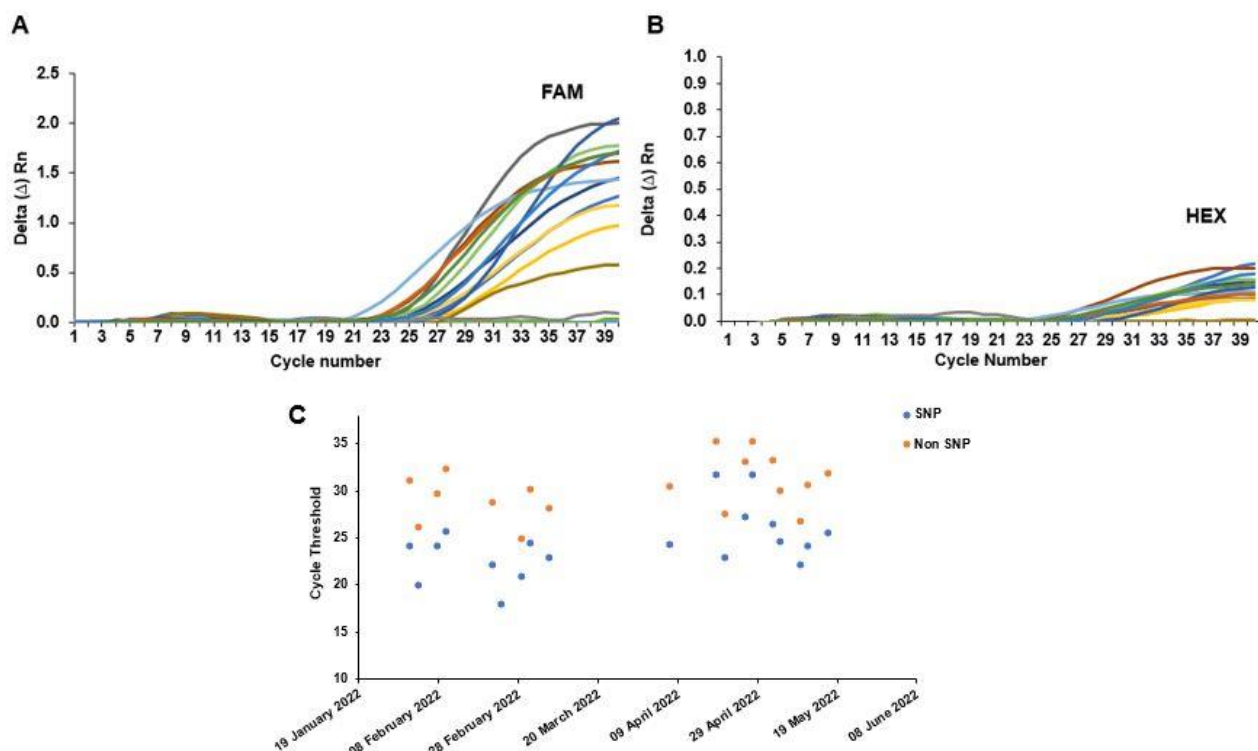


Figure 3-15: Amplification plot for Cranfield University samples. (A) SNP probe FAM fluorophore tested. (B) non-SNP probe HEX fluorophore tested. (C) Mean cycle threshold limits for SNP probe and non-SNP probes.

Sequencing of tested samples

Sequencing is the gold-standard method for variant identification. In order to further validate the developed method, all samples tested in this study have been sent for sequencing with collaborators at Sheffield University. Samples were sequenced using the SubARTIC protocol on Oxford Nanopore. This protocol focuses on the spike region of the SARS-CoV-2 genome. It presents good sensitivity and has been easily adopted for the identification of variants of concern in the UK. In addition to the 15 samples sequenced, two negative controls were tested to ensure accuracy. Sequences were assembled and analysed with the DRAGEN COVID Lineage (Illumina, v.3.5.3).

The S-gene was targeted for sequencing rather than the N-gene for various reasons. Currently there are many well-established protocols on S-gene sequencing for variant/SNP identification and not many around the N-gene. A comprehensive list of all variations in SARS-CoV-2 S-gene has been put together by various groups, making it easier for comparison. Other reasons include the size of the genes, the S-gene is larger providing much more information on the sample.

In total, 15 samples were sent for sequencing. Of these samples, 5 were from quarantine hotels from June 2021 and 10 were from Cranfield's wastewater treatment plant from April to May 2022. During the collection of the samples from June 2021, the dominant variant was delta (seen in Figure 3-13) based on GISAID data. The samples from April-May 2022 the predominant variant was Omicron, but Delta was still circulating (Figure 3-13) based on GISAID data. Sequencing results are in agreement with this information from GISAID (Table A-11). The SNP-PCR assay was designed to targets SNPs of VOCs and samples tested during December – May 2022 showed that VOCs were present in sample more than the original sequence (i.e., Wuhan). Omicron and Delta share mutations in the S-gene (target for sequencing) and in many cases it may appear to be Omicron but can also be Delta. Whole genome sequencing would have been ideal however, wasn't an available option. The available sequencing information shows presence of Omicron and Delta and supports the idea that the SNP-PCR assay can be used to identify variants of concern.

In Table A-10, the raw read counts for each sample sequenced can be seen. Raw reads are the reads that have been mapped to a particular gene transcript. The higher the read count, the more accurate the mapping of the gene is. Depth of coverage for each mapped read tells us how well mapped the gene actually is. For the specific protocol used,

depth >50,000 indicates good mapping and <50,000 would mean poor mapping. DNA/RNA concentration, presence of inhibitor, protocols and primers used can all influence the final result. The reads of the base pairs are then compared to reference sequences. The current list of mutations for the S gene can be seen in Table A-9. Each mutation corresponds to a variant of concern. Some mutations are shared (i.e., N501Y) and can appear to be multiple variants.

3.5 Conclusion

By testing wastewater samples, a comprehensive health report on the population can be gathered. Wastewater-based epidemiology (WBE) has been used in the past for other viral outbreaks such as Polio virus. With a proven track record, WBE could be used as an early warning system for novel SARS-CoV-2 variants of concern and for future pandemics (Hui et al., 2020). Real-time data is crucial in pandemics, WBE offers this data much quicker than methods being used throughout the pandemic. Several countries opted for using WBE data for monitoring the spread of the virus and for policy adjustments (Ahmed et al., 2020; Baldovin et al., 2021; National Institute for Public Health and the Environment, 2020; Westhaus et al., 2021). Currently, WBE is extremely promising and is rapidly evolving.

In this study, a simple yet effective variant of concern screening method was developed. In order to differentiate between VOC, a two probe PCR assay was developed. Viruses mutate rapidly and most mutations are known as single nucleotide polymorphisms (SNPs). These SNPs can be unique to specific VOC or shared between strains. As the aim was develop a method specific to the Delta variant, each SNP selected was unique to that variant. Current assays using this approach target the spike gene which encode for the spike protein (Zhang et al., 2023 ; Specchiarello et al., 2023). Although it is essential for the viral life cycle, it is also the gene that has suffered the most mutations throughout the pandemic, currently over 60 mutations have been recorded (Magazine et al., 2022). Mid 2020, the Alpha variant appeared and resulted in the S-gene target failure (SGTF). This resulted in negative PCR results when in fact the sample was positive. With this in mind, it was clear that the selected target needed to be more of a conserved region. The selected gene was the nucleocapsid gene (N-gene). This gene also encodes for an essential protein in the viral life cycle; however, it is less prone to mutations as it is not used in the process of binding to the ACE2 receptor for infection (Dutta et al., 2020) . SNPs present in the N-gene generally are unique to specific variants making this an ideal target for variant differentiation. Two probes were designed

overlapping the same location, one probe containing the SNP and the other containing the original sequence. As it is only one base-pair mutation, there will be competition for binding and the probe that provides the highest match will bind. There may be some non-specific binding, but this would not be enough to result in same cycle thresholds for both probes.

The gap in cycle threshold between the two probes allows for VOC identification. Generally, the higher the cycle threshold the lower the viral load and vice-versa. In this PCR assay, the viral load is the same for both probes as it is the same sample tested in one pot. The probes will have different cycle thresholds dependent on what strain or strains are present in sample. There is the possibility of looking into further quantification such as being able to identify percentages of each variant present in a specific sample (i.e., 60% Delta VOC, 30% Omicron VOC and 10% others). A melting curve analysis was also approached and provides another possible route. Coupling the SNP-PCR method with a melt curve analysis could also assist in the process of identification of VOCs. This is due to variants presenting different melt curves, a result of unique SNPs. The melt curve was done with artificial gene sequences therefore it would be necessary to test this again with RNA extracted from real samples.

In addition to developing a SNP-specific PCR method, a comparison between wastewater concentration methods was done. As the goal is to use the SNP assay for WBE, it was essential to perfect the sample preparation as well. Wastewater is a complex matrix that contains many inhibitors as well as historical RNA/DNA in solid parts. By comparing various methods, it was possible to identify the ideal processes needed to ensure best outcome. PEG-precipitation was the selected concentration method, it is cheaper than the compared ultrafiltration method. In addition to being cheaper, this method also includes a step removing inhibitors using TRIzol and chloroform. The RNA extraction method for both were the same with the exclusion of the first step. For ultrafiltration, the lysis step is still required. For the PEG concentrated samples, the lysis step prior to RNA extraction is not needed as TRIzol/chloroform acts as lysis whilst also separating inhibitors, other proteins, DNA and RNA. Selecting a method that provides higher RNA yield has more accurate results and provides better understanding. Current literature reinforces that PEG is the best option given that the samples were of larger volume and not frozen as discussed in the results (Farkas et al., 2022 and Lucansky et al., 2023).

As wastewater is a complex matrix, it is expected that multiple variants are present in sample. Sequencing results confirm this, and these results can be seen Table A-11. As many mutations are shared it is expected that the percentages of variants isn't fully accurate. Some mutations allocated as "Omicron" in Table A-10 once were mutations predominantly Delta had. The sharing of mutations may make it appear that there is more of one variant than the other. These sequencing results do tell us that during the collection of these samples Delta and Omicron were both present in sample. The developed PCR assay targeted SNPs that were unique to specific to VOCs. The tests conducted using the SNP-PCR assay confirmed with high accuracy the presence of VOC in comparison to non-variant (Wuhan). Sequencing confirmed low presence of the reference genome Wuhan.

The COVID-19 pandemic has been an unprecedented challenge and has shown mankind that our response and preparedness is crucial. In 2023, the spread of SARS-CoV-2 has continued and booster vaccination campaigns only attracting around half the eligible population (NHS England, 2023), it is likely that we have to learn how to live with this virus. With the possibility of additional variants appearing at any time, a variant specific detection method is very attractive. Many groups around the world have worked to develop variant-specific detection methods. In Specchiarello et al., (2023), a comparison between three popular assays was done: ThermoFisher TaqPath COVID-19 RT-PCR (non-SNP-specific), Seegene Novaplex SARS-CoV-2 Variant assay (SNP-specific) and Allplex VariantII (SNP-specific). Reference method used was the gold-standard Sanger sequencing. The results obtained showed that in 12 samples, no amplification was obtained with Sanger sequencing, likely due to low viral load, but were correctly identified with one of the SNP- assays. This and other data presented in this study show the high ability of a SNP-based assay for variant identification.

The developed SNP-specific assay developed shows high sensitivity, specificity and can be used for routine testing. Beyond the implementation in locations such as universities, hospitals, prisons, the use of a SNP-specific assays can be further optimized to be implemented in biosensors for onsite detection, as mentioned in Chapter 2 of this thesis. This is demonstrated by Zhang et al., (2023), where a multiplexed electrical detection assay based on a paperclip-shaped (PNprobe) nucleic acid probe functionalized field-effect transistor (FET) biosensor was developed for SNP detection. The developed biosensor significantly amplified the target and the PNprobe allowed for a thermodynamic stability difference between variants. In this study, it was possible to detect key mutations in seven SARS-CoV-

2 variants presenting an accurate of 97.1%. Limitations with this study include the use of simulated throat swabs instead of real clinical samples. Even with a few limitations, this is promising as this can be used as a tool for future pandemics and is very cost-effective meaning it can be used even in remote locations.

Another possible route for the SNP assay developed is multiplexing to detect multiple variants and even other pathogens. Every year Influenza A, measles and even respiratory syncytial virus (RSV) spread and cause overloading in our hospitals. A multiplexed assay to detect all the mentioned pathogens at once is not only time-efficient but also allows for a quicker response. Monoplex assays only have one target (i.e., Influenza A) and often have high analytical sensitivity. A limitation with multiplex assays, is that they are not always comparable with monoplex assays in sensitivity and specificity. A group in Canada managed to develop a multiplex assay targeting the above-mentioned pathogens and maintain similar sensitivity and specificity to its monoplex assays (Hayes et al., 2023). The comparison done shows minimal differences when testing wastewater samples with known concentrations of viral load. The assay could be improved however, the assay does present robustness and it very cost effective due to less reagent being using and less run time needed when compared to monoplex assays. A limitation to this study is that the primers-probes used to detect SARS-CoV-2 were not variant specific, this demonstrates the need to develop multiplex assays for variant-specific SNP detection in wastewater alongside other pathogens.

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4 Overall Discussion

SARS-COV-2 and its variants of concern (VOCs) continue to present a threat to the United Kingdom and many other countries. Some VOCs are known to escape our vaccines (Alquaraan et al., 2023; Willett et al., 2020) and even result in more severe disease in certain groups such as venerable individuals and the elderly. Currently, there is a lack of methods that can accurately and rapidly identify specific VOC. The gold standard method is sequencing, which is expensive and timely. This method is not widely available in most countries. GISAID data (2022) suggests that throughout the pandemic less than 10% of samples were sequenced. This may have resulted in a faster spread of variants due to slower response. In order to provide a positive or negative result methods such as PCR and lateral flow test are used. The challenge is that these methods only provide a negative or positive result without providing any information on viral variants.

In chapter 1, an introduction to the COVID-19 pandemic and its challenges were presented. The SARS-CoV-2 variants of concern were discussed in detail along with supporting literature. With the increased worry around vaccine effectiveness, it's clear that novel ways to identify and monitor these variants is needed. With this in mind, wastewater-based epidemiology (WBE) was presented as a possible tool to help gather vital information. WBE and its history was discussed; success stories such as the use of WBE for Polio (Tebbens et al., 2010) and for illicit drugs (Van Nuijs et al., 2011) were presented. Alongside this, analysis of multiple studies using WBE for various applications was cross examined. The drawbacks of WBE of SARS-CoV-2 were evaluated as well. One being that we limited in understanding how the virus and its RNA remain in sewers, whether its viable or not. Infectious SARS-CoV-2 in wastewater is difficult to recover and only results in a very low concentration, despite several attempts.

A comprehensive health report can be obtained by using WBE however, methods should be adjusted for specific targets. It allows for cost-effective population-level detection which can be used by governments as WBE is an objective approach. Current methods for SARS-CoV-2 detection were detailed such as RT-PCR and lateral flow tests; and individual challenges debated about each technique using relevant

literature to support. Whilst most methods are highly accurate in detecting the virus, even in complex matrix, they cannot differentiate between variants. Only some methods, such as sequencing, can provide this information however, come at a high cost, require complex sample preparation and are not widely available. The aims and objectives of this thesis along with the thesis structure, were presented in chapter 1. Extensive literature review showed a lack of accessible methods that can detect and differentiate viral variants in complex sample types such as wastewater. With this in mind, the aim of this thesis was to develop a PCR-based method for SNP detection. SNPs can be unique to variants which allows for variant differentiation and PCR is commonly used for wastewater testing. WBE allows us to know more on the variant dissemination and general population health.

An Education and Public Outreach (EPO) Book Chapter (chapter 2) was completed and allowed further discussion around the possibilities of WBE for pandemics. The intended target audience was school children interested in the sciences. WBE was presented as a public health tool that could help us with the COVID-19 pandemic. As discussed in chapter 1, there are many challenges around gathering population health reports, particularly in underdeveloped countries. Some challenges include the neglect of those who live in rural areas (i.e., far from testing facilities) and the lack of technologies in many countries due to high cost (i.e., sequencing). To address these two challenges the use of biosensors for wastewater testing was presented as an attractive solution. The history, development and mechanisms of action of biosensors were presented to the reader. Biosensors for WBE can be used anywhere in the world with little resources and are very affordable. Alternatives to PCR, such as LAMP, can be used. With the data collected in chapter 3, it is possible to start designing a LAMP-based method for SNP detection. This method can be implemented to paper-based biosensors used for WBE thus, allowing us to gather a health reports from neglected areas. Future perspectives include developing a biosensor for detecting SARS-CoV-2 variants of concern which can be used for WBE.

In chapter 3, Development of a PCR-Based Method for Single Nucleotide Polymorphism Detection, the proposed aim set in chapter 1 is approached. Most PCR methods consist of using forward and reverse primers and one probe containing a fluorophore, all specific to the target. A single nucleotide mismatch can result in a negative result and this scenario was seen during the pandemic when the first viral variants appeared (Clark et al., 2022). The alpha variant spread rapidly without being

detected due mutations in its genome along the location where primers and probes bonded (Imaizumi et al., 2022). This continued to happen with other variants, so the approach of this project was to use two probes instead of one. It's difficult to guess where mutations are going to occur, we know that mutations are more likely in the S gene due to its important role in the viral life cycle (European Centre for Disease Prevention and Control, 2020). For this reason, the S gene was not selected as a target. Upon bioinformatic analysis, it was seen that mutations occurring in the N gene were less likely and more unique. Mutations in the N gene generally were variant specific and maintained for longer periods.

Using bioinformatic analysis, unique SNPs were selected for the current variants at the time (Delta and Omicron). For each SNP two probes were designed overlapping the same region, one containing the mutation and the other no mutation. Forward and reverse primers were the same for both variant and non-variant. In a complex matrix where there might be more than one type of variant, there will be competition for probe binding which will allow for variant differentiation. One nucleotide mutation is sufficient to cause a difference in binding. Although there is expected to be some non-specific binding it's not seen enough to result in a high cycle threshold. Primers and probes were produced by Thermofisher and tested. Initial tests used artificial gene sequences for both variant and non-variant (Wuhan), both prepared to have the same viral load. A series of experiments were done to optimize the assay such as testing multiple concentrations of primer/probe, different ratios of primer/probe and performing limit of detection assays. Once the protocol was established, the two probe in one pot design was tested. It was possible to see a gap in cycle threshold between the two probes which allowed for VOC identification. Initial tests done with artificial sequences showed that the two probes were highly accurate at detecting a variant or non-variant. A melt curve analysis showed that the presence of a SNP results in a different curve, also allowing variant identification. The pairing of the SNP PCR assay with a melt curve analysis is one of the many future prospects. There is also the need to further develop the assay for quantification, such as being able to identify variants in a set of percentages for complex samples (i.e., 40% Delta VOC and 60% Omicron VOC).

Wastewater samples from quarantine hotels in June/July 2021 and wastewater samples from Cranfield's Wastewater Treatment Plant over the course of several months were obtained. These samples were tested using the SNP PCR assay and it was possible to see trends that matched data from wastewater treatment plants of

cities nearby (Milton Keynes). The SNP PCR method showed that in the majority of these samples VOC were present, mostly Delta. Data from Public Health England and GISAID showed that during the collection of the wastewater samples tested, the predominant variants circulating were Delta and Omicron. This matched the results the developed assay showed. To ensure that the samples tested had the indented targets, samples were sent for sequencing with a collaborator at the University of Sheffield. The partial sequencing (S-gene only) confirmed the presence of Omicron and Delta in all samples. The results obtained using the SNP-PCR assay confirm the presence of specific variants and sequencing assisted in further validating this claim.

As the intended use of the SNP PCR assay was for WBE, a comparison between wastewater concentration methods was also done. Sample preparation is very important when working with wastewater as it can impact the end results if not done properly. By comparing PEG-precipitation to ultrafiltration, it was determined that PEG-precipitation was the best method going forward. Not only is it cheaper long term, but it also includes a step to remove inhibitors and resulted in higher RNA yield. This was also in line with recent literature which shows that PEG generally is a better option for wastewater samples in higher volume, with solid particles and for samples that had not been frozen. For the quarantine hotels specifically, ultrafiltration might have performed better due to the samples being frozen for a long period and of lower volume compared to the University samples. Limitations of this study include the partial sequencing of the SARS-CoV-2 genome. To be able to fully quantify VOCs in samples a whole genome sequencing is needed. For the purpose of confirming the presence of SARS-CoV-2 in the samples, the S-gene sequencing alone was sufficient. Samples collected from quarantine hotels were sequenced over a year after collection and therefor resulted in low depth. Low depth samples indicate that the mapping of the genome might not be as accurate. Whole genome sequencing would be needed to increase the quality of the quarantine hotel samples genome mapping and allow for better interpretation.

The develop SNP assay showed high sensitivity and specificity to specific viral variants. This assay can be used for routine testing and should be implemented in locations where there may be individuals at risks such as care homes, hospitals, et cetera. The assay should be further optimized to expand its testing horizons. A future prospect of this project includes the development of a SNP-LAMP (Hyman et al., 2022) assay. LAMP is a potent alternative to PCR and presents many benefits that PCR and

other methods cannot offer. By transferring knowledge obtained by developing the SNP-PCR method, a SNP-LAMP method can be developed and implemented on paper-based biosensors as demonstrated by Zhang et al., (2023). Paper-based biosensors present even more benefits as they may be used in rural areas for rapid detection. Benefits of a biosensor capable of detecting VOC in wastewater is further discussed in chapter 2. Another future prospect is coupling the SNP-PCR method with other methods such as melt curve analysis, this will be an extra layer in the process of identifying VOC. The melt curve analysis done in the study shows that variants have distinct peaks and can be very easily identified. A drawback to this approach is unfortunately, this would require the use of expensive equipment and trained personnel.

Perhaps the most interesting and beneficial future prospect is multiplexing the SNP-PCR assay. Multiplexing can be done to include multiple variants of concern in test or even test for multiple pathogens (Hayes et al., 2023). With a high percentage of the population being vaccinated for COVID-19, it is expected that cases may come down however, every year we continue to see many cases of Influenza, Measles and RSV. A multiplex assay that detects all these pathogens in one test would be of great benefit to the population. This assay coupled with WBE can help us prevent outbreaks and respond in a timely manner.

APPENDICES

Appendix A

Table A-1: Table showing all primers and probes used in this study.

Target	Name	Sequence 5' → 3'	Fluorophore	Tm°	GC%	Amplicon size (bp)
Nucleocapsid gene	N1* (Reference - all variants)	Forward: GACCCCAAAATCAGCGAA AT		64.9	45	
		Reverse: TCTGGTTACTGCCAGTTGAATCTG		66.2	45.8	73
		Probe: ACCCCGCATTACGTTTGGTGGACC-BHQ1	FAM	75.4	58.3	
Nucleocapsid gene	N3* (Reference - all variants)	Forward: GGGAGCCTTGAATACACCAAAA		55.4	45.5	
		Reverse: TGTAGCACGATTGCAGCATTG		56.3	47.6	72
		Probe: AYCACATTGGCAGCCGCAATCCTG-BHQ1	FAM	64.3	56.2	
Nucleocapsid gene	N-Gene-F-Delta	Forward: GTTCCTCATCACGTAGTCGCA		65.2	52.3	
	N-Gene-R-Delta	Reverse: CGCCATTGCCAGCCATTCTA		70	55	
	N-Gene-P-DeltaSNP	Probe: TCAACTCCAGGCAGCAGTA T GGGA-BHQ1	FAM	72.6	54.1	91
	N-Gene-P-Delta	Probe: TCAACTCCAGGCAGCAGTAGGGGA-BHQ1	VIC	73.6	58.3	
Nucleocapsid gene	N-Gene-F-Omicron	Forward: CAGACTATTACCTGGGG		50	52.9	
	N-Gene-R-Omicron	Reverse: GCCCCACCATTTCTGGTTACT		57.3	55	
	N-Gene-P-OmicronSNP	Probe: CGCTTTACGTG G GGCG-BHQ1	FAM	68.9	57	94
	N-Gene-P-Omicron	Probe: CGCTTTACGTGAGGCG-BHQ1	VIC	62.5	53.9	
Nucleocapsid gene	N-Gene-F-T7	Forward: AATTCTAATACGACTCACTATAGGG CCAAATTGGCTACTACCGAAGAGCTAC		78.5	42.3	900†
	N-Gene-R-T7	Reverse: CACAGTTTGCTGTTTCTTCTGTCTCTGCGG		75.7	50	

*CDC, Centers for Disease Control and Prevention; N, nucleocapsid protein gene; RT-PCR, real-time reverse transcription PCR; SARS-CoV-2, severe acute respiratory coronavirus 2.

† Approximate number of bp. Actual size will depend on variant due to insertions and deletions.

Base pairs in bold red are targeted SNPs present in VOC.

Base pairs in bold green indicate a T7 promoter region

>Wuhan_N_Gene

ATGTCTGATAATGGACCCCAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTG
GCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATA
CTGCGTCTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCTCGAGGACAAGGCGTTCC
AATTAACACCAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGT
GACGGTAAAATGAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTC
CCTATGGTGCTAACAAAGACGGGCATCATATGGGTGCAACTGAGGGAGCCTTGAATACACCAAAAGATCACA
TTGGCACCCGCAATCCTGCTAACAATGCTGCAATCGTGCTACAACCTCCTCAAGGAACAACATTGCCAAAAGG
CTTCTACGCAGAAGGGAGCAGAGGGCGGCAGTCAAGCCTCTTCTCGTTCCCTCATCACGTAGTCGCAACAGTTCA
AGAAATTCAACTCCAGGCAGCAGTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCT
CTTGCTTTGCTGCTGCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAG
GCCAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGC
ATACAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGGAACT
AATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTC
GGAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTG
GATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCC
ACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAACTCAAGCCTTACCGCAGAGACAGAAGA
AACAGCAAACGTGACTCTTCTTCCTGCTGCAGATTTGGATGATTTCTCCAAACAATTGCAACAATCCATGAGC
AGTGCTGACTCAACTCAGGCCTAA

Figure A-1: Nucleocapsid gene sequence for Wuhan SARS-CoV-2 (Reference sequence)

>Delta_B.1.617.2_N_Gene

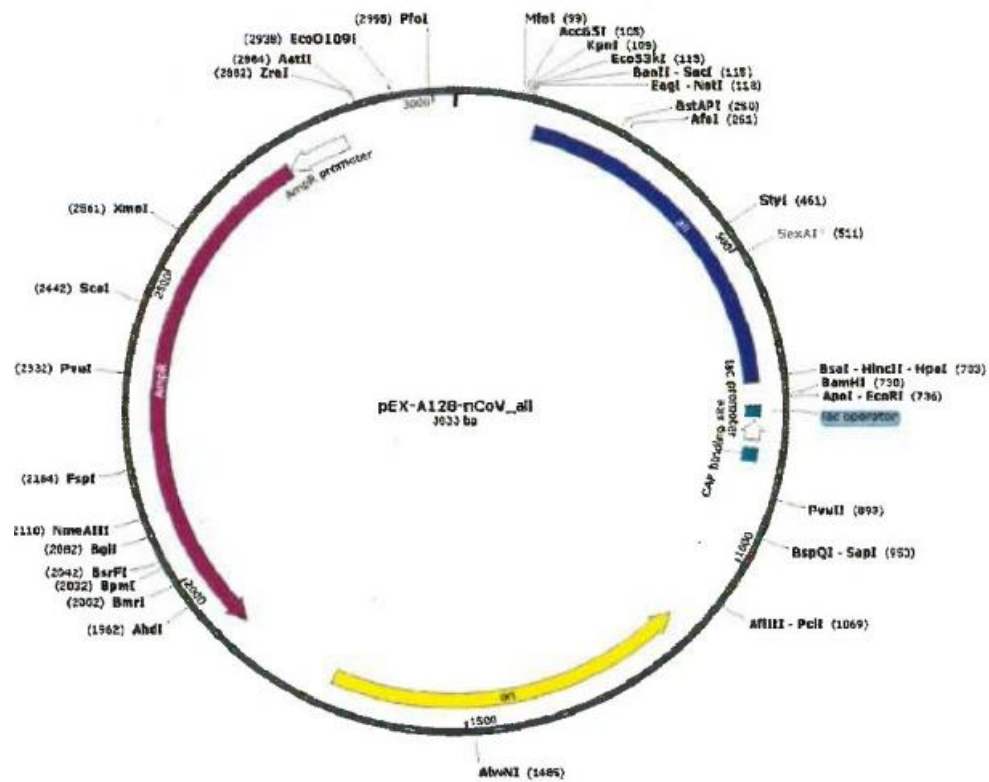
ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTG
GCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATA
CTGCGTCTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGGCCTTAAATTCCCTCGAGGACAAGGCGTTCC
AATTAACACCAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGT
GACGGTAAAATGAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTC
CCTATGGTGCTAACAAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAAAGATCACA
TTGGCACCCGCAATCCTGCTAACAATGCTGCAATCGTGCTACAACCTTCCTCAAGGAACAACATTGCCAAAAGG
CTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCA
AGAAATTCAACTCCAGGCAGCAGTATGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTC
TTGCTTTGCTGCTGCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGG
CCAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTAAGTCCCACTAAAGCA
TACAATGTAAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAACCCAAGGAAATTTGGGGACCAGGAACCTA
ATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCAGCGCTTCAGCGTTCTTCG
GAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGG
ATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGTGAATAAGCATATTGACGCATACAAAACATTCCCA
CCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTTATGAACTCAAGCCTTACCGCAGAAACAGAAGAA
ACAGCAAACGTGACTCTTCTTCCTGCTGCAGATTTGGATGATTTCTCCAAACAATTGCAACAATCCATGAGCA
GTGCTGACTCAACTCAGGCCTAA

Figure A-2: Nucleocapsid gene sequence for Delta (B.1.617.2) SARS-CoV-2 variant of concern.

>Omicron_B.1.1.529_N_Gene

ATGTCTGATAATGGACCCCAAATCAGCGAAATGCACTCCGCATTACGTTTGGTGGACCCTCAGATTCAACTG
GCAGTAACCAGAATGGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGTCTT
GGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCTCGAGGACAAGGCGTTCCAATTAACAC
CAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAA
AATGAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGT
GCTAACAAAGACGGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAGATCACATTGGCACC
CGCAATCCTGCTAACAATGCTGCAATCGTGCTACAACCTTCTCAAGGAACAACATTGCCAAAAGGCTTCTACGC
AGAAGGGAGCAGAGGGCGGCAGTCAAGCCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCA
ACTCCAGGCAGCAGTAAACGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTGC
TGCTGCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAACTG
TCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGCATACAATGT
AACACAAGCTTTCGGCAGACGTGGTCCAGAACAACCCAAGGAAATTTTGGGGACCAGGAACTAATCAGACA
AGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCAGCGCTTCAGCGTTCTTCGGAATGTCG
CGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAA
GATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCCACCAACAGA
GCCTAA

Figure A-3: Nucleocapsid gene sequence for Omicron (B.1.1.529.9) SARS-CoV-2 variant of concern.



MCS of pEX-A128-nCoV_all

GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGTCG
GGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGAAAGG
caattgGGTACCgagctcGCGGCCGCAAGC>AllWHOCDGenes>ACC
TGCTTTTGCTCGCTTGGATCCgaattcAAAGGTGAAATTGTTATCCGCT
CACAATTCACACAACATACGAGCCGGAAGCATAAAGGTAAAGCCTG

Figure A-4: Positive control plasmid containing all SARS-CoV-2 genes (Wuhan reference sequence).

Table A-2: Table of influent wastewater samples tested in this study showing collection dates, location, storage conditions, volume and concentration (ng/uL).

Sample Number	Date	Location	Sample type	Eluted and stored in	Volume	Measured concentration (ng/uL)
1	07 April 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	5.48
2	19 April 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	8
3	21 April 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	4.44
4	26 April 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	3.78
5	28 April 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	6.44
6	03 May 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	5.64
7	05 May 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	2.23

8	10 May 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	5.32
9	12 May 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	5.32
10	17 May 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	2.16
11	17 June 2021	Quarantine Hotel 1	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	16
12	17 June 2021	Quarantine Hotel 2	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	20.8
13	17 June 2021	Quarantine Hotel 3	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	3.08
14	17 June 2021	Quarantine Hotel 4	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	>1.0
15	17 June 2021	Quarantine Hotel 5	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	5.76
16	01 February 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	19

17	03 February 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	0.92
18	09 February 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	9.68
19	11 February 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	25.6
20	24 February 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	6.2
21	27 February 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	>1.0
22	01 March 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	28.8
23	03 March 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	1.11
24	08 March 2022	Cranfield WWTP	Extracted RNA	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	75 uL	2.01

Table A-3: Cranfield's Wastewater Treatment Plant daily flow data provided by Alpheus.

Date	Inlet flow (m3/d)	Date	Inlet flow (m3/d)	Date	Inlet flow (m3/d)
01/03/2022	918	01/04/2022	457	02/05/2022	401
02/03/2022	778	02/04/2022	435	03/05/2022	454
03/03/2022	918	03/04/2022	396	04/05/2022	477
04/03/2022	766	04/04/2022	405	05/05/2022	455
05/03/2022	715	05/04/2022	527	06/05/2022	417
06/03/2022	597	06/04/2022	502	07/05/2022	390
07/03/2022	554	07/04/2022	497	08/05/2022	380
08/03/2022	569	08/04/2022	495	09/05/2022	442
09/03/2022	562	09/04/2022	463	10/05/2022	454
10/03/2022	546	10/04/2022	398	11/05/2022	546
11/03/2022	521	11/04/2022	406	12/05/2022	503
12/03/2022	514	12/04/2022	450	13/05/2022	473
13/03/2022	456	13/04/2022	461	14/05/2022	380
14/03/2022	462	14/04/2022	464	15/05/2022	413
15/03/2022	495	15/04/2022	446	16/05/2022	500

16/03/2022	510	16/04/2022	389	17/05/2022	491
17/03/2022	812	17/04/2022	371	18/05/2022	496
18/03/2022	761	18/04/2022	382	19/05/2022	493
19/03/2022	599	19/04/2022	397	20/05/2022	456
20/03/2022	524	20/04/2022	465	21/05/2022	397
21/03/2022	493	21/04/2022	498	22/05/2022	403
22/03/2022	517	22/04/2022	490	23/05/2022	441
23/03/2022	510	23/04/2022	473	24/05/2022	472
24/03/2022	488	24/04/2022	464	25/05/2022	489
25/03/2022	477	25/04/2022	443	26/05/2022	460
26/03/2022	465	26/04/2022	460	27/05/2022	457
27/03/2022	407	27/04/2022	488	28/05/2022	399
28/03/2022	409	28/04/2022	482	29/05/2022	398
29/03/2022	457	29/04/2022	466	30/05/2022	473
30/03/2022	481	30/04/2022	446	31/05/2022	488

Table A-4: QIAamp Viral RNA Mini Kit components

Solution Name	Mixture	Exposure Limit	Hazards	Hazard codes
Buffer AVL	Not disclosed on SDS – Confirmed no hazardous ingredients.	Contains no substances with occupational exposure limit values.	N/A	N/A
Buffer AW1 (Wash 1)	Guanidine hydrochloride (concentration not provided – lower concentration)	Contains no substances with occupational exposure limit values.	Harmful	H302, H332, H315, H319
Buffer AW2 (Wash 2)	Guanidine hydrochloride (concentration not provided – higher concentration)	Contains no substances with occupational exposure limit values.	Harmful	H302, H332, H315, H319
Buffer AVE	Not disclosed on SDS – Confirmed no hazardous ingredients.	Contains no substances with occupational exposure limit values.	N/A	N/A
Buffer AE	10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0.	Contains no substances with occupational exposure limit values.	N/A	N/A

Table A-5: Reagents used for gel electrophoresis

Solution Name	Mixture	Exposure limit	Hazards	Hazard codes
Agarose	This product contains no substances which at their given concentration, are considered to be hazardous to health.	Contains no substances with occupational exposure limit values.	N/A	N/A
TAE (Tris-acetate-EDTA) Buffer	10X TAE Buffer pH 8.5	Contains no substances with occupational exposure limit values	N/A	N/A
SYBR Safe DNA Gel Stain	Dimethyl sulfoxide (95-100) SYBR® Safe 98 (0.1-1)	Contains no substances with occupational exposure limit values	N/A	N/A
Invitrogen Loading Buffer	This product contains no substances which at their given concentration, are considered to be hazardous to health	Contains no substances with occupational exposure limit values	N/A	N/A
Invitrogen DNA Ladder	This product contains no substances which at their given concentration, are considered to be hazardous to health	Contains no substances with occupational exposure limit values	N/A	N/A

Table A-6: Zymoclean Gel DNA Recovery Kit reagents.

Solution Name	Mixture	Exposure limit	Hazards	Hazard codes
ADB Buffer	Sodium iodide <30% mixed with non-hazardous substances not stated in SDS	Long-term value: NIC-0.015** mg/m ³ , (0.01*) ppm	N/A	N/A
DNA Wash Buffer (Concentrate)	This product contains no substances which at their given concentration, are considered to be hazardous to health	The product does not contain any relevant quantities of materials with critical values that have to be monitored at the workplace.	N/A	N/A
DNA Elution Buffer	This product contains no substances which at their given concentration, are considered to be hazardous to health	The product does not contain any relevant quantities of materials with critical values that have to be monitored at the workplace.	N/A	N/A

Table A-7: Monarch RNA Cleanup Kit Reagents

Solution Name	Mixture	Exposure limit	Hazards	Hazard codes
Binding Buffer	Guanidine Thiocyanate, Sodium Acetate, Ethylenediamine tetra acetic acid. Exact concentrations are withheld as trade secret.	Contains no substances with occupational exposure limit values	Harmful if swallowed Causes severe skin burns and eye damage	H314, H302, H318
Wash Buffer	This product contains no substances which at their given concentration, are considered to be hazardous to health	Contains no substances with occupational exposure limit values	N/A	N/A

Table A-8: Data from Cranfield's WWTP for the March to May 2022

Pre-Primary Settlement Tank							
Date	Time	BOD	Nitrate	Ammonia	TSS	pH	Kjeldahl Nitrogen
		mg/l	mg/l	mg/l	mg/l		mg/l
02/03/2022	10:08	100	<0.1	28	330	8	34
09/03/2022	14:35	280	0.15	53	180	7.8	53
16/03/2022	08:30	770	0.92	27	1100	8	33
23/03/2022	09:00	220	0.52	56	220	7.6	68
30/03/2022	09:00	240	0.46	32	310	7.7	57
06/04/2022	08:00	300	0.34	51	370	7.4	70
13/04/2022	09:00	340	<0.1	50	470	7.6	62
21/04/2022	07:00	460	0.31	53	330	7	64
27/04/2022	08:00	160	0.98	53	72	8	61
04/05/2022	08:00	230	0.21	50	110	7.4	54
11/05/2022	08:00	120	<0.1	34	120	7	34
18/05/2022	08:00	140	0.49	51	230	7	58
25/05/2022	09:00	450	0.31	54	1200	7.3	75

Table A-9: Variant Identification for Sequenced Samples

Covid Variant	Mutation	Nucleotide	Position	Wuhan Base Pair	Variant Base Pair
Alpha.B.1.1.7.Kent	N501Y	A23063T	23063	A	T
Alpha.B.1.1.7.Kent	A570D	C23271A	23271	C	A
Alpha.B.1.1.7.Kent	P681H	C23604A	23604	C	A
Alpha.B.1.1.7.Kent	T716I	C23709T	23709	C	T
Alpha.B.1.1.7.Kent	S982A	T24506G	24506	T	G
Alpha.B.1.1.7.Kent	D1118H	G24914C	24914	G	C
Beta.B.1.351.South.Africa	D80A	A21801C	21801	A	C
Beta.B.1.351.South.Africa	D215G	A22206G	22206	A	G
Beta.B.1.351.South.Africa	K417N	G22813T	22813	G	T
Beta.B.1.351.South.Africa	E484K	G23012A	23012	G	A
Beta.B.1.351.South.Africa	N501Y	A23063T	23063	A	T
Beta.B.1.351.South.Africa	A701V	C23664T	23664	C	T
Delta. B.1.617.2.India	T19R	C21618G	21618	C	G
Delta. B.1.617.2.India	L452R	T22917G	22917	T	G
Delta. B.1.617.2.India	T478K	C22995A	22995	C	A
Delta. B.1.617.2.India	P681R	C23604G	23604	C	G
Delta. B.1.617.2.India	D950N	G24410A	24410	G	A
Gamma.P.1.Brazil.2	T20N	C21621A	21621	C	A
Gamma.P.1.Brazil.2	P26S	C21638T	21638	C	T
Gamma.P.1.Brazil.2	K417T	A22812C	22812	A	C
Gamma.P.1.Brazil.2	E484K	G23012A	23012	G	A
Gamma.P.1.Brazil.2	N501Y	A23063T	23063	A	T

Gamma.P.1.Brazil.2	T1027I	C24642T	24642	C	T
Omicron_BA.1	A67V	C21762T	21762	C	T
Omicron_BA.1	T95I	C21846T	21846	C	T
	No				
Omicron_BA.1	change	T22673C	22673	T	C
Omicron_BA.1	G446S	G22898A	22898	G	A
Omicron_BA.1	G496S	G23048A	23048	G	A
Omicron_BA.1	T547K	C23202A	23202	C	A
Omicron_BA.1	N856K	C24130A	24130	C	A
Omicron_BA.1	L981F	C24503T	24503	C	T
Omicron_BA1,2,4,5	G142D	G21987A	21987	G	A
Omicron_BA1,2,4,5	G339D	G22578A	22578	G	A
Omicron_BA1,2,4,5	S371F	C22674T	22674	C	T
Omicron_BA1,2,4,5	S373P	T22679C	22679	T	C
Omicron_BA1,2,4,5	S375F	C22686T	22686	C	T
Omicron_BA1,2,4,5	K417N	G22813T	22813	G	T
Omicron_BA1,2,4,5	N440K	T22882G	22882	T	G
Omicron_BA1,2,4,5	S477N	G22992A	22992	G	A
Omicron_BA1,2,4,5	T478K	C22995A	22995	C	A
Omicron_BA1,2,4,5	E484A	A23013C	23013	A	C
Omicron_BA1+2	Q493R	A23040G	23040	A	G
Omicron_BA1,2,4,5	Q498R	A23055G	23055	A	G
Omicron_BA1,2,4,5	N501Y	A23063T	23063	A	T
Omicron_BA1,2,4,5	Y505H	T23075C	23075	T	C
Omicron_BA1,2,4,5	D614G	A23403G	23403	A	G

Omicron_BA1,2,4,5	H655Y	C23525T	23525	C	T
Omicron_BA1,2,4,5	N679K	T23599G	23599	T	G
Omicron_BA1,2,4,5	P681H	C23604A	23604	C	A
Omicron_BA1,2,4,5	N764K	C23854A	23854	C	A
Omicron_BA1,2,4,5	D796Y	G23948T	23948	G	T
Omicron_BA1,2,4,5	Q954H	A24424T	24424	A	T
Omicron_BA1,2,4,5	N969K	T24469A	24469	T	A
Omicron_BA1+2	No				
	change	C25000T	25000	C	T
Omicron_BA1+2	No				
	change	C25584T	25584	C	T
Omicron_BA.2,4,5	T19I	C21618T	21618	C	T
Omicron_BA.2,4,5	V213G	T22200G	22200	T	G
Omicron_BA.2,4,5	T376A	A22688G	22688	A	G
Omicron_BA.2,4,5	D405N	G22775A	22775	G	A
Omicron_BA.2,4,5	R408S	A22786T	22786	A	T
Omicron_BA.4+5	L452R	T22917G	22917	T	G
Omicron_BA.4+5	F486V	T23018G	23018	T	G

Table A-10: Sequenced Samples Raw Read Counts

Sample number	Date Collected	Raw Read Counts
1	07 April 2022	7869
2	19 April 2022	736765
3	21 April 2022	93560
4	26 April 2022	344632
5	28 April 2022	106515
6	03 May 2022	274051
7	05 May 2022	67696
8	10 May 2022	117040
9	12 May 2022	44853
10	17 May 2022	7313
11	17 June 2021	2473
12	17 June 2021	1180
13	17 June 2021	4560
14	17 June 2021	2778
15	17 June 2021	18374

Table A-11: Presence of VOC in Cranfield's Wastewater Samples (Sequenced probability)

Sample Number	Depth	Probability of VOC in sample
1	<50,000	67.39% Omicron; 8.70% Delta; 23.91% Other VOCs
2	>50,000	62.29% Omicron; 8.20% Delta; 29.51% Other VOCs
3	>50,000	60.0% Omicron; 9.09% Delta; 30.91% Other VOCs
4	>50,000	63.33% Omicron; 8.33% Delta; 28.34% Other VOCs
5	>50,000	63.69% Omicron; 8.62% Delta; 27.69% Other VOCs
6	>50,000	65.45% Omicron; 7.27% Delta; 27.28% Other VOCs
7	>50,000	67.22% Omicron; 9.30% Delta; 23.48% Other VOCs
8	>50,000	62.71% Omicron; 8.47% Delta; 28.82% Other VOCs
9	>50,000	63.16% Omicron; 8.77% Delta; 28.07% Other VOCs
10	<50,000	52.94% Omicron; 11.76% Delta; 35.30% Other VOCs