



Qingxin Hui

CRISPR/Cas-enabled paper microfluidic device to detect SARS-CoV-2 and its variants for wastewater-based epidemiology

Faculty of Engineering and Applied Sciences

PhD in Water

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Academic Year: 2021 - 2024

Primary supervisor: Professor Zhugen Yang

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## List of Publications

### Peer-Reviewed Articles

1. Wang, Y., Pan, Y., Han, W., Rossi, C.S., **Hui, Q.**, Guo, Y., Mojisola Christiana Owoseni, McAdam, E., Yong, Y.-C., Wang, B. and Yang, Z. (2024). CRISPR-enabled sensors for rapid monitoring of environmental contaminants. *TrAC Trends in Analytical Chemistry*, pp.118128–118128. doi:<https://doi.org/10.1016/j.trac.2024.118128>.
2. Pan, Y., Mao, K., **Hui, Q.**, Wang, B., Cooper, J. and Yang, Z. (2022). Paper-based devices for rapid diagnosis and wastewater surveillance. *TrAC. Trends in analytical chemistry*, 157, pp.116760–116760. doi:<https://doi.org/10.1016/j.trac.2022.116760>.
3. **Hui, Q.**, Pan, Y. and Yang, Z. (2020). Paper-based devices for rapid diagnostics and testing sewage for early warning of COVID-19 outbreak. *Case Studies in Chemical and Environmental Engineering*, [online] 2, p.100064. doi:<https://doi.org/10.1016/j.cscee.2020.100064>.

### Book Chapters

1. **Hui, Q.**, Pan, Y., Cullen, D. and Yang, Z. (2025). Advanced sensors enabled rapid and on-site wastewater surveillance for SARS-CoV-2 and beyond. *Sample Handling and Trace Analysis of Pollutants*, pp.719–738. doi:<https://doi.org/10.1016/b978-0-323-85601-0.00005-9>.
2. Pan, Y., Li, W., **Hui, Q.** and Yang, Z. (2023). Biosensors for bacteria detection. *Elsevier eBooks*, pp.81–123. doi:<https://doi.org/10.1016/b978-0-323-90222-9.00011-x>.

## Abstract

Wastewater-based epidemiology (WBE) has been demonstrated to be a powerful tool for monitoring public health through the detection of biomarkers, such as drugs and pathogens, in wastewater. During the COVID-19 pandemic, it evolved into a significant supplementary approach to clinical diagnostics, offering capabilities as an early warning system, enabling population-level infection monitoring, and tracking down the virus including the variants of concern. However, current methods for wastewater analysis are heavily dependent on the centralized laboratory which requires expensive equipment, specialized personnel, and result in slow turnaround. This thesis addresses these critical challenges by developing innovative approaches for the detection of SARS-CoV-2 and its variants, combining molecular diagnostics with field-deployable paper microfluidic devices. A comparative analysis of polyethylene glycol (PEG) precipitation and ultrafiltration for concentrating SARS-CoV-2 RNA from wastewater led to the development of an optimized protocol suitable for routine analysis. This protocol was applied for long-term monitoring of viral loads (N-gene) in a local wastewater treatment plant, providing valuable insights into infection trends. Additionally, a CRISPR/Cas12a-based fluorescent assay was developed to detect SARS-CoV-2 and its variants with high specificity and sensitivity, able to detect as low as 5 copies  $\mu\text{L}^{-1}$ . To enable on-site diagnostics, a portable paper microfluidic device was designed, integrating the optimized concentration methods and CRISPR/Cas12a assays. The device demonstrated a rapid detection of wastewater within 90 minutes for SARS-CoV-2 even without laboratory settings. This research findings advances WBE by bridging the gap between laboratory-based techniques and in-field testing. The technology provides a scalable, cost-effective platform for rapid and onsite monitoring SARS-CoV-2 and other pathogens, contributing significantly to global health management, particularly in resource-limited settings.

**Keywords:** Wastewater-based epidemiology; SARS-CoV-2 and Variants, CRISPR/Cas; Paper microfluidic device; Isothermal amplification

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## **List of Abbreviations**

AMR - Antimicrobial Resistance

ATP - Adenosine Triphosphate

AVL - Viral Lysis Buffer

BHQ - Black Hole Quencher

BIP - Backward Inner Primer

BoHV-1 - Bovine Herpes Virus-1

CDC - Centers for Disease Control and Prevention

cDNA - Complementary DNA

CFU - Colony-Forming Unit

CoSHH - Control of Substances Hazardous to Health

COVID-19 - Coronavirus Disease 2019

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

C<sub>t</sub> - Cycle Threshold

dH<sub>2</sub>O - Distilled Water

DNA - Deoxyribonucleic Acid

dNTP - Deoxynucleoside Triphosphates

dsDNA - Double-Stranded DNA

ELISA - Enzyme-Linked Immunosorbent Assay

FAM - Fluorescein Amidites

FDA - Food and Drug Administration

FIP - Forward Inner Primer

gRNA - Guide RNA

HAV - Hepatitis A Virus

HCl - Hydrochloric Acid

HRP - Horseradish Peroxidase

HTS - High-Throughput Sequencing

IDT - Integrated DNA Technologies

IgG - Immunoglobulin G

IgM - Immunoglobulin M

LAMP - Loop-Mediated Isothermal Amplification

LB - Loop Backward Primer

LF - Loop Forward Primer

LFA - Lateral Flow Assay

LOD - Limit of Detection

MCE - Mixed Cellulose Esters

MERS - Middle East Respiratory Syndrome

MHV - Mouse Hepatitis Virus

MRSA - Methicillin-Resistant Staphylococcus Aureus

NaCl - Sodium Chloride

NaOH - Sodium Hydroxide

NEBuffer - New England Biolabs Buffer

NGS - Next-Generation Sequencing

NTC - No-Template Control

PAM - Protospacer Adjacent Motif

PCCO - Polypropylene Copolymer

PCR - Polymerase Chain Reaction

PDMS - Polydimethylsiloxane

PEG - Polyethylene Glycol

Phi6 - Pseudomonas Virus Phi6

PON - Point-of-Need

RdRp - RNA-Dependent RNA Polymerase

RNA - Ribonucleic Acid

ROX - Carboxyrhodamine

RT-LAMP - Reverse Transcription Loop-Mediated Isothermal Amplification

RT-qPCR - Reverse Transcription Quantitative Polymerase Chain Reaction

SARS - Severe Acute Respiratory Syndrome

SARS-CoV-2 - Severe Acute Respiratory Syndrome Coronavirus 2

SDS - Safety Data Sheets

SNP - Single-Nucleotide Polymorphism

TMB - 3,3',5,5'-Tetramethylbenzidine

UKHSA - UK Health Security Agency

UV - Ultraviolet

VOC - Variant of Concern

WBE - Wastewater-Based Epidemiology

WWTP - Wastewater Treatment Plant

ZIKV - Zika Virus

# Chapter 1: Introduction

## 1.1 Context

During the outbreak of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), it was highlighted that there was an urgent need for novel assessment approaches that could reveal the epidemiological scope of the disease (Bogler *et al.*, 2020). Traditional surveillance approaches relied on individual clinical diagnosis, but the overwhelmed health system lacked diagnostic capacity, and a large proportion of the population was missed due to incubation periods or asymptomatic infection (Polo *et al.*, 2020). As a complementary approach, wastewater-based epidemiology (WBE) has emerged as an important surveillance tool for monitoring infection and transmission pathways at the population level, as SARS-CoV-2 has been found in the feces and urine of infected patients (Daughton, 2020; Jones *et al.*, 2020).

Also, it is believed that the true standout of WBE is the capability of early warning for pandemic, as wastewater analysis shows great promise to be employed as an early warning system in two ways (Zhu *et al.*, 2021). The first is to predict the early stage of the outbreak before the clinical confirmation reports. Due to the lack of noticeable symptoms like fever and dry cough, asymptomatic and presymptomatic virus carriers can blend in with the community, but detecting viruses from wastewater can identify them indiscriminately as long as they exhibit viral RNA shedding (Jones *et al.*, 2020). Thus, if a positive viral load in wastewater is found in an ongoing wastewater survey in a community with no previous cases, it may indicate that the virus has been introduced in the region (Bibby *et al.*, 2021). Intervention through the issuance of warnings based on the availability of this epidemiological information can inform the public of the potential threat and reduce the chances of stealth transmission. In addition, sharp increases in viral loads in wastewater can be regarded as a signal that a significant outbreak is imminent, allowing healthcare systems to take steps to prepare in advance and avoid the possibility of overburdening facilities. Because the viral load in wastewater is highly positively correlated with the number of

people infected, and there are reports of viral peaks in wastewater earlier than in clinical testing due to the lag time in clinical testing caused by the latency period of the virus (Trigo-Tasende *et al.*, 2023; Singer *et al.*, 2023).

Despite the great potential of WBE, there are several challenges to its practical implementation. First, wastewater samples are inherently complex, containing a variety of biological and chemical components, the presence of which can interfere with the recovery and detection of viral RNA (Melvin *et al.*, 2021). In addition, the lower concentration of viral particles in wastewater than in clinical samples requires additional concentration and extraction methods to bring the viral RNA to the point of assay (Hart and Halden, 2020). Given these issues with wastewater samples, robust methods need to be developed to ensure that long-term surveillance can be achieved in wastewater for WBE implementation.

The detection of SARS-CoV-2 variants in wastewater adds another layer of complexity. During COVID-19, several SARS-CoV-2 variants had different genetic profiles, and specific mutations affected the transmissibility and neutralizing antibody resistance of the variants (Tao *et al.*, 2021). While effective, conventional diagnostic methods using sequencing, are expensive and time-consuming, making them unsuitable for use in early warning systems for wastewater (Smyth *et al.*, 2022). The development of CRISPR/Cas12a-based assays is a significant step-forward in molecular diagnostics. The CRISPR/Cas system was first discovered for gene editing because of its high specificity (Hsu, Lander and Zhang, 2014). During the COVID-19 outbreak, assays for SARS-CoV-2 were developed using Cas12 or Cas13 enzymes to provide rapid, highly specific and sensitive clinical detection of the virus (Broughton *et al.*, 2020; Patchsung *et al.*, 2020). The CRISPR/Cas system was further used for single nucleotide polymorphisms (SNPs) detection by designing a highly specific guide RNA (gRNA) that targets the SNP site in the DNA or RNA sequence (Yang *et al.*, 2022). However, harnessing the CRISPR/Cas system for variant detection in environmental monitoring, particularly wastewater analysis, is still in its infancy.

Finally, the conventional wastewater analysis mode relies on the collection of wastewater samples to the laboratory followed by reverse transcription

quantitative polymerase chain reaction (RT-qPCR) for analysis. Although the gold standard method is highly sensitive and specific, it requires well-equipped laboratories, trained personnel, and expensive infrastructure, limiting its applicability in decentralized and resource-limited settings (Yang, 2023). Rapid field testing is therefore necessary to overcome these limitations, allowing sensitive detection of low concentrations of pathogens in wastewater while providing real-time monitoring to generate data sets large enough for early warning systems (Mao *et al.*, 2021). Paper-based microfluidic devices were originally developed for point-of-care diagnostics (Martinez *et al.*, 2010), and their characteristics were low-cost, sensitive, specific, user-friendly, rapid, robust, equipment-free and available to end-users (Peeling *et al.*, 2006). As technology experienced rapid advances, the paper-based microfluidic devices have been expanded for environmental monitoring (Kung *et al.*, 2019). Also, the combination with isothermal amplification techniques allowed the paper-based microfluidic devices for nucleic acid detection. For instance, it has been demonstrated for the detection of *Escherichia coli* and *Streptococcus pneumonia* (Choi *et al.*, 2016), human papillomavirus (HPV) 16 virus (Rodriguez *et al.*, 2016) and malaria (Xu *et al.*, 2016). Furthermore, the paper-based microfluidic device has a potential to be further integrated with CRISPR/Cas technology to enable the specific detection of SARS-CoV-2 and its variants from wastewater and benefit WBE.

## **1.2 Aims and Objectives**

The overall aim of this PhD research is to enhance the capabilities of WBE for SARS-CoV-2 detection by conducting wastewater analysis at Cranfield University, developing an advanced CRISPR/Cas12a-based diagnostic, and developing a field-deployable paper microfluidic detection device.

The following objectives have been set to achieve this goal:

1. To develop and optimize a routine laboratory analytical approach for the detection of SARS-CoV-2 in wastewater and to apply the approach to the study of WBE on the Cranfield University campus.

2. To develop a CRISPR/Cas12a-based fluorescent assay for the sensitive and specific detection of SARS-CoV-2 and its variants, including Delta and Omicron.
3. To design and fabricate a paper-based microfluidic device integrated with CRISPR/Cas12a-based assay for on-site SARS-CoV-2 detection in wastewater.

These objectives aim to address critical technology gaps in wastewater surveillance by enhancing methodological efficiency, ensuring robust variant detection, and enabling diagnostics at decentralised settings.

### **1.3 Thesis Plan**

This thesis is structured into six chapters, each addressing a specific objective, as shown in Figure 1.1. The structure and content of the chapters are summarized below:

#### **Chapter 1: Introduction**

This chapter provides an overview of the research context, outlines the aims and objectives of the study, and presents the structure of the thesis. It establishes the significance of the research in the broader context of molecular assays, public health and wastewater surveillance.

#### **Chapter 2: Background and Literature Review**

This chapter discusses the state-of-the-art in wastewater-based epidemiology, molecular diagnostics, and microfluidic technologies. It critically evaluates current methods for SARS-CoV-2 detection and identifies gaps in existing research. The review highlights the potential of integrating advanced molecular diagnostics with portable platforms to enhance WBE.

#### **Chapter 3: Optimization of concentration methods for SARS-CoV-2 detection in wastewater and insights from wastewater surveillance at Cranfield University**

This chapter focuses on the comparative analysis and optimization of concentration methods critical in wastewater analysis, including PEG precipitation and ultrafiltration. It establishes a detailed methodology for detecting

SARS-CoV-2 from wastewater in the laboratory. Using the established methodology, WBE was applied to the Cranfield University campus to obtain longitudinal monitoring data, demonstrating the efficacy of the optimized methodology in providing actionable insight into viral dynamics.

#### **Chapter 4: Development of a CRISPR/Cas12a-based fluorescent detection method for SARS-CoV-2 and its Delta and Omicron variants**

This chapter describes the development of a sensitive and specific CRISPR/Cas12a-based SARS-CoV-2 assay. Based on the establishment of the use of CRISPR/Cas technology for the detection of SARS-CoV-2, it focuses on the use of highly specific CRISPR technology to achieve the detection of single-point mutations, thus enabling the differentiation of mutants (including delta and omicron) for detection.

#### **Chapter 5: Development of a paper microfluidic device for on-site SARS-CoV-2 on-site detection from wastewater**

This chapter describes a portable microfluidic device's design, fabrication and optimization. The device integrates concentration and molecular detection methods for on-site SARS-CoV-2 monitoring. Key innovations include multiplexed detection of SARS-CoV-2 variants with colourimetric assay of CRISPR/Cas12a coupled LAMP. The field suitability of the device was emphasized by a performance evaluation using spiked real wastewater samples.

#### **Chapter 6: Overall Discussion and Conclusions**

The final chapter summarizes the thesis findings, discusses the limitations in research, and outlines future research directions. It highlights the contributions of the study to advancing molecular diagnostics and WBE, addressing current limitations, and paving the way for further development.

This thesis addresses critical challenges in wastewater monitoring by developing innovative methods for SARS-CoV-2 detection. By optimizing viral concentration techniques, conducting wastewater surveillance, advancing molecular

diagnostics, and developing portable diagnostic devices, this research contributes to the field of WBE and public health monitoring.

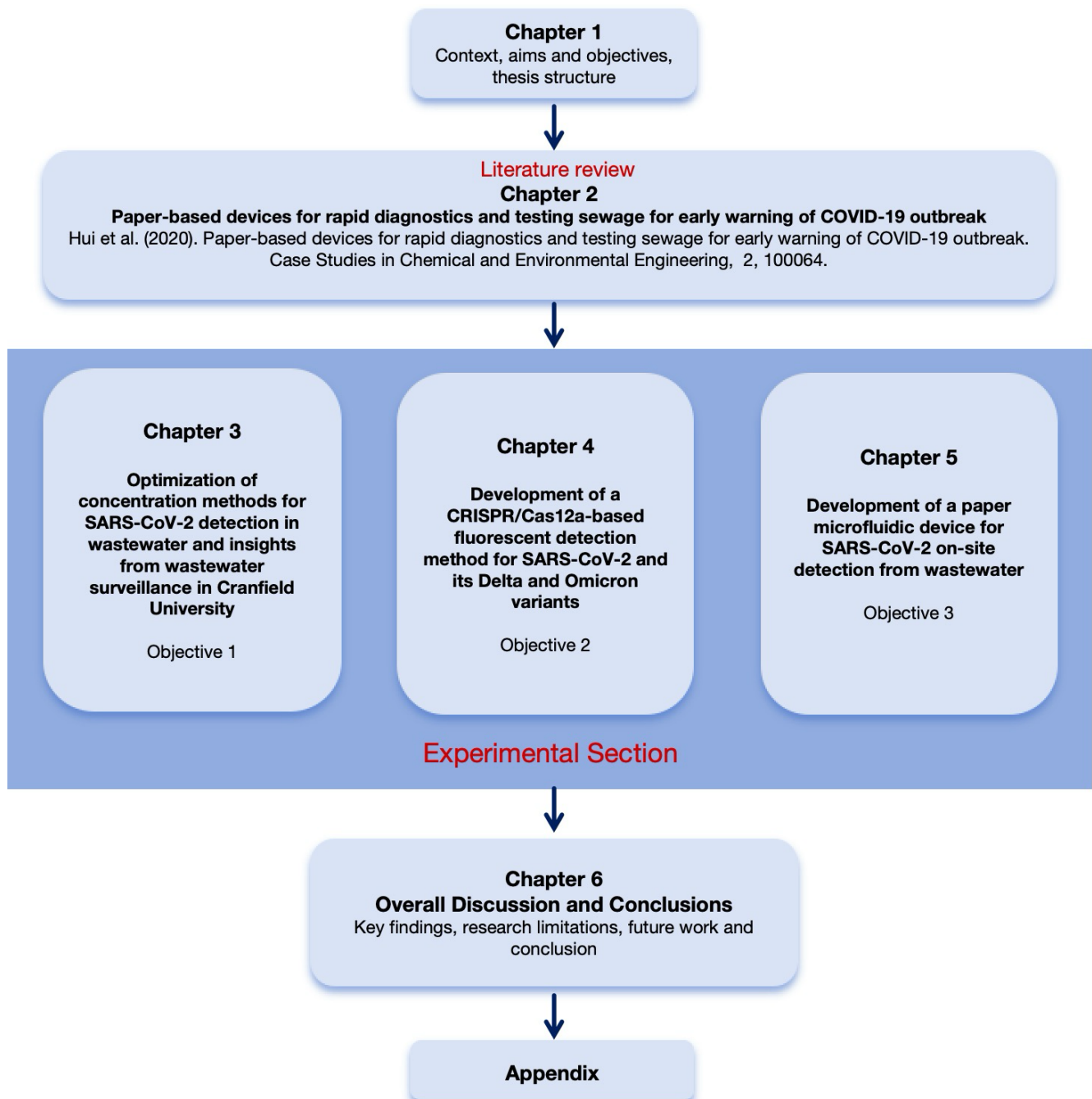


**Figure1.1 Thesis plan**

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## **Chapter 2: Paper-based devices for rapid diagnostics and testing sewage for early warning of COVID-19 outbreak**

### **Abstract**

Coronavirus disease (COVID-19), caused by SARS-CoV-2, evolved into a global pandemic in 2020, and the outbreak has taken an enormous toll on individuals, families, communities and societies around the world. The effective strategy is to implement rapid case identification based on a rapid testing to respond to this public health crisis. Currently, the available technologies used for rapid diagnostics include reverse transcription polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), enzyme linked immunosorbent assay (ELISA) and next generation sequencing (NGS). Still, due to their different limitations, they are not well suited for rapid diagnosis in a variety of locations. Paper-based devices are alternative approaches to achieve rapid diagnosis, which are cost-effective, highly selective, sensitive, portable, and easy-to-use. In addition to individual virus screening, wastewater-based epidemiology has emerged as an effective early warning tool for outbreaks within populations. By detecting viral genome sequences in wastewater, it provides insights into the spread and distribution of the virus, as SARS-CoV-2 can be shed through the feces and urine of infected individuals. In this chapter, we describe paper-based device as a low-cost and rapid sensor for both diagnosis and testing of sewage for early warning of outbreak. More importantly, the device has great potential for real-time detection in the field, without any advanced facilities or well-trained and skilled personnel, and provides early warning or timely intervention of an outbreak of pandemic.

**Keywords:** Paper-based devices; Rapid diagnosis; Wastewater-based epidemiology; COVID-19; Early warning system

This Chapter is directly adapted from a published review paper with format modifications for consistency with the thesis format. Hui *et al.* (2020). Paper-based devices for rapid diagnostics and testing sewage for early warning of COVID-19 outbreak. *Case Studies in Chemical and Environmental Engineering*, 2, 100064.

## 2.1 Introduction

The entire world has been faced with a vast pandemic, coronavirus disease 2019 (COVID-19) since 2019, and this global pandemic is still an unsolved problem and has caused a significant crisis for global public health. COVID-19 is a new respiratory infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the family of coronaviruses. There are some other infectious diseases caused by coronaviruses in history, like severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) (Bhalla *et al.*, 2020). One defining characteristic of COVID-19 is that it spreads quickly across the world, and the outbreak with near exponential growth has put an overwhelmed strain on health systems around the world. Although countries around the world have different public health responses to this global disease because of the different stages of the outbreak, a typical effective strategy is to identify cases through rapid testing and isolate infected patients. Therefore, it is of vital importance to adopt COVID-19 testing that could accurately and rapidly detect infected individuals to reduce transmission and control outbreaks.

In a short period of time, COVID-19 has evolved into a global pandemic that has had a profoundly negative impact on individuals, families and societies around the world (WHO, 2020). There have been profound changes in people's daily lives, such as telecommuting, maintaining social distance, and the inability to freely go out, and many family reunions events have been cancelled due to the ban on people gathering, like funerals and weddings. More importantly, the COVID-19 epidemic has plunged economies around the world into recession because of the adoption of lockdown measures to control virus transmission. Cultural activities in society, such as global conferences and events on fashion and sports, have been disrupted by infectious diseases. In order to alleviate the unprecedented pressures on individuals, families and societies at this difficult time, there is an urgent need for developing personal rapid diagnosis and large-scale screening tools for infections, as well as seeking effective early warning system for effective intervention and management.

## 2.2 Current state-of-art for COVID-19 diagnostics

Generally, there are mainly two categories of analytical techniques for COVID-19 detection, and the first type is the molecular approaches for the detection of viral nucleic acids. Reverse transcription polymerase chain reaction (RT-PCR) is the most widely used molecular detection technique and is regarded as the gold standard for diagnosis of SARS-CoV-2 virus (Carter *et al.*, 2020). The first step of RT-PCR assay is to collect biological fluids that may contain virus strains from the upper and lower respiratory tract, which is followed by several filtration and separation steps to isolated RNA (Udugama *et al.*, 2020). Then, the transcriptase enzyme is used to converse viral RNA to complementary DNA (cDNA). The next step is the PCR reaction where DNA undergoes amplification with DNA polymerase. During the amplification, the TaqMan probe with a fluorescent molecule and a quencher molecule can detect specific viral sequences and generate fluorescence signal. After dozens of amplification cycles, the system can detect the intensity of the fluorescent signal, which is proportional to the virus concentration in the sample.

As a predominant diagnostic method for managing COVID-19 pandemic, RT-PCR is sensitive, specific and reliable, but it still has some unavoidable limitations. To perform the RT-PCR test, well-trained professionals, various specialists and expensive instruments are necessary, which is a concern in the developing regions and countries in short supply (Afzal, 2020). Also, the analysis time of RT-PCR lasts for four to six hours, and the turn-round time from sample to result usually exceeds 24 hours, which may be longer when the number of infectious cases dramatically increases (Kashir and Yaqinuddin, 2020). More importantly, some research indicated that the diagnosis of SARS-CoV-2 by RT-PCR might be false negative at some times (Liu *et al.*, 2020; Chen *et al.*, 2020). There are many possible reasons for false negative results, and the primary one is that RNA is easy to degrade, so special handling at a low temperature is needed in collection, storage, transfer, purification and processing (Afzal, 2020).

Another molecular method that uses isothermal amplification to detect viral nucleic acids is reverse transcription loop-mediated isothermal amplification (RT-

LAMP). RT-LAMP depends on its ability to amplify the complementary DNA of coronavirus at a constant temperature, and the amplification needs four primers with high specificity to detect target sequences (Kashir and Yaqinuddin, 2020). The LAMP reaction is accompanied by magnesium pyrophosphate precipitation when viral sequences are amplified, and this byproduct can increase the turbidity of the sample (Carter *et al.*, 2020). Therefore, it is possible to measure the turbidity change or employing intercalating dyes that can cause visible colour change based on the increase in turbidity to monitor the amplification in real time. Since the amplification condition is at a constant temperature, this approach does not require high-cost reagents or equipment, which may reduce some financial pressures and perform the testing outside the laboratories (Zhang *et al.*, 2020). Besides, due to the high specificity and high sensitivity, Lamb *et al.* suggest the use of RT-LAMP test significantly shortens the detection time to about 30 minutes, which is beneficial for rapid detection (Lamb *et al.*, 2020).

Apart from molecular methods for detecting viral nucleic acids, an alternative diagnostic strategy involves immunological assays that detect antibodies specific to SARS-CoV-2 antigens, and these assays are used to monitor disease progression and identify past infections (Carter *et al.*, 2020). Enzyme-linked immunosorbent assay (ELISA) is currently the most common antibody detection method in use. It uses a multi-well plate coated with viral proteins, which can specifically bind antibodies from blood samples (Kilic *et al.*, 2020). As the other antibodies labelled with enzymes are introduced subsequently, a colourimetric or fluorescent-based signal is produced.

The next-generation sequencing (NGS), also known as high-throughput sequencing (HTS), is another method for implementing COVID-19 diagnosis. This technology can determine the genome sequence of more than 1 million base pairs in a single sequencing, thus it can be used to detect the gene sequence of a virus in a short time. Previously, whole-genome sequencing method was used to identify the transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) in community and hospital, and this technology showed significantly high precision and traceability in patients compared to other standard infection-control

techniques (Harris *et al.*, 2013). In 2020, the FDA approved one product named Illumina COVIDSeq Test. This amplicon-based NGS test employs 98 DNA fragments to cover the roughly 30 kilobases in the SARS-CoV-2 genome, and the detection is usually completed within 12 hours (First NGS-based COVID-19 diagnostic, 2020). Although the sequencing technique has high accuracy in virus diagnosis, it requires a relatively long detection time, and the instruments and reagents used require high costs.

### **2.3 Wastewater-based epidemiology for early warning of COVID-19 outbreak**

Although these mentioned assay techniques are evolving with improved approaches and can be well applied in COVID-19 diagnosis, it is still an enormous challenge for the globe to screen every suspicious infection in such a critical time. To address this issue, it is believed that wastewater-based epidemiology (WBE) is an alternative method for predicting virus spread and early warning pandemic by detecting pathogens in wastewater (Mao *et al.*, 2020). Gormely *et al.* reported that the sanitary plumbing system was one potential spread route for SARS-CoV-1 if infected patients were found in the community (Gormely *et al.*, 2017), and similarly SARS-CoV-2 virus can be transmitted by aerosol or water droplets. Additionally, SARS-CoV-2 biomarkers can be detected in the sewer system, because the virus can be isolated from the infected patients' feces and urine (Holshue *et al.*, 2020). According to the results of various studies, which investigated fecal samples from infected patients, the positive rate of SARS-CoV-2 in patients' feces varied from 15-83% (Foladori *et al.*, 2020). Especially, one study highlighted that the virus in feces persisted positively even after the nasopharyngeal testing had become negative (Xu *et al.*, 2020). Consequently, the analysis of wastewater in communities is a potential method to track infected people, and the epidemiology of the communities can be monitored via sewage pipe networks.

The concept of wastewater-based epidemiology is to give comprehensive health information on communities, and the schematic diagram of WBE is presented in Figure 2.1 (Sims and Kasprzyk-Hordern, 2020). Initially, WBE was used to estimate drug abuse in the community. By analyzing certain target drug residues in sewage, it is possible to estimate and monitor the trends of illegal drug consumption (Zuccato *et al.*, 2008). Other biological indicators in wastewater, such as pharmaceuticals, and personal care products, markers of population size, and industrial chemicals, can also be quantitatively and qualitatively determined to obtain epidemiologic data for addressing public health issues (Choi *et al.*, 2018). Similarly, viruses are another important biomarker for wastewater -based epidemiology. Since viruses cannot grow outside the host cells, the level of viruses in wastewater is directly proportional to the concentration of viruses in the excrement of the corresponding population (Xagorarakis and Brien, 2019). Through the analysis of sewage, the changes in virus concentration and diversity can be obtained, which are useful information for assessing the temporal and spatial patterns of virus infection in the community. Significantly, adopting this method in the early stage of the viral outbreak can determine where the virus starts to develop and spread (Bhalla *et al.*, 2020). Correspondingly, early warning and intervention can be taken based on these findings. Considering all these merits of WBE, Bivins *et al.* call for global collaboration to improve detection approaches for COVID-19 in wastewater (Bivins *et al.*, 2020).

## Wastewater-Based Epidemiology

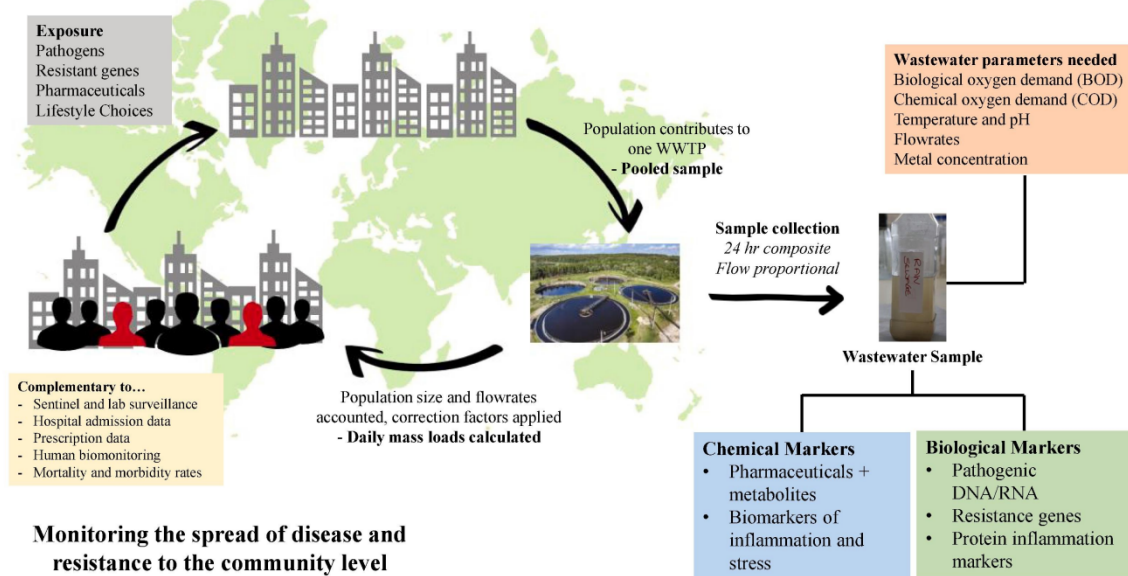


**Figure 2.1 The schematic diagram of WBE (Sims and Kasprzyk-Hordern, 2020)**

During the COVID-19 pandemic, there are several techniques for sewage analysis, and PCR-based methods have been the prime techniques in the application. Many countries reported the presence of SARS-CoV-2 RNA in sewage by using RT-PCR assays because as mentioned before, this diagnostic method provides high specificity and sensitivity. The Netherlands tested three nucleocapsid genes (N1–N3) and one the envelope gene (E) of SARS-CoV-2 in the sewage samples from six cities and airports (Medema *et al.*, 2020). The results showed that the target RNA was not detected before the first confirmed infection case in February. Nevertheless, due to the prevalence of COVID-19 in March, the target nucleic acid signal increased significantly. A study conducted in Massachusetts using RT-PCR also showed that the observed viral titers were higher than the expected based on the confirmed clinic cases in March (Wu *et al.*, 2020). One explanation for this result may be that the clinic testing is limited in diagnosing asymptomatic patients. Therefore, using RT-PCR for viral detection in sewage can be regarded as an early warning tool for the presence and prevalence of COVID-19 infection and a supplementary method for clinical testing.

Although PCR-based methods are mainstream ones for sewage analysis, some disadvantages of PCR assay earlier mentioned limit its global application. When it comes to WBE, the ideal wastewater analysis method is to perform rapid virus detection at the wastewater collection outlets so that the transmission of the virus can be monitored in real time and early warnings can be implemented in time. Since PCR-based methods are time-consuming and need sophisticated equipment and centralized laboratories, it is necessary to develop other methods that have high specificity and sensitivity, but also have the advantages of low cost, rapidness, accuracy and simplicity (Bhalla *et al.*, 2020). One promising method that can be applied for WBE is a paper-based microfluidic device. This portable diagnostic device offers the potential for a medical diagnosis that can be used in resource-limited areas and urgent situations.

## **2.4 Paper-based device**

Paper has been used as a diagnostic tool for a long time, and the most common examples are test papers, such as pH test strips and urine test strips. The urine test strips are designed to detect some metabolic products in urine, which are common pathological indicators (e.g., protein, glucose, and salt) (Sher *et al.*, 2017). Due to the different concentrations of metabolic products, various colour changes happened on the paper, and this can be read by comparing to the standard test strips.

With the improvement of rapid analysis methods, lateral flow assay (LFA) was introduced into paper-based diagnostic procedures and updated paper-based devices. One typical LFA device consists of four parts, involving sample pad, conjugate pad, nitrocellulose membrane and absorbent pad (Ngom *et al.*, 2010). There are two types of LFA assay frequently used and they are competitive assay and sandwich assay. The former one is used to test low molecular weight or single epitope analytes, while the sandwich format is more suitable for analytes presenting several epitopes. When using competitive assay, if there is no analyte in the sample, unbound antibodies will bind to immobilized

molecules on a sheet of the plastic backing, causing visual signal. On the contrary, a visual change can be observed on sandwich assay paper when the analyte is present in the sample. LFA, as a paper-based assay, gain popularity in the detection of drugs, toxins, pesticides, bacteria and viruses due to its simplicity and rapidity.

Currently, the latest paper-based devices refer to microfluidic devices that take advantage of paper as a substrate for various bioassays, and paper-based microfluidic devices are powerful tools for pathogen analysis and determination of infection transmission. The reason for choosing paper as the substrate is that it is affordable, light, easily accessible and disposable (Mao *et al.*, 2020). To fabricate paper-based microfluidic devices for different detection aims, paper made up with various material are picked up, such as cotton cellulose and nitrocellulose (Sher *et al.*, 2017). Also, there are several production approaches for paper-based microfluidic devices, including photolithography, polydimethylsiloxane (PDMS) plotting, inkjet printing, wax printing, wax screen printing, wax dipping, and plasma treatment.

When the biomolecules to be tested are nucleic acids, the frequently used approach is wax printing. The wax printer can print this analytical paper with wax channels that can restrict and guide samples and reagents, and the entire detection process of genetic materials, from extraction, enrichment, purification, elution, amplification, to visual detection are combined on this paper (Mao *et al.*, 2020). It is not required to have external power or pump supply for conducting paper-based testing, instead only a few simple folds of the device are necessary to complete the detection. Due to the simple operation and ease of fabrication, paper-based microfluidic devices can potentially be an alternative to PCR for rapid and accurate diagnosis of infectious disease with a fast turnaround time when there is a lack of skilled professions and high-cost equipment.

Efforts have been made to improve paper-based microfluidics and studies showed that this technique meets prime demands of field-based diagnostics and can be used for infectious disease prevention, control and precise diagnosis. Kaarj *et al.* developed a platform utilizing RT-LAMP, a more straightforward

amplification method requiring a constant temperature, to assay Zika virus (ZIKV), and the whole process was completed on a wax printed paper microfluidic chip as shown in Figure 2.2 (Kaarj *et al.*, 2018).

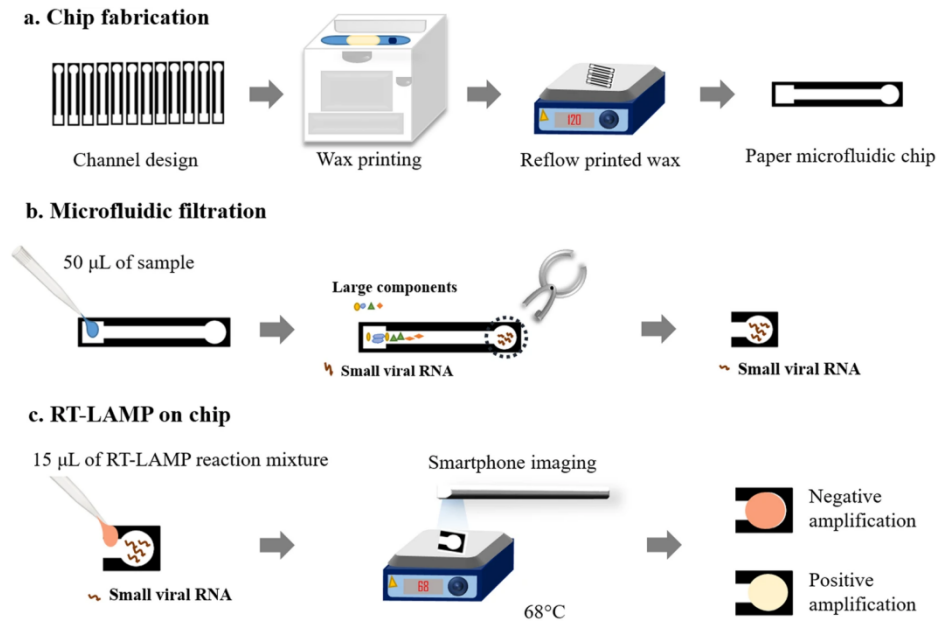


**Figure 2.2 The schematic diagram of paper microfluidic RT-LAMP assay for ZIKV**

When the sample (urine or blood plasma) was added to the device, the paper fibres had a capability to filter large size molecules as a pre-treatment. In addition, the viral RNA with strong negative charges spontaneously moved to the end of the channel, while proteins and other cell fragments remained because of the strong negative polarity of cellulose fibres in paper. The target nucleic acids were amplified on a simple hot plate, and the amplification results can be obtained by observing the intensity of the added pH indicator dye. The LAMP-based paper devices have also been used to diagnose rotavirus A, the most common pathogen that contributes to gastroenteritis in children (Ye *et al.*, 2018). The nucleic acid extraction and thermostatic RNA amplification were completed on a simple paper disc, and the entire process only took about half an hour. The positive amplification results of rotavirus A can be read immediately with the naked eye, that is, the rose-red on the glass fibre paper.

The previous studies were to complete the detection of pathogens using paper-based microfluidic devices in the laboratory. In addition, one study

practically used this point-of-care diagnostic tool in rural Uganda to quickly diagnose malaria for residents (Reboud *et al.*, 2019). The testing could give results in approximately 50 minutes by analyzing multiplexed RNA sequences of pathogens, which greatly shortened the diagnosis time compared with the high-cost PCR analysis. Besides, the sensitivity of this diagnostic origami device was higher than expected, 98% of people with malaria can be detected, while the detection rates of other field-based, benchmark techniques, including optical microscopy and industry-standard rapid immunodiagnostic tests, were 86% and 83% respectively. Like PCR assay, these field-based techniques required experienced personnel to perform.

Paper-based microfluidic devices were demonstrated to be rapid, affordable, portable and easy to handle in field-based diagnosis, and were feasible for use in resource-poor areas. Another advantage of the paper analytical tool is that it is thin and lightweight, thus this device tends to be easy to stack, transport and store (Mao *et al.*, 2020). Additionally, after using paper-based microfluidic device to complete the diagnosis, it can be disposed of by incineration, which possibly reduces the risk of causing contamination. As a result, this novel analytical device with various merits has excellent potential to be applied to respond to the COVID-19 pandemic, thereby enabling rapid and on-site virus detection for immediate diagnosis and wastewater-based epidemiology investigation.

## **2.5 Paper-based device for diagnostics and testing wastewater for early warning of COVID-19**

### **2.5.1 Rapid diagnosis for COVID-19**

One characteristic of COVID-19 is its ability of spreading quickly around the world, and a critical solution to this public health issue is to offer rapid and accurate screening for infected patients whether they have symptoms or not. Based on a large-scale screening of infection cases carried out early in the epidemic, confirmed cases can be treated in time, reducing severe cases, easing the burden on the medical system and managing the epidemic. Since paper-based microfluidic devices have been developed to diagnose infectious diseases before, it holds a great potential to meet the demand for rapid diagnosis for COVID-19 as well.

The first type of rapid diagnosis is a nucleic acid-based assay, and it is possible to design an analysis tool that can identify nucleic acids in a short time by applying various isothermal amplification techniques to paper-based devices. Some paper-based devices have been focused on LAMP detection of nucleic acids in infectious diseases, which could be adopted to detect COVID-19 nucleic acids.

For instance, Xu *et al.* presented a paper-origami device for multiplex detection of *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium pan* (Xu *et al.*, 2016). DNA extraction, LAMP, and fluorescence detection was integrated for target detection from whole blood. Origami-paper based LAMP assay can identify between *Plasmodium falciparum* and *Plasmodium vivax* infections, which can guide appropriate treatments for corresponding infections. A paper microfluidic device has been developed for multiplex detection of three bovine infectious reproductive diseases in semen samples and tested in a rural India farm (Yang *et al.*, 2018). Pathogen DNA was extracted from bovine herpes virus-1 (BoHV-1), *Brucella* and *Leptospira*, then amplified by LAMP and detected fluorescently (Figure 2.3). The detection limits for three pathogens were as low

as 50 *Leptospira* organisms, 50 CFU *Brucella*, and 1 TCID<sub>50</sub> BoHV-1, which make paper microfluidic devices suitable for on-site diagnosis of infectious diseases.

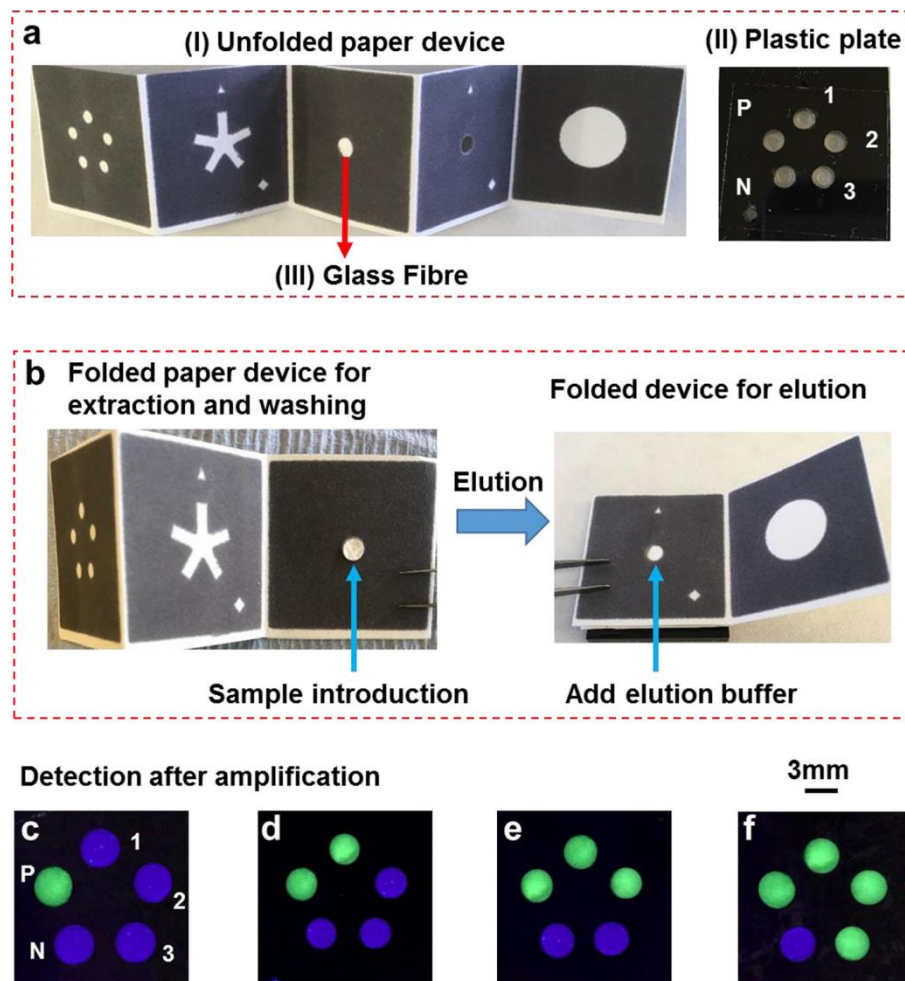


**Figure 2.3 The schematic diagram of the paper device for the detection of BoHV-1, *Brucella* and *Leptospira* (Yang *et al.*, 2018)**

Choi *et al.* developed an integrated paper-based biosensor for nucleic acid testing (2016). The Fast Technology Analysis (FTA) card and glass fibre were integrated into a lateral flow strip for sample addition, washing, paper-based LAMP, visual detection or smartphone quantification. A handheld battery-powered heating device was used for on-site LAMP tests. Detection limits of 10–1000 CFU mL<sup>-1</sup> were measured for *Escherichia coli* in spiked drinking water, milk, blood and spinach. This integrated paper-based biosensor is suitable for

transformation into a device for rapid on-site RT-LAMP detection of COVID-19 nucleic acids.

Recently, rapid progress has been made in RT-LAMP enabled isothermal amplification methods for COVID-19 diagnosis (Thompson and Lei, 2020). The Abbott recently launched the ID NOW COVID-19 assay for clinical use that can detect the RNA-dependent RNA polymerase (RdRp) gene from nasopharyngeal and throat swabs through isothermal amplification and get a LOD of 125 genome equivalents mL<sup>-1</sup> in less than 15 minutes (Basu *et al.*, 2020). Another team adopted the RT-LAMP and the clustered regularly interspaced short palindromic repeats (CRISPR) technique to detect SARS-CoV-2 in respiratory swabs within 40 minutes, and the diagnosis result can be known using the LFA (Broughton *et al.*, 2020). With the presence of the virus, the target capture line on the LFA shows positive results. This work has expanded the possibility of CRISPR-based DETECTR technology for on-site COVID-19 diagnosis with the investigations on portable microfluidic cartridges and lyophilized reagents. Therefore, it is promising to use paper-based devices such as LFA to detect the LAMP reaction products, and the whole process can be completed on a paper device for a point-of-care coronavirus disease diagnosis. Notably, the emergence of the paper-based devices for rapid diagnosis could be a massive improvement for places where resources are limited, and the public health system is overwhelmed.

In addition to detecting nucleic acids to screen infected patients, paper-based devices can also be used to detect antibodies to help infectious disease diagnosis. Singh *et al.* reported a colourimetric paper-based sensor to detect pan malaria and *Plasmodium falciparum* based on a dye-based chromogenic reaction (Singh *et al.*, 2019). The instrument-free technique could be implemented for quantitative and qualitative determination of malaria by visual readout or a camera integrated software. In another work, a deployable bioplasmonic paper-based device was fabricated to detect anti-ZIKV-NS1 Immunoglobulin G (IgG) and IgM using LSPR (Jiang *et al.*, 2017). The device was validated for target detection in serum samples, indicating great potential for effective determination of anti-ZIKV-NS1 IgG and IgM in the complex physiological environment with

multiple interfering substances. When it comes to COVID-19, a variety of lateral flow test strips have been employed for COVID-19 diagnosis, such as Cellex qSARS-CoV-2 IgG/IgM Rapid Test, Wondfo SARS-CoV-2 antibody test, Mammoth Biosciences SARS-CoV-2 DETECTR and SGTi-flex COVID-19 IgM/IgG (Sheridan, 2020). For example, a lateral flow immunoassay was presented for combined detection of SARS-CoV-2 IgG and IgM in human blood in less than 15 minutes during various infection stages, shown in Figure 2.4 (Li *et al.*, 2020). The detection device comprises of a sample pad, a conjugate pad, a nitrocellulose membrane, an absorbent pad and an adhesive card. The clinical efficacy uses was demonstrated by performing clinical tests on different venous and fingerstick blood samples with a sensitivity of 88.66% and a specificity of 90.63%.

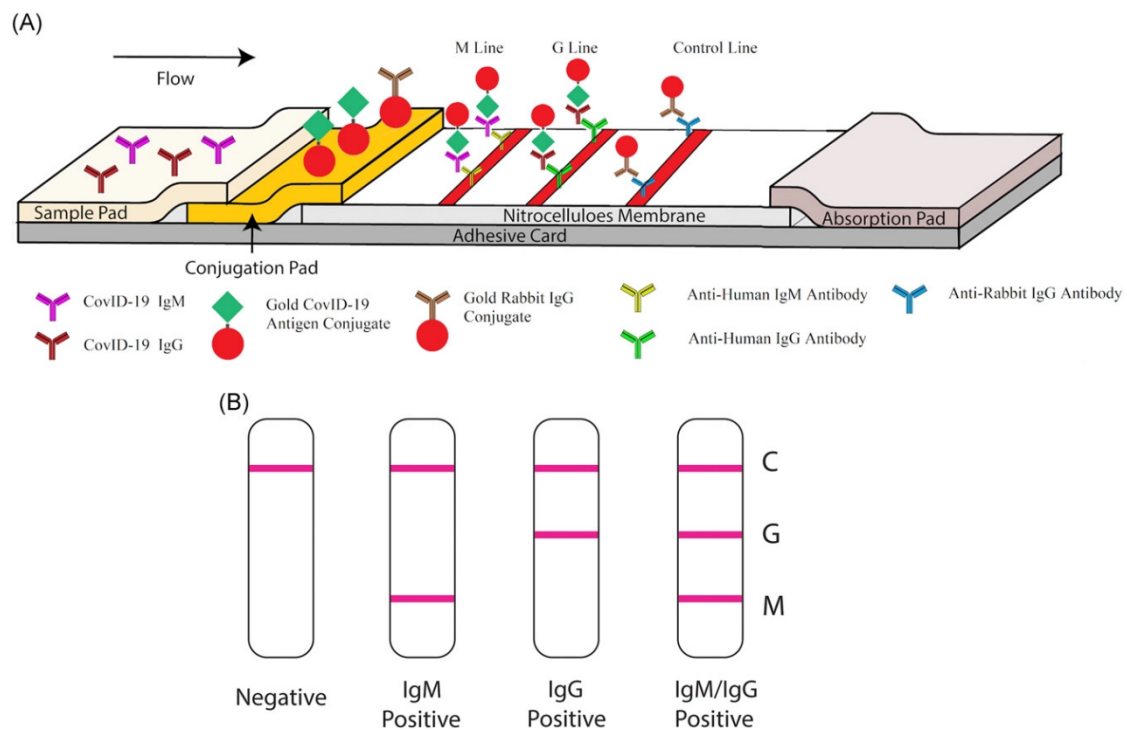


**Figure 2.4 The schematic illustration of SARS-CoV-2 IgG and IgM test (Li *et al.*, 2020) (A) The detection device. (B) Different test results.**

Kasetsirikul *et al.* designed a colourimetric paper-based assay to detect SARS-CoV-2 humanized antibody, which is a cheap and accessible serological assay (Kasetsirikul *et al.*, 2020). This development combined a common

serological assay method, ELISA, with the paper-based devices, by coating the recombinant SARS-CoV-2 nucleocapsid antigen on the paper analysis tool. The target antibodies from human serum could be captured by the recombinant antigen on the device, forming an immunological complex. By adding anti-human IgG-HRP to the device, the complex formation could be read out through a colourimetric reaction, 3,3',5,5'-tetramethylbenzidine substrate with horseradish peroxidase (TMB/HRP), and the entire process took 30 minutes, which was much shorter than a conventional ELISA assay (usually one to two hours).

### **2.5.2 Analysis of SARS-CoV-2 for early warning of outbreak**

The sewage analysis is a cost-effective and highly efficient method to determine the infectious cases, monitor the virus transmission and manage the pandemic. As mentioned before, PCR is still the main method used to detect SARS-CoV-2 in wastewater. However, this kind of detection method that requires sophisticated instruments, complicated operation and long-time analysis is not suitable for real-time detection on the wastewater site. As an alternative approach, the paper-based device is suggested for on-site inspection of wastewater. Despite the fact that various kinds of substances are contained in wastewater, it is believed that the paper-based device is capable of detecting pathogens from sewage.

Yang *et al.* reported a “sample-to-answer” tool to analyze the wastewater in communities, tracking biomolecular agents and genetic information (Yang *et al.*, 2017). The platform still took advantage of LAMP as a method of nucleic acid amplification. After removing solid impurities in the original wastewater and extracting and enriching DNA, it performed quantitative monitoring of the target genetic material. After the amplification, the amplicons were conjugated with fluorescein isothiocyanate (FITC) and biotins, which could further be captured by the streptavidin conjugated with coloured particles on the LFA, leading to the visual result. The time to finish this assay at the sample collection point was 45 minutes, and no expensive equipment or experienced operators were required.

Similar paper devices may also become sewage analysis tools for the COVID-19 outbreak. In view of the high selectivity, high sensitivity, rapid response time and cost-effectiveness of this technique, it is believed that paper-based devices have the capability to be applied to the on-site detection of wastewater, providing real-time and continuous information. Based on this information, the local epidemiology of SARS-CoV-2 can be analyzed to better manage the infection situation in the community, such as early warning of epidemics or isolation of infected people.

## **2.6 Conclusions and Future Outlook**

In summary, during the COVID-19 outbreak, isolating the infected people through effective diagnostic methods is a universal method to slow the spread of the disease, and the main method used today is RT-PCR. Compared with RT-PCR, paper-based device is an analytical tool that can provide rapid and accurate biomolecular detection. More importantly, this detection method does not require laboratory-grade instruments and professional operators to complete it. The development of this cheap and rapid diagnosis method has brought hope to control the epidemic in some resource-limited regions and developing countries.

Besides, wastewater-based epidemiology is a new and promising monitoring mechanism for managing epidemics at the community and population level because the analysis of sewage can obtain the infection situation inside the area and facilitate for early warning of infectious, so as to carry out effective prevention and intervention. In order to continuously detect viral RNA in sewage in real time, an on-site detection method is needed. The paper-based device, such a portable analytical tool, can meet the requirements for wastewater-based epidemiology, and detect biomolecules in sewage containing complex matrices. Based on the analysis results, it is possible to give an early warning of infectious disease, identify SARS-CoV-2 carriers, determine the infection status in the community, and finally control the epidemic through timely measure and intervention.

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# **Chapter 3: Optimization of concentration methods for SARS-CoV-2 detection in wastewater and insights from wastewater surveillance in Cranfield University**

## **Abstract**

Wastewater-based epidemiology (WBE) has shown to be effective to track the prevalence of pathogens in communities and identify outbreaks for early warning; however, there is currently no global standard concentration method for wastewater analysis, from concentration to molecular testing. This chapter focuses on the optimization of viral concentration methods for the detection of SARS-CoV-2 in wastewater samples collected at a local wastewater treatment plant of Cranfield University, to ensure the viral RNA can be detected with the molecular method. Two primary concentration methods, polyethylene glycol (PEG) precipitation and ultrafiltration, were compared to determine the most effective technique for wastewater concentration. From the comparison, PEG precipitation consistently demonstrated higher recovery efficiencies. Further optimization of the PEG method included reducing the precipitation time, determining the need for large particle removal and investigating viral extraction techniques. The longitudinal monitoring of local wastewater provides important information for understanding the dynamics of virus transmission, these optimizations increase the utility of wastewater analysis. In addition, the WBE methods can also offer control of outbreak earlier than standard epidemiological paradigms that depend on reports for clinical data. This study demonstrates the utility of real-time WBE as an early warning system and shows how the detection of SARS-CoV-2 in wastewater can be used to provide timely viral surveillance on a university campus.

**Keywords:** Wastewater-based epidemiology; PEG precipitation; Ultrafiltration; Wastewater surveillance

### 3.1 Introduction

Wastewater-based epidemiology (WBE) has become a powerful public health tool, particularly for tracking infectious diseases such as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) during the Coronavirus disease 2019 (COVID-19) pandemic. WBE was first put forward for non-invasively monitoring communal drug use through the quantification and detection of drugs in sewage (Zuccato *et al.*, 2005). As this approach continues to evolve, it could also become an effective method for investigating antimicrobial resistance (AMR) by monitoring antimicrobial use or assessing the level of exposure to carcinogens, pollutants and toxins in specific populations (Coutu *et al.* 2013; Golovko *et al.* 2014; Rousis *et al.* 2017). WBE has recently been introduced into infectious diseases, and a study of eight pathogenic viruses (norovirus, astrovirus, rotavirus, adenovirus, Aichi virus, par echovirus, hepatitis A virus [HAV] and hepatitis E virus) suggested that routine testing of wastewater for these viruses may be an early indicator of a potential pandemic (Hellmér *et al.*, 2014).

The COVID-19 outbreak has highlighted the importance of widespread monitoring of infection prevalence, and WBE has been proposed again as an alternative surveillance method for clinical diagnosis to measure the emergence and spread of SARS-CoV-2 in communities. It began with the discovery that SARS-CoV-2 was present in urine and faeces samples from infected individuals (Holshue *et al.*, 2020). Subsequent studies have found that fecal samples may be positive even if the patient has no respiratory symptoms (Xiao *et al.*, 2020). Given that fecal samples can contain significant concentrations of viral particles (ranging from 0.8 to 7.5 log<sub>10</sub> gene copies per gram) (Wölfel *et al.*, 2020), it was expected that examination of the virus in pandemic wastewater would provide additional epidemiological data for prevalence surveillance.

Unlike traditional surveillance techniques that rely on consent for clinical testing, WBE has the ability to collect data from entire populations, including those without symptoms or who refuse to be tested (Wu *et al.*, 2020). During the COVID-19 pandemic, WBE provided a way to detect viral spreading patterns in real time. Thus, it became a crucial part when it came to making response plans in many countries, particularly in places where clinical testing was unavailable (Bueno *et al.*, 2022). Targeted interventions were announced based on the information gained from wastewater analysis, like vaccination programs or localized closure.

While WBE is useful, there are many technical challenges to overcome, especially the low concentration of the virus extracted from the complex wastewater matrices. In wastewater, there are diverse components which may hamper the identification and measurement of viral RNA, like organic debris, bacteria, sludges, and chemical contaminants (Corpuz *et al.*, 2020). One critical task at the beginning of the wastewater analysis is to enrich virus particles from enormous volumes of wastewater into smaller, more manageable samples. The sensitivity and accuracy of assays used for downstream detection are significantly influenced by the concentration technique selected (Lu *et al.*, 2020). There are several techniques available for SARS-CoV-2 enrichment, like polyethylene glycol (PEG) precipitation, aluminum hydroxide precipitation, ultrafiltration, electronegative filtration, and ultracentrifugation (Mohammad Dehghan Banadaki *et al.*, 2024). Various studies have not been unanimous as to which method is the most efficient for recovering viral RNA from wastewater, due to the variability in the physical and chemical composition of wastewater (Rusiñol *et al.*, 2020). The two most widely used enrichment techniques for SARS-CoV-2 detection from wastewater are PEG precipitation and ultrafiltration. Both approaches have unique advantages and disadvantages depending on the type of sample being tested, the viral load in the sample, and the overall goal of the surveillance effort (Mailepessov *et al.*, 2022).

At the same time, the COVID-19 pandemic posed a challenge for virus surveillance because of the rapid spread of the virus and the large number of asymptomatic carriers. Successful cases of using WBE to track the spread of SARS-CoV-2 at the community level, such as on university campuses, in long-term care facilities and in urban neighborhoods, are worthy of consideration and learning (Betancourt *et al.*, 2021; Akingbola *et al.*, 2022). One of the major features of this Cranfield university's wastewater treatment plant is that is independent, making it an ideal setting for local wastewater monitoring. The campus has residential halls to host 1,200 residents, with other staff and students commuting to and from work during the weekdays. As the wastewater treatment plant is isolated, there is no risk of complexities with industrial wastewater, groundwater infiltration, and mixing of wastewater from different areas. By conducting localized studies, the results of viral RNA detection in wastewater can be more convincingly linked to the population living within the campus district. Thus, it may provide a better understanding of how WBE can be used to obtain data on the dynamics of SARS-CoV-2 transmission on campus.

The aim of this study is to compare the effectiveness of PEG precipitation and ultrafiltration techniques in concentrating SARS-CoV-2 from wastewater samples collected at Cranfield University. In particular, the objective is to determine which approach is more efficient in recovering RNA and more suitable for routine monitoring of viral infections in wastewater. The study will also investigate how the PEG precipitation technique can be optimized to reduce processing times and improve viral RNA recovery. The evaluation of WBE approach in monitoring SARS-CoV-2 transmission on campus for two years, from 2022 to 2023, is the next aim. In order to assess whether WBE can be used as an early warning system for a COVID-19 outbreak at Cranfield University, this study will use the optimized concentration method to treat wastewater samples collected on campus and obtain changes in viral concentrations over this period and finally compare wastewater analyzes with clinical case data.

## **3.2 Materials and Methods**

In this study, solutions are presented in either volume/volume (v/v) or weight/volume (w/v) format. The chemical, reagent, and solution compounds utilized in this research have been subjected to comprehensive risk assessment and have been assigned a CoSHH classification. The investigation was conducted using water from a Veolia PURELAB Chorus 1 Complete system. The water has been deionised, double-distilled, and ultrapure (type 1 laboratory grade), and is free of microorganisms, pyrogens, and both DNase and RNase. If this water was unavailable for use in laboratory experiments, Thermo Fisher Scientific, UK (UK) provided Invitrogen nuclease-free water as an alternative.

### **3.2.1 The collection of wastewater samples**

The ISCO 3700 sampler was employed for the purpose of gathering samples from the wastewater treatment plant situated in Cranfield as shown in Figure 3.1. The sampler was operated for a period of 12 hours, with 250mL of wastewater collected at hourly intervals. Prior to any form of treatment, the wastewater for all samples was obtained from the balancing tank. Following a 12-hour collection period, the samples of influent were combined and mixed thoroughly, and a 500ml sample was then collected from the mixture. Subsequently, the samples were transported to the laboratory on ice and stored at 4°C until processing.



**Figure 3.1 (a) Cranfield University wastewater sample collection point  
(b) ISCO 3700 sampler and 250 mL collection bottles for sampler**

### **3.2.2 PEG precipitation approach for virus concentration**

The wastewater samples were first homogenized by inverting the collection bottle multiple times to ensure an even particle distribution. Then, four 50-mL aliquots were centrifuged at 3,000g for 30 minutes at 4°C using a Thermo Fisher Scientific, UK Megafuge 8R centrifuge. Afterward, 150 mL of the supernatant was carefully transferred to a 250 mL polypropylene copolymer (PCCO) bottle, ensuring the pellet remained undisturbed. The pH of the samples was measured and adjusted to a range between 7.0 and 7.6, if necessary, by adding either 1M NaOH or 1M HCl.

Each 150 mL sample was then mixed with 50 mL of a solution containing 40% polyethylene glycol 8000 (PEG 8000) and 8% sodium chloride (NaCl) (Sigma-Aldrich). The mixture was thoroughly mixed by inverting the container several times and incubated at 4°C for approximately 24 hours. The following day, the samples underwent further centrifugation at 10,000g for 30 minutes at 4°C. After centrifugation, 1 mL of the supernatant was retained, and the remainder was discarded into a waste container containing 70% ethanol. The pellet-supernatant mixture was then centrifuged again at 12,000g for 10 minutes

at 4°C. The supernatant was carefully removed using a pipette, leaving the pellet for further analysis. The pellet was resuspended in 800 µL of TRIzol reagent (Thermo Fisher Scientific, UK) and vortexed for 15 seconds to ensure thorough mixing. It was expected that no significant number of macromolecular particles, like suspended solids and plastic fragments, remained at this point. Next, 0.2 mL of chloroform (Thermo Fisher Scientific, UK) was added to the solution to promote cell lysis. The samples were then thoroughly mixed by shaking or vortexing and incubated at room temperature for 2-3 minutes.

The samples were subsequently centrifuged at 12,000 xg for 15 minutes at 4°C, resulting in the separation of the mixture into three phases: a lower red phenol-chloroform phase containing proteins, an opaque interphase containing DNA, and a clear upper aqueous phase containing RNA. The clear upper aqueous phase was carefully transferred to a new tube, while the remaining phases were discarded. The RNA samples were either kept on ice for immediate purification or stored at -20°C for future use.

### **3.2.3 Ultrafiltration approach for virus concentration**

The wastewater samples were initially homogenized by inverting the collection bottle on several occasions, with the intention of ensuring an even distribution of the sample contents. Subsequently, four 50-mL aliquots were subjected to centrifugation at 3,000g for 30 minutes at 4° C using a Thermo Scientific Megafuge 8/8R centrifuge. Subsequently, the supernatant was meticulously gathered without dislodging the pellet.

Subsequently, 15 mL of the collected supernatant was transferred to a 10 kDa ultracentrifugal filter tube (Amicon, Merck, UK) for the ultrafiltration process. Subsequently, the tubes were subjected to centrifugation at 3,000g for a period of 45 minutes to one hour at 4° C. Following centrifugation, the flow-through was discarded and an additional 15 mL of the sample was added to the top of the

filter. The centrifugation step was repeated under identical conditions (3,000g for 45 minutes to 1 hour at 4° C).

Finally, the flow-through was discarded once more, and the concentrated sample remaining above the filter was transferred with care to an RNase-free Eppendorf tube. At this stage, the samples were either refrigerated until RNA extraction and purification could be carried out without delay or stored at -20° C until further processing.

### **3.2.4 RNA extraction and purification**

The RNA extraction and purification process was conducted in accordance with the instructions provided by the manufacturer of the QIAamp® Viral RNA Mini Kit. Before the commencement of the procedure, the biosafety cabinet was subjected to a thorough sterilisation process, which involved exposure to UV light for a period of at least 20 minutes, followed by a cleaning of surfaces with a 70% ethanol solution. Furthermore, additional decontamination of equipment, including centrifuges and pipettes, was conducted using RNA Zap (Thermo Fisher Scientific, UK), thus ensuring an RNase-free environment.

In the case of samples concentrated via ultrafiltration, the RNA extraction protocol was initiated from the outset, whereas those concentrated using the PEG precipitation method followed the protocol after the initial incubation step. In preparation for RNA extraction, 800 µL of AVL Buffer containing carrier RNA (provided in the QIAamp® Viral RNA Mini Kit) was added to a 1.5 mL microcentrifuge tube. Subsequently, 200 µL of the corresponding sample was introduced to the tube, and the mixture was vortexed for 15 seconds to ensure thorough homogenisation. Subsequently, a brief centrifugation was performed to collect any liquid that may have adhered to the tube lid. The mixture was incubated at room temperature for 10 minutes, allowing the lysis buffer to inactivate any RNases and ensuring effective viral RNA release.

For samples concentrated by polyethylene glycol precipitation, 800  $\mu\text{L}$  of 100% ethanol can be added directly to 200  $\mu\text{L}$  of sample, thus avoiding the lysis step. The mixture was then vortexed for 15 seconds and briefly centrifuged. Subsequently, the lysate-ethanol mixture was subjected to RNA purification via QIAamp Mini columns. 630  $\mu\text{L}$  of the mixture was initially added to the QIAamp Mini column, which was placed in a 2 ml collection tube. The column was then centrifuged at 8000 rpm for one minute and the flow-through was discarded. This process was repeated until all samples have been processed by the column.

To prevent impurities contamination, the column was then transferred to a new collection tube, and 500  $\mu\text{L}$  of AW1 Buffer was added to the system before the next step. The column was then centrifuged at 8,000 RPM for one minute, after which the sewage was discarded. This was followed by a second wash with 500  $\mu\text{L}$  of AW2 buffer and centrifugation at 13,000 RPM for 3 minutes. The flow-through was then discarded, and the column was placed in a new collection tube.

The final stage of the process was the elution of the purified RNA. 60  $\mu\text{L}$  of AVE buffer, equilibrated to room temperature, was added to the column. After one minute of incubation at room temperature, the RNA was centrifuged at 8,000 RPM for one minute to facilitate the elution of the RNA. The flow-through, which was the extracted viral RNA, was then stored at  $-20^{\circ}\text{C}$  until further downstream analysis could be performed. The scheme of both Ultrafiltration and PEG precipitation approaches is summarized in the Table 3.1.

**Table 3.1 The scheme of the Ultrafiltration and PEG precipitation**

|                | <b>Ultrafiltration</b>           | <b>PEG precipitation</b> |
|----------------|----------------------------------|--------------------------|
| Initial stage  | Centrifugation                   | Centrifugation           |
| Virus lysis    | AVL buffer                       | TRIzol/Chloroform        |
| RNA extraction | QIAamp kit starting with ethanol |                          |

### 3.2.5 The detection of SARS-CoV-2 from extracted RNA

After RNA extraction and purification, SARS-CoV-2 RNA was detected using real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The assay was performed using TaqMan™ Fast Virus 1-Step Master Mix from Thermo Fisher Scientific, UK. Primers and probes used in this assay were the same as those specified in the CDC 2019 nCoV Real- Time RT-PCR diagnostic panel. More details on primers and probes can be found in Table 3.2 below. Primers and probe were purchased from Sigma as lyophilized powder. On receipt, they were resuspended in nuclease-free distilled water (dH<sub>2</sub>O) to give a stock concentration of 100 µM. Before use in the assay, these stock solutions were further diluted to a working concentration of 20 µM.

**Table 3.2 Assay primer/probe sequences for the US CDC RT-PCR panel for detection of SARS-CoV-2**

| Name              | Location    | Sequence                                |
|-------------------|-------------|-----------------------------------------|
| N3-Forward (N3-F) | 28697–28718 | GGGAGCCTTGAATACACCAAAA                  |
| N3-Reverse (N3-R) | 28768–28748 | TGTAGCACGATTGCAGCATTG                   |
| N3-Probe (N3-P)   | 28720–28743 | 5'-FAM-AYCACATTGGCACCCGCAATCCTG-BHQ1-3' |

The PCR reaction mixture consisted of 5 µL TaqMan® Fast Virus 1-Step Master Mix, 8.75 µL nuclease-free dH<sub>2</sub>O, 0.5 µL forward primer (N3-F), 0.5 µL reverse primer (N3-R), 0.25 µL probe (N3-P) and 5 µL RNA target sample. The total reaction volume was 20 µL. RT-qPCR amplification was performed on the QuantStudio 3 PCR machine (Thermo Fisher Scientific, UK, UK) using the specific cycling conditions listed in Table 3.3. These conditions were optimized to ensure accurate and sensitive detection of SARS-CoV-2 RNA, following the standard protocols recommended for TaqMan assays.

**Table 3.3 Conditions for RT-PCR panel for detection of SARS-CoV-2**

| Step                  | Temperature | Duration   | Cycle |
|-----------------------|-------------|------------|-------|
| Reverse transcription | 50          | 5 minutes  | 1     |
| Initial denaturation  | 95          | 10 minutes | 1     |
| Denaturation          | 95          | 15 seconds | 40    |
| Annealing             | 60          | 1 minute   | 40    |

### **3.2.6 The comparison between PEG precipitation and ultrafiltration**

To evaluate the efficacy of PEG precipitation versus ultrafiltration for the concentration of SARS-CoV-2 from wastewater, samples collected on 06/01/2022, and 11/01/2022, were subjected to both methods. Each wastewater sample was processed in triplicate for both the PEG precipitation and ultrafiltration methods. The experimental workflow for both methods included sample concentration, viral RNA extraction and purification, and RT-qPCR analysis, as detailed in Sections 2.2 to 2.5.

After purification of the RNA, RT-qPCR was used to detect the extracted viral RNA, and the results were recorded the RT-qPCR cycle threshold ( $C_t$ ) for both methods.  $C_t$  value refers to the number of cycles needed to replicate enough DNA/RNA to be detected (crosses a threshold line). In order to assess the relative efficiency of each concentration method in recovering viruses from the wastewater, the  $C_t$  values obtained from the RT-qPCR results of the two methods were compared. Lower  $C_t$  values indicate a higher concentration of viral RNA, suggesting a more efficient viral concentration.

### **3.2.7 The optimization of the PEG precipitation method**

#### **3.2.7.1 The investigation of Viral Extraction Techniques**

This study investigated the effect of using TRIzol/Chloroform extraction and QIAamp Viral RNA Mini Kit in a PEG precipitation method on viral RNA recovery. Wastewater samples collected on 19/04/2022, 21/04/2022 and 28/04/2022 were tested. Each wastewater sample was divided into three experimental groups of 50 ml each, each with a different extraction protocol, to assess the effect on RNA recovery and detection.

In the control group, a complete concentration and extraction process was performed, including TRIzol/chloroform extraction and QIAamp Viral RNA Mini Kit purification steps. This group served as a control and represented the fully optimized extraction method. Concentration and extraction were performed without the use of chloroform and TRIzol and RNA was extracted using only the QIAamp Viral RNA Mini Kit in Experimental Group A. In Experimental Group B, the concentration process was performed without subsequent purification using the QIAamp Viral RNA Mini Kit and relied solely on the TRIzol/chloroform extraction to recover RNA. After extraction, RNA from each group was analyzed and analyzed as described in Section 2.5. After extraction, the extracted RNA from each group was analyzed by RT-qPCR as described in section 2.5. The  $C_t$  obtained from the RT-qPCR results was recorded and the  $C_t$  values were converted to concentration ( $\log_{10}$ ) using a standard curve and compared between the three groups.

To generate the standard curve for quantification of SARS-CoV-2 RNA, a sixfold tenfold serial dilution of the N gene standard RNA (New England Biolabs) was prepared. The dilution series ranged from  $10^8$  to  $10^3$  copies  $\mu\text{L}^{-1}$ . The N-gene standard was initially diluted to provide a stock solution with a concentration of  $10^8$  copies  $\mu\text{L}^{-1}$ . Subsequent tenfold dilutions were then performed by adding 10 microliters of the previous dilution to 90 microliters of nuclease-free water,

resulting in a series of standards. Each dilution was then subjected to RT-qPCR using the same N3 primers and probe used for the wastewater samples, and each dilution was performed with triplicate. The  $C_t$  values obtained for each standard concentration were plotted against the logarithm of the known concentrations to generate the standard curve.

### **3.2.7.2 The investigation of the large solid removal**

The focus of this investigation was to determine whether the omission of large solids removal, specifically the initial centrifugation step, would affect the overall yield of viral RNA. The wastewater samples used in this study were the same as those used in the previous viral extraction technique study. Each wastewater sample was divided into two groups of 50 mL each. The control group followed the full concentration and extraction protocol, including the first centrifugation step. In this step, 50 mL aliquots were centrifuged at 3,000g for 30 minutes at 4°C using a Thermo Fisher Scientific, UK Megafuge 8R centrifuge. This centrifugation was designed to remove large solids from the wastewater samples, potentially reducing the presence of inhibitors and improving the efficiency of subsequent RNA extraction. The experiment group omitted the initial centrifugation step and proceeded directly to the PEG precipitation method without removing solids. This approach was designed to assess whether bypassing the solids removal step would have any effect on the efficiency of viral RNA recovery. Following the concentration and extraction procedures, RNA from both groups was subjected to RT-qPCR analysis as described in sections 2.5. Cycle  $C_t$  values obtained from the RT-qPCR assays were recorded, using the standard curve to convert  $C_t$  values to concentration ( $\log_{10}$ ), and compared between the two groups.

### **3.2.7.3 The investigation of the precipitation time**

This investigation was conducted in two separate experiments, each focusing on different precipitation times at 4°C, to determine the optimal incubation period for maximizing RNA yield.

In the first experiment, the effect of longer-precipitation times was assessed by comparing precipitation periods of 1 day and 7 days. Wastewater samples collected on 21/06/2022, 23/06/2022 and 05/07/2022 were used for this investigation. After the initial processing steps, including homogenization and centrifugation as described in the previous sections, the PEG solution was added to each sample (50ml). The samples were then incubated at 4°C for either 1 day or 7 days to allow viral precipitation. After the appropriate incubation period, the samples were processed for RNA extraction and purification as described in sections 2.3 to 2.4. The RNA was then analyzed by RT-qPCR to determine  $C_t$  values, and the  $C_t$  values were further converted into the RNA concentration using standard curve, which provide a measure of the efficiency of viral RNA recovery for each precipitation time.

The second experiment focused on comparing shorter precipitation times, investigating the effect of 0 hours (immediate processing), 2 hours and 24 hours of precipitation at 4°C. The wastewater samples, collected on 19/07/2022, 21/07/2022 and 09/08/2022 - were used for consistency of comparison. After the addition of the PEG solution, the samples were subjected to the specified precipitation times. The 0-hour group was processed immediately after PEG addition without any incubation. The 2-hour and 24-hour groups were incubated at 4°C for the respective periods. At the end of the precipitation periods, RNA extraction and purification were performed as described above, followed by RT-qPCR analysis to compare the RNA concentration.

### **3.2.8 Analysis of real wastewater samples analysis**

Wastewater samples collected from 2022 to 2023 were analyzed using established wastewater concentration, extraction and RT-qPCR methods. On average, six to eight bottles of wastewater were collected monthly and stored at 4°C until analysis. From these collections, four samples per month were selected for testing, typically equivalent to one sample per week. A total of 96 samples were prepared and systematically labelled for analysis. The labelling system was defined by the month of collection and the order of selection within that month. For example, the second sample selected in April was labelled as 4-2. Samples collected in 2023 were labelled sequentially, with months numbered from 13 to 24. Detailed information on the specific sample collection dates and their corresponding labels is provided in the Appendix.

For each sample, 50 mL of raw wastewater was processed using the optimized PEG precipitation method for virus concentration. After concentration, RNA was extracted using the QIAamp Viral RNA Mini Kit, following the section 2.4. Detection of SARS-CoV-2 RNA was then performed by RT-qPCR using the N3 primers and probe as described in the section 2.5. To quantify the viral concentration in each sample, the RT-qPCR results were analyzed against a standard curve.

### **3.3 Results and Discussion**

#### **3.3.1 Comparison of PEG precipitation and ultrafiltration for viral RNA recovery from wastewater**

The detection of pathogens in wastewater involves a number of steps, including sampling, virus concentration, viral RNA purification, and detection. The concentration step is a crucial part of this workflow and must be carefully selected and optimised, as it plays a significant role in determining nucleic acid recovery from complex and heterogeneous wastewater samples. Among various concentration methods, ultrafiltration and PEG precipitation are widely used due to their effectiveness. However, comparative studies evaluating these methods, such as those by Lucansky *et al.* (2023) and Farkas *et al.* (2022), have reported different results. These differences are influenced by several factors, such as the choice of nucleic acid extraction kit, the composition of wastewater sample, and the particular protocol used. Therefore, it remains a challenge to determine which of these two methods is the most suitable for general application for virus concentration from wastewater. This highlights the need to find a better solution by comparison in each research scenario and to optimize the method in that case.

Ultrafiltration is a widely used method for concentrating viruses from wastewater. The mechanism of it is to use membranes with specific pore sizes to separate viruses from wastewater (Forés *et al.*, 2021). This technique uses semi-permeable membranes with pore diameters typically in the range of 1 to 100 nanometers. These pore sizes allow smaller molecules, such as water and dissolved compounds, to pass through while being large enough to retain viruses. Viruses, typically ranging from 20 to 300 nanometers in diameter, are captured on the membrane surface or within its structure (Rusiñol *et al.*, 2020). Meanwhile, smaller substances like nutrients and salts pass through the membrane, resulting in a filtrate.

By combining the ultrafiltration with centrifugation, the efficiency of this approach could be further improved. Before ultrafiltration, it is common to use low-speed centrifugation (1000 to 3000g) to remove large particles and suspends solids (Ye *et al.*, 2016). This is because these components may clog the membrane during the ultrafiltration process, leading to lower concentration efficiency. The liquid above the sediment, or supernatant, is then collected for ultrafiltration. During this process, the sample volume is reduced, resulting in an increase of the virus concentration. After ultrafiltration, the concentrated filtrate is centrifuged at high speed (20,000 to 100,000 x g). This high-speed centrifugation allows more liquid to pass through the membrane, further decreasing the volume of concentrated retentate.

PEG precipitation is another common method for concentrating viruses from wastewater. PEG is a hydrophilic polymer and has a compact molecular structure (Farkas *et al.*, 2021). When large amounts of PEG are added to a wastewater sample, the PEG molecules interact with the water, effectively reducing the solubility of other components, including viruses (Lu *et al.*, 2020). This reduction in solubility causes the viruses to precipitate out of solution. In addition, since PEG occupies a large volume in solution, it can exclude smaller molecules from this volume, resulting in a repulsive volume effect (Cohn *et al.*, 1946). This exclusion effect increases the local concentration of smaller molecules like viruses, promoting their aggregation and subsequent precipitation. Also, viruses can form complexes with PEG through hydrophobic and electrostatic interactions, which has lower solubility compared to the original viruses and lead to increased aggregation and precipitation (Vajda, 1978). The efficiency of this precipitation process can be further enhanced by adding sodium chloride (NaCl) (Barril *et al.*, 2021). The salt ions neutralize the charges on the virus particles, reducing the electrostatic repulsion and facilitating further precipitation.

In order to compare the effectiveness of these two concentration methods, wastewater samples collected from the Cranfield wastewater treatment plant

were employed as the template. As wastewater is produced continuously throughout the day from a wide range of activities, collecting a single sample at a specific time may not provide a representative profile of virus concentrations. To overcome this problem, samples were taken during the busiest 12 hours of the day at the university, when wastewater flows were expected to peak. These samples were then combined to form a composite sample to more accurately represent the overall wastewater profile for the day. This composite sample was then used to evaluate both concentration methods. To improve the reliability and reproducibility of the results, the experiment was performed in triplicate.

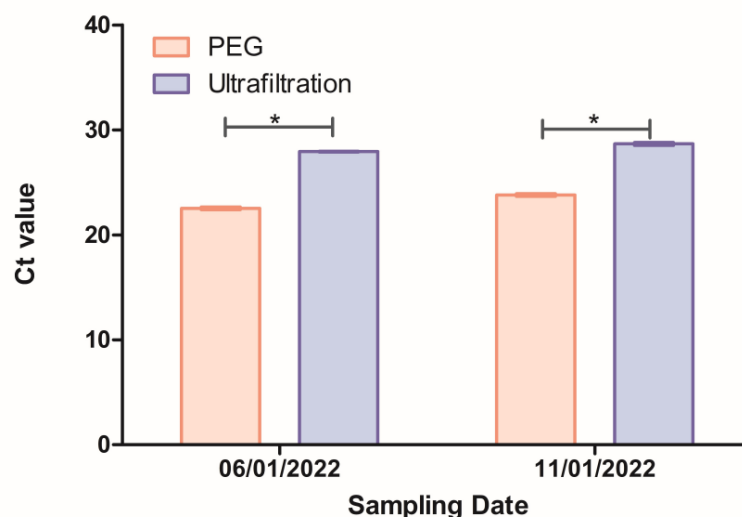


**Figure 3.2 The comparison results between PEG precipitation and ultrafiltration**

Figure 3.2 shows the results between ultrafiltration and PEG precipitation. The qPCR was used to measure the amount of SARS-CoV-2 RNA, and the results are expressed as  $C_t$  values. Lower  $C_t$  values indicate higher levels of viral RNA, which is consistent with a better recovery efficiency of the technique used. The results of our qPCR analysis show that the PEG approach consistently produced lower  $C_t$  values than the ultrafiltration approach on both sampling days. This indicates that the viral RNA recovered from the wastewater samples was more effectively recovered by PEG precipitation. With p-values well below the

generally accepted significance level of 0.05, an independent t-test was used to perform a thorough statistical analysis and validate this finding. This indicates that the differences between the approaches are statistically significant.

The performance of concentration techniques varies depending on the volume and content of the sample, according to recent research. In a UK study, Farkas *et al.* (2022) evaluated five widely used techniques on 115 unspiked wastewater samples. The most successful techniques overall were beef extract-enhanced PEG precipitation and Amicon ultrafiltration. In particular, PEG precipitation worked better with larger sample volumes, although ultrafiltration was more effective with smaller volumes. As this work used fresh samples, which may have contributed to the greater RNA recovery with both methods, this highlights the importance of sample freshness. Furthermore, these results are consistent with the work of Lucansky *et al.*, who also found that PEG slightly outperformed ultrafiltration, despite using a different RNA extraction kit. Table 3.4 summarizes the advantages and disadvantages of each of these two approaches.

**Table 3.4 The comparison between PEG precipitation and ultrafiltration**

| Method          | Principle      | Advantage                         | Disadvantage                            |
|-----------------|----------------|-----------------------------------|-----------------------------------------|
| Ultrafiltration | Size exclusion | Time saving, Minimal chemical use | Expensive, Membrane Fouling             |
| PEG             | Precipitation  | Cost-effective, High recovery     | Toxic reagents (TRIzol), Time consuming |

In addition to recovery efficiency, practical considerations were taken into account for each approach. As historical RNA/DNA may be present in these solids and could potentially confound qPCR data, both methods require an initial centrifugation step to remove suspended solids (Graham *et al.*, 2021). During the

TRIzol step, proteins, DNA and RNA can be separated using the PEG technique. As they are similar in size to the target RNA, many PCR inhibitors that can normally pass through the ultrafiltration membrane are removed by this separation process, which is very beneficial. A lysis phase during RNA extraction is also no longer necessary thanks to the TRIzol step in the PEG approach, which isolates nucleic acid and cuts down on extraction time from several hours to around half that for each sample. On the other hand, the complete RNA extraction procedure is required as ultrafiltration does not lead to nucleic acid separation. Though the PEG approach takes 6 to 24 hours to complete, ultrafiltration may be completed in 3 to 4 hours, making it a simpler and faster process overall.

The PEG approach performed better in this laboratory setting for concentrating SARS-CoV-2 from wastewater when all parameters were considered, including practical execution, time requirements and efficiency of viral RNA recovery. Therefore, PEG precipitation is the preferred approach for further investigation due to its higher recovery efficiency and lower  $C_t$  values obtained despite the longer processing time.

### **3.3.2 The optimization of PEG precipitation technique**

As the PEG precipitation method was chosen to concentrate the viruses in the wastewater, the method had to be optimized before the real wastewater analysis. The initial focus was on improving the RNA extraction process to ensure maximum efficiency. This was followed by optimizing the removal of large solids in the initial stage, which was essential to minimize potential interferences. Finally, optimization efforts focused on reducing the precipitation time, as the original protocol required overnight settling, which was time-consuming and a significant drawback.

The first investigation aimed to determine the need for two extraction steps, comprising TRIzol extraction and the QIAamp® Viral RNA Mini Kit, in the PEG precipitation technique for viral RNA concentration. Since both steps serve the

same function, the experimental process could potentially be simplified and time saved, by removing one of them without compromising RNA recovery efficiency. The results are illustrated in Figure 3.3 (a), which shows the viral RNA concentrations (log10) for each group over three sampling dates: 19/04/2022, 21/04/2022 and 28/04/2022.

According to the results, it is important to maintain these two procedures for optimal RNA recovery from the wastewater. The control group using both extraction steps consistently had the highest concentration of RNA detected in all samples. Group A using only the QIAamp® kit had a lower RNA concentration compared to the control, indicating that the lack of the TRIzol step resulted in less efficient RNA recovery. The increased efficiency of RNA purification using the QIAamp® kit after the TRIzol step may be due to the ability of TRIzol to remove proteins and inhibitors. TRIzol lyses cells and disrupts cell membranes when in contact with biological materials. The chaotropic component of TRIzol, guanidine isothiocyanate, is essential for denaturing proteins and deactivating RNases, enzymes that break down RNA (Zheng *et al.*, 2022).

After lysis, the sample is usually centrifuged to aid phase separation. Because of the different solubilities of the molecules in TRIzol, the centrifuge separates the sample into three phases (Trujillo *et al.*, 2021). Viral RNA is present in the upper, clear, aqueous phase because RNA is hydrophilic and prefers the polar environment created by guanidine isothiocyanate. Since DNA is less soluble in water, it becomes trapped between the aqueous phase and the organic phase below, which is found in the intermediate phase. Proteins and lipids that have been denatured are present in the bottom phase, which is a pink or red phenol-chloroform layer. These hydrophobic molecules are separated from the RNA by the organic solvents' phenol and chloroform. Therefore, the combination of guanidine isothiocyanate, phenol and chloroform ensure that RNA, DNA, proteins and lipids can be efficiently separated and purified from complex

wastewater samples and protect the integrity of RNA by excluding non-RNA molecules in other layers.

Group B, which relied only on TRIzol extraction, did not detect any RNA across the three samples. The absence of detectable RNA suggests that while TRIzol is effective at lysing cells and removing contaminants, it alone is insufficient for the complete extraction, purification and recovery of viral RNA, particularly in complex samples like wastewater. In addition, the RNA after the TRIzol extraction step was concentrated into approximately 200 to 400  $\mu\text{L}$  of aqueous phase, while the RNA after the kit step was eluted into approximately 60 to 80  $\mu\text{L}$  of eluent. Even without considering the effect of the kit on RNA extraction and purification, the RNA concentration should increase after the kit extraction step due to volume compression. Silica membranes may play an important role in purifying viral RNA and increasing its concentration during the use of QIAamp® kits (Alamin *et al.*, 2022). In the presence of chaotropic agents, the silica surface forms a complex with RNA, allowing it to bind tightly to the membrane, while other contaminants such as proteins and polysaccharides do not. When washing buffer containing ethanol is used to remove contaminants, only pure RNA remains bound to the membrane, while other contaminants are washed away (Hjelmsø *et al.*, 2017). The purified RNA is then eluted from the silica surface using low-salt elution buffer or nuclease-free water for collection, as these can break the bond between the RNA and the silica membrane.

In summary, the results demonstrate the necessity of using both TRIzol extraction and the QIAamp® Viral RNA Mini Kit in the PEG precipitation protocol. The combination of these two steps significantly enhances RNA recovery, as evidenced by the higher concentrations observed in the control group. The dual-step process appears to be essential for removing inhibitors and efficiently purifying RNA, which is critical for the sensitivity and accuracy of downstream analyzes such as qPCR. The results clearly indicate that maintaining both

extraction steps in the protocol is vital for achieving the highest possible yield of viral RNA from wastewater samples.

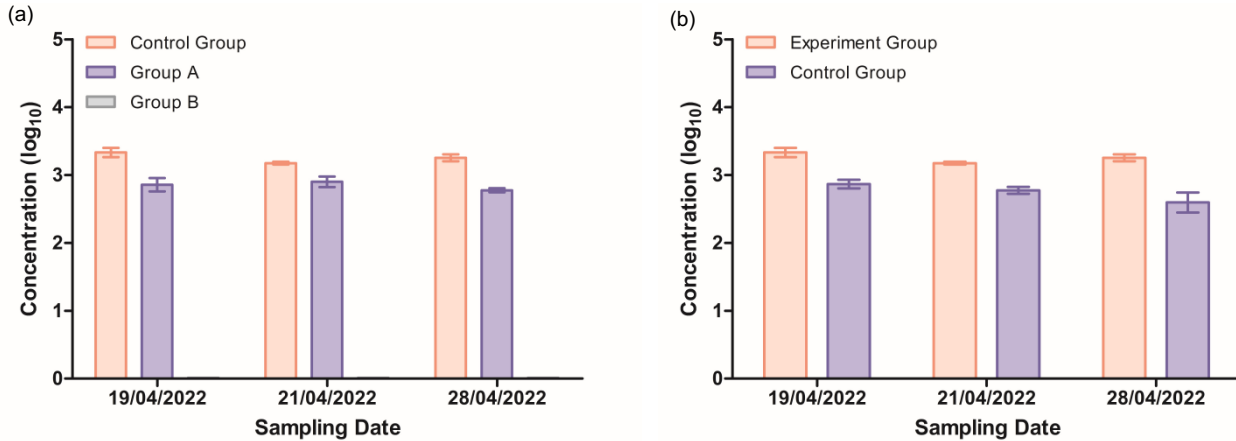


**Figure 3.3 The optimization of PEG precipitation approach (a) Extraction method (b) Large particle removal; Results represent the mean +/-SD of three independent replicates.**

The next stage in the optimization process focused on evaluating the need for the large particle removal step in the PEG precipitation protocol. This step, which involves initial centrifugation to remove large contaminants, was originally retained to compare the PEG method with ultrafiltration. When it comes to the ultrafiltration approach, the filtration membrane may be blocked if the first centrifugation is omitted, resulting in reduced concentration efficiency. In addition, previous studies reported there might be historical RNA presenting in the large, suspended solids (Graham *et al.*, 2021). The remaining of historical RNA may cause a decline accuracy of the final qPCR assay. Conversely, other evidence also suggested that by retaining the sludge that may adsorb the virus, viral RNA recovery in the final detection may be improved. Symonds *et al.* (2014) found that viruses can be adsorbed on highly aggregated sludge surfaces due to electrostatic and hydrophobic interactions. In addition, Strubbia *et al.* (2019) showed that the use of an ultrasonic step before PEG precipitation increased the recovery of norovirus particles by releasing them from organic matter.

In view of the inconsistency of previous views, an experiment was designed to evaluate the necessity of large particle centrifugation during PEG protocol. The experiment group omitted the initial centrifugation step, while the control group retained it. Figure 3.3 (b) displays the findings of three separate sample dates used to quantify the recovery viral RNA concentrations (log10) for both groups. Across all three sampling dates, the experiment group (which omitted the centrifugation step) yielded higher viral RNA concentrations than the control group. These findings suggest that the RNA recovery efficiency of PEG precipitation protocol increases when the large-particle centrifugation phase is skipped. This result supports the theory that recovering viral RNA is improved by remaining the sludge, where viral particles may adsorb to solids. The findings are consistent with the research conducted by Strubbia *et al.* (2019), which showed that recovery efficiency may be increased by interfering with the interactions between viruses and organic materials. Another study performed particle-associated virus-liquid/solid fraction analysis and showed that SARS-CoV-2 viruses could not be detected in the supernatant after initial pre-spinning when the wastewater contained large amounts of dissolved solids (Sapula *et al.*, 2021). Viral particles that would ordinarily be discarded with the big solids during centrifugation may be captured by the PEG approach by omitting the centrifugation step. Therefore, the large-particle removal step may not be necessary for the PEG precipitation protocol and may, in fact, reduce RNA recovery.

Since the original protocol required a 24-hour precipitation period, the last optimization step concentrated on shortening the precipitation duration in the PEG technique for viral concentration. This prolonged duration can be considered a drawback, especially in situations when rapid processing is in need. Two studies evaluating longer and shorter precipitation intervals were carried out to find a compromise between time efficiency and RNA recovery in order to explore the requirement of long precipitation periods.

The first experiment compared the effect of extended precipitation times by incubating wastewater samples for 1 day and 7 days at 4°C after the addition of PEG for precipitation. After the addition of PEG, the samples were incubated under the specified conditions and viral RNA was extracted and purified on the nominated days. The viral RNA was then detected by RT-qPCR and the RNA concentration was further measured using a standard curve as previous ones.

As shown in Figure 3.4 (a), in all three samples tested, the concentration of RNA recovered from the wastewater samples after the 7-day precipitation period was consistently higher than that from the 1-day incubation period. For example, from the sample collected on 21/06/2022, the RNA concentration of the 7-day group reached to approximately  $10^{3.8}$  copies  $\mu\text{L}^{-1}$ , while the 1-day group averaged approximately  $10^{3.2}$  copies  $\mu\text{L}^{-1}$ . A similar trend was observed on samples collected on 23/06/2022 and 55/07/2022, where the 7-day precipitation produced significantly higher viral RNA concentrations than the 1-day group. After one week of incubation, it was observed that all three samples became more turbid, and more particles were observed at the bottom of the tube after centrifugation. This also suggests that if the precipitation time is extended from one day to seven days, more particles can be collected, and more viral RNA can be recovered from the original wastewater sample.

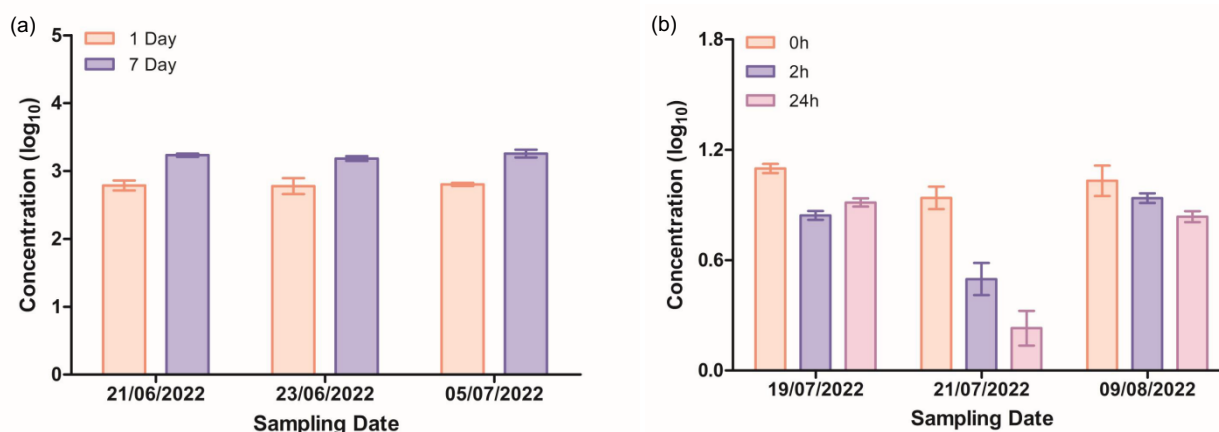


**Figure 3.4 The investigation of PEG precipitation time (a) The comparison between 1 day and 7 days (b) The comparison between 0h, 2h and 24 h; Results represent the mean +/-SD of three independent replicates.**

The prolonged precipitation time improves RNA recovery, which supports the hypothesis that longer incubation times allow more viral particles to aggregate and precipitate with PEG (Krsek and Wellington, 1999; Roose-Amsaleg, Garnier-Sillam and Harry, 2001). Farkas *et al.* (2017) found that increased incubation times resulted in higher DNA concentrations when qPCR quantification was used, but less prolonged PEG incubation resulted in inhibited quantification by digital PCR. It has been suggested that PCR inhibitors may concentrate with nucleic acids during PEG precipitation (LaMontagne *et al.*, 2002; Miura *et al.*, 2011). Despite the high RNA recovery observed in the 7-day incubation group, extending the incubation period from one to seven days appeared unnecessary, as a one-day incubation yielded sufficient RNA.

In addition, other studies have reported comparable or higher recoveries of viral RNA for final qPCR identification during PEG treatment when precipitation times were controlled between 0 and 2 hours. Zheng *et al.* developed a rapid, high-throughput, and sensitive PEG-precipitation method for SARS-CoV-2 wastewater surveillance, and an evaluation of different incubation and

centrifugation times was made to examine the potential of PEG-precipitation method to decrease turnaround time (2023). The results after overnight incubation were comparable to those from the standard PEG precipitation approach without the incubation step, suggesting that the incubation step could be eliminated to streamline the process and increase sample capacity. Because the PEG precipitation technique requires a longer incubation time and results in a low throughput, another study also explored whether shortening the incubation time of PEG precipitation would reduce recovery (Torii *et al.*, 2022). The results showed that overnight incubation did not improve the recovery rate after spiking mouse hepatitis virus (MHV) into the raw wastewater. In contrast, overnight incubation has a negative impact on the recovery rate of the whole process. Compared with the three schemes with longer precipitation time, the MHV recovery rate of the process without any incubation time is relatively higher. A similar study was carried out using wastewater spiking with *Pseudomonas* virus phi6 (Phi6) to evaluate the role of long incubation in the PEG precipitation approach (Flood *et al.*, 2021). Although the recovery rate of Phi6 by PEG method without overnight incubation was lower than that by PEG method with overnight incubation, the difference was not statistically significant.

The second optimization experiment aimed to investigate the effect of different precipitation times - 0 hours (immediate processing), 2 hours and 24 hours - on the recovery of viral RNA using the PEG precipitation method. Wastewater samples were collected on 19/07/2022, 21/07/2022 and 09/08/2022 and, after processing with PEG, were subjected to the specified precipitation times. The viral RNA concentrations were then quantified by RT-qPCR and the results are shown in Figure 3.4 (b), illustrating the differences in RNA recovery based on precipitation time.

The 0-hour group, whose samples were analyzed immediately after PEG addition without incubation, appeared to have the highest RNA recovery of all samples. In the 0-hour group of samples collected on 19/07/2022, the

concentration of detected SARS-CoV-2 virus RNA was approximately  $10^{1.2}$  copies  $\mu\text{L}^{-1}$ , which was significantly higher than the concentrations of the 2-hour and 24-hour groups (approximately  $10^{1.0}$  copies  $\mu\text{L}^{-1}$  and  $10^{1.1}$  copies  $\mu\text{L}^{-1}$ , respectively). Identical trends were observed in two other samples, with the 0-hour group consistently outperforming the other two groups. According to these results, rapid processing could potentially prevent viral RNA loss or degradation, as longer incubation times could introduce elements such as RNA decay or reduced viral particle agglomeration, which would reduce recovery efficiency.

Additionally, the difference between the 2-hour and 24-hour groups was slight in samples collected on 19/07/2022 and 09/08/2022, but that was more obvious in sample collected on 21/07/2022. Of the three samples, the sample collected on 21 July 2022 had the lowest concentration of detectable viral RNA, and the concentration of detectable RNA became significantly lower as the processing time increased. This may indicate that while a short period of precipitation (2 hours) may recover acceptable RNA when the virus concentration in the wastewater is low, further extension of the incubation time to 24 hours does not provide any additional benefit and may even reduce the efficiency of RNA recovery. The results presented suggest that in this particular situation, rapid processing would provide a superior recovery plan. This finding is particularly valuable for optimizing protocols in high-throughput laboratories, where time efficiency is critical without sacrificing the quality of viral RNA recovery. Besides, the disagreement with some previous studies highlights the importance of context-specific optimization when using PEG precipitation methods for wastewater analysis, as it may be caused by variations in sample composition, viral load or processing conditions.

### **3.3.3 Wastewater surveillance at Cranfield University campus**

Wastewater samples were collected weekly at Cranfield wastewater treatment Plant since the start of the pandemic. Cranfield University is a

University in Bedfordshire, England, with a median residential population of 1,200 and a further 5,000 staff and students on weekdays. The University has an independently operated, self-contained sewer system which receives wastewater primarily from the University's technical buildings, student accommodation and residences. The sewer carries wastewater from large buildings, including residences, campus businesses and the University Estate, but does not contain industrial wastewater. Wastewater treatment at Cranfield is a multi-stage process consisting of: a screen to remove coarse material from the water, a flow equalization tank (the "balancing tank") which also acts as a settling tank, a set of primary lamella clarifiers, a trickling filter, a second set of lamellas, a set of two trickling filters and the final outfall, where the treated wastewater is discharged into a small river called Chicheley Brook. Since the flow equalization basin collects a representative sample of wastewater from across campus, samples were routinely collected after the wastewater settles in the equalization basin. The flow equalization basin is cleaned and dislodged weekly to maintain optimal function.

Cranfield University provides an exclusive and self-contained setting for wastewater monitoring. Because the campus has its wastewater treatment system, it is easier to monitor and test samples from a specific population. Because of its isolation, the sewage system is less complicated than larger urban systems, which can be complicated by factors such as groundwater infiltration, industrial pollution and inflow from neighboring areas. By ensuring that the SARS-CoV-2 RNA found in the wastewater was representative of the university population, this localized sampling scheme provided a clear picture of the presence of the virus and the dynamics of its transmission within the campus.

Wastewater samples from the university's separate sewerage system were treated using an optimized PEG precipitation method to concentrate the virus. RNA was extracted using the QIAamp Viral RNA Mini Kit and analyzed by RT-qPCR against the SARS-CoV-2 N gene. Results were quantified by comparing

$C_t$  values to a standard curve generated from known concentrations of the N gene to accurately estimate viral levels. Continuous monitoring of viral load was possible by collecting wastewater samples for two years. By continuously labelling samples, structured analyzes can be performed to provide a monthly snapshot of viral prevalence. Weekly sampling ensures reliable and continuous detection of changes in viral load.

Figure 3.5 shows the variation of SARS-CoV-2 RNA concentration in wastewater over 96 weeks, from the beginning of 2022 to the end of 2023. The concentration of viral RNA in the wastewater was calculated from the standard curve and converted to the initial concentration in the original sample, taking into account the concentration ratio during wastewater enrichment process. There were clear peaks in viral concentration in the early weeks of 2022, corresponding to the end of the Omicron wave that affected the UK. Viral concentrations during these peaks reach around 20,000 copies/mL, reflecting high levels of SARS-CoV-2 circulation within the university community. These peaks in viral RNA are likely to be associated with the resumption of face-to-face activities on campus following the relaxation of COVID-19 restrictions, leading to increased viral transmission. This is followed by a slow decline as vaccination campaigns increased and public health regulations were introduced. A second set of smaller peaks are seen in 2022, when the UK government relaxed regulations, particularly in weeks 20 to 40. These peaks most likely correlate with localized epidemics, possibly triggered by the relaxation of restrictions and increased social contact. The result shows that viral transmission continued in 2023, with viral RNA detected consistently throughout the year, but at low levels. This reflects persistent but manageable levels of infection, consistent with the shift to COVID-19 as an endemic virus rather than an acute pandemic threat (Looi, 2023). Towards the end of 2023, a marked decline in virus concentration is observed, indicating successful containment strategies and lower transmission

rates. This figure provides an essential overview of the transmission dynamics of SARS-CoV-2 within Cranfield University over the course of two years.

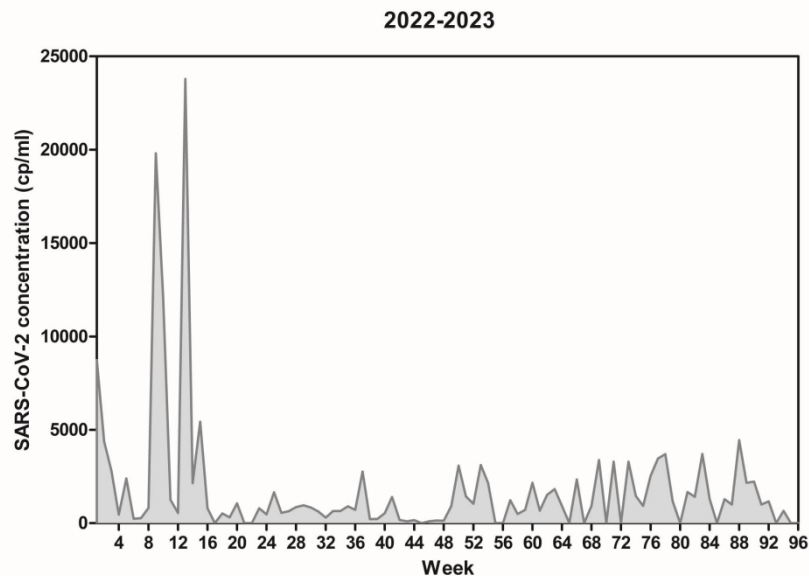


**Figure 3.5 The quantified concentration of N gene of SARS-CoV-2 in Wastewater at Cranfield University (2022-2023)**

WBE also addresses some of the limitations of clinical reporting, especially during times when public compliance with testing drops, or when there are testing shortages (Ahmed *et al.*, 2020). In order to understand the role of WBE in COVID-19 outbreak more comprehensively, the data gained from wastewater was compared to clinical reported numbers and presented in Figure 3.6. The figures provided showcase the value of comparing WBE data with clinical case reports to gain a more accurate understanding of viral transmission. The number of clinically reported cases is taken from the NHS Detection and Tracking (England) Weekly Statistics, which updates the number of new cases in England each week since the start of the pandemic (GOV.UK, 2024). The number of infected cases in England is counted daily and specific information can be found in the appendix. For consistency with the epidemiological information on wastewater, the daily case counts have been further processed to weekly case counts by calculating the average number of cases for the week.

Figure 3.6 (a) compares SARS-CoV-2 concentrations in wastewater (measured in copies/mL) with the number of confirmed clinical cases in the population throughout 2022. According to both datasets, the first half of the year is broadly consistent, with increases in reported cases, particularly in weeks 1-12, coinciding with peaks in viral loads in wastewater. The Omicron strain was spreading at this time, causing a sharp increase in cases throughout the UK (Wei *et al.*, 2024). As the UK was still recovering from the Omicron outbreak in early 2022, increased emphasis was placed on surveillance and clinical testing. Vaccination and booster programs were still being promoted, and social isolation techniques were being used to prevent the spread of the virus (Elliott *et al.*, 2022). Early increases in clinical cases and viral levels in wastewater indicated that the Omicron strain and its substrains had spread widely, causing an infectious epidemic across the country (Wise, 2022).

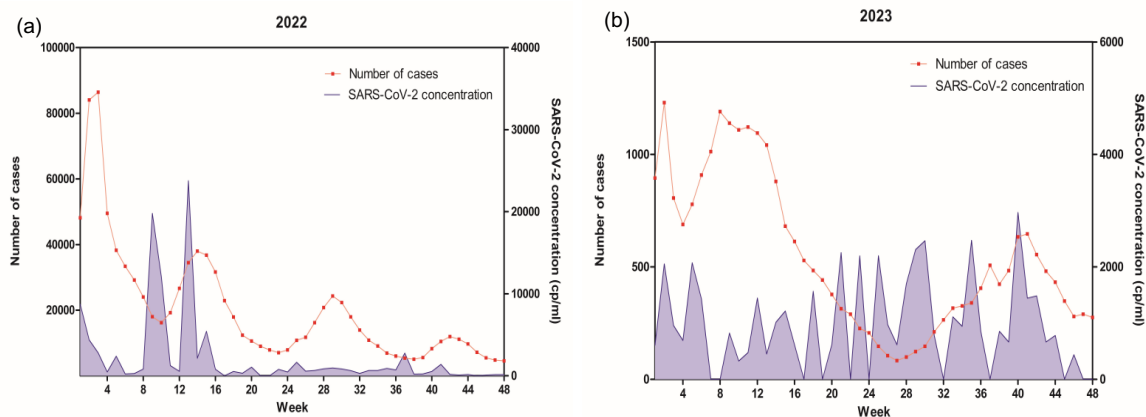


**Figure 3.6 Comparison of clinically reported cases in England and changes in SARS-CoV-2 concentrations in wastewater at Cranfield University**

Several key observations also highlight that peaks in SARS-CoV-2 concentrations in wastewater often precede peaks in the number of cases, indicating the early detection potential of WBE. The number of reported cases increases significantly in the first four weeks of 2022, reaching a peak of around 80,000 cases in the second week. It's interesting to note that the concentration of the virus in the wastewater may peak before this spike in cases, and had already begun to decline by the time the total number of reported cases reached its peak. This implies that the peak of virus shedding into the sewage occurred before the peak of symptomatic cases recorded. This early increase in wastewater concentration shows the potential of WBE can detect viral transmission in the community before clinical testing shows a corresponding increase in the number of cases. SARS-CoV-2 concentrations in wastewater increased twice between weeks 8 and 16, which also preceded the peak in the number of cases. The first peak in wastewater occurred at week 9 before the number of clinically reported cases increased slightly beginning at week 10. Similar to the previous example, a second peak in wastewater occurred at week 12, which also preceded the maximum number of reported cases at week 14,

suggesting that the data on significant increases in viral concentrations obtained in the WBE were once again an early marker of viral transmission.

Other studies also show that WBE can detect the presence of SARS-CoV-2 before clinical case reports, because people start shedding viral RNA in their feces before they become symptomatic or seek medical care (Randazzo *et al.*, 2020; Medema *et al.*, 2020). Both symptomatic and asymptomatic individuals are thought to shed virus particles in feces that enter sewage treatment plants. Remarkably, 18-45% of individuals infected with COVID-19 are asymptomatic; however, they are still capable of spreading the disease and have the potential to negatively impact COVID-19 containment efforts (Yang, Gui and Xiong, 2020). SARS-CoV-2 RNA is present in wastewater from both asymptomatic and symptomatic individuals, so WBE could be used for COVID-19 monitoring to predict the overall pandemic state (Kumar *et al.*, 2021). In many studies, peaks in wastewater virus concentrations have been observed days or even weeks before comparable increases in clinical cases, demonstrating the usefulness of WBE for early detection (Trigo-Tasende *et al.*, 2023).

During the COVID-19 pandemic, WBE was widely used as an outbreak early warning tool. By monitoring levels of viral RNA in wastewater, public health officials were able to track trends in infection rates in the population, even as clinical testing rates declined due to reduced adherence or asymptomatic infections. A study conducted in Toronto for a large homeless shelter demonstrated the effectiveness of wastewater surveillance as an early warning system for outbreaks (Akingbola *et al.*, 2022). Wastewater samples were regularly collected and tested for SARS-CoV-2 RNA, and the first positive wastewater signal in August 2021 led to immediate action by health authorities, including increased testing of residents, which later confirmed clinical cases. The outbreak was officially declared on 2 September, just days after the first wastewater signal. Similar studies have been conducted at the University of Arizona, where wastewater monitoring allowed the university to detect the

presence of SARS-CoV-2 before clinical symptoms appeared (Betancourt *et al.*, 2021). Because of the early detection, more widespread epidemics could be avoided through targeted testing, isolation and treatment. The study shows how WBE can be used as a preventative strategy to stop the spread of viruses in closed spaces.

The number of clinical cases began to decline, particularly after the peak of the Omicron outbreak, but two more increases could be observed during the weeks of 24-36 and the weeks of 40-44. The UK government's initiatives to extend the enhanced vaccination program have been associated with this decline. Although the number of reported cases has decreased significantly, relatively low but persistent levels of viral RNA are still found in wastewater at weeks 20-40. As vaccination rates rise and the public health situation stabilizes, the UK government is beginning to lift the COVID-19 restriction: by mid-2022, mandatory testing standards was relaxed, meaning that people did no longer have to disclose test results unless they have symptoms or were very ill (BBC, 2022). This policy change may have contributed to the decline in recorded cases, although wastewater monitoring was still able to detect low levels of viral RNA. As the virus concentration in wastewater has remained stable at low levels, this suggests a more subdued phase of the outbreak.

Figure 3.6 (b) shows a reduced association between wastewater virus concentrations and clinical cases in 2023 compared to 2022. Although there are still periodic spikes in wastewater virus loads, the very low number of confirmed cases reported throughout the year suggests a decoupling of infection levels as measured by clinical tests. Based on clinical testing data, the peak incidence in England in 2023 was no more than 1,500 cases, but the lowest level of reported infections in 2022 was also more than 5,000 cases. This may be the result of a change in public health policy in 2023, which treats COVID-19 as an endemic virus rather than an acute pandemic threat. These trends may have been influenced by the UK's transition to 'living with COVID', where fewer people

undergo clinical testing even if they were infected, because the focus was on high-risk individuals rather than mass testing (UK Health Security Agency, 2023). Despite the limited number of clinical cases, the wastewater data spikes seen in weeks 20-40 of 2023 suggest that viral transmission was still ongoing. This lends further credence to the argument that wastewater surveillance is necessary to identify latent chains of transmission and infectious diseases that are missed when clinical testing declines. In addition, continuous detection of SARS-CoV-2 RNA in wastewater is also necessary to identify emerging viral variants or potential outbreaks of the virus, which may not be immediately detected by clinical reported case alone. These results are consistent with global patterns where WBE has identified new outbreaks or persistent viral presence even when clinical case numbers appeared to be low.

To gain a deeper understanding of the local spread of SARS-CoV-2 and to assess the effectiveness of WBE on a smaller scale, this study also compared virus concentrations at Cranfield University with those in the nearby towns of Milton Keynes and Bedford. While data from larger areas such as England can provide insights into broader patterns of transmission, focusing on smaller, geographically proximate sites including Milton Keynes and Bedford allows for a more nuanced analysis of local viral circulations, public health responses and socio-environmental factors affecting transmission. Because Milton Keynes and Bedford are so close to the Cranfield campus, it is possible to determine whether the patterns seen in the sewage from the site are representative of the surrounding areas. These towns are suitable for a localized comparison as they have comparable environmental factors, population concentrations and access to healthcare resources. Furthermore, by comparing the two towns, it was possible to determine whether the virus concentrations in the university's wastewater were caused by internal variables specific to the campus population, or whether they follow more general regional trends observed in nearby towns.

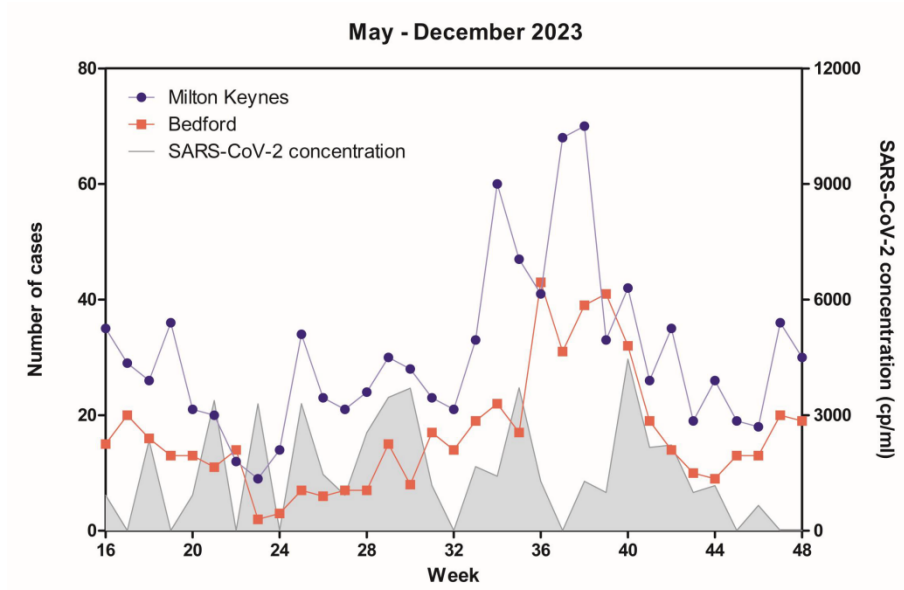


**Figure 3.7 The comparison between SARS-CoV-2 concentration changes and reported cases in Milton Keynes and Bedford**

The Figure 3.7 presents these comparisons, showing the number of COVID-19 cases in Milton Keynes and Bedford alongside the SARS-CoV-2 concentration in wastewater, from May to December 2023. The number of COVID-19 cases for Milton Keynes and Bedford was taken from the UKHSA data dashboard, with lower tier local authorities selected for area type. As the dashboard only provided data from 1 May 2023 when processing the results (currently only data from September 2023 is available), the daily data was downloaded and further processed into weekly data by calculating an average. The specific information can be found in the appendix. The grey shaded area represents the concentration (cp/mL) of SARS-CoV-2 RNA in the wastewater from Cranfield campus, and the blue and red lines represent the number of reported cases in Milton Keynes and Bedford, respectively. The case data are plotted against the left y-axis, while the viral RNA concentration in wastewater is plotted on the right y-axis. Virus concentrations and the number of reported cases fluctuated in each week of the 32-week observation period, with differences between the two towns, but the general trends were similar.

Milton Keynes generally had a higher number of cases throughout the period compared to Bedford. Peak numbers of cases occurred in weeks 33-35 and 37-38, with weekly numbers as high as 60-70 cases. In contrast, Bedford reported fewer cases, generally fluctuating between 0 and 20 cases per week, with only a slight increase between peak weeks 35-39, peaking at roughly 40 cases. However, the increase in viral RNA concentration in wastewater remained modest, with levels reaching only about 4,000 cp mL<sup>-1</sup>, which is much lower than what is typically associated with larger outbreaks. This pattern indicates that while there was some transmission of the virus, it may not have been widespread enough to generate significant viral shedding into the wastewater. This contrast between increasing case numbers and relatively low levels of SARS-CoV-2 in wastewater suggests that the viral load in wastewater did not increase in parallel with reported cases. A comparison of the number of cases and concentrations of SARS-CoV-2 in wastewater from Week 32 to Week 40 provides important new information on the dynamics of viral transmission and the utility of WBE. This finding suggests that the epidemic at that time may have been less severe or more localized than previous epidemics, as viral shedding in wastewater did not indicate significant levels of community transmission at Cranfield University.

The difference between the number of observed cases and the concentration of viral RNA in wastewater highlights the importance of using WBE in conjunction with clinical reporting. While the number of cases provides a useful indication of asymptomatic infections and testing patterns, wastewater monitoring provides a broader picture of viral transmission in the community, including asymptomatic populations who may not seek testing. In this case, an increase in case numbers without a corresponding significant increase in viral RNA concentrations suggests that the outbreak is relatively contained, and that viral transmission may not be as widespread as the case data themselves suggest.

### 3.4 Conclusions

This study provided an example of using WBE as a surveillance tool for tracking SARS-CoV-2 transmission within Cranfield University campus. The study initially focused on the comparison between PEG precipitation and ultrafiltration methods for virus concentration from wastewater. The results indicate that PEG precipitation is a more effective technique for concentrating SARS-CoV-2 from wastewater than ultrafiltration. This is because the RT-qPCR results of the wastewater have lower  $C_t$  values after concentration with PEG precipitation, reflecting higher viral RNA recovery. In this setting, the superior RNA recovery efficiency of the PEG precipitation approach is more appropriate for the following study.

Further optimization was made to improve the practicality of the PEG precipitation method for long-term wastewater surveillance. The initial large particle removal step is omitted for higher viral RNA recovery. Since viruses may adsorb to organic solids in wastewater and the removal of these organic solids may inadvertently reduce the amount of viral RNA recovered. Another optimization involved the RNA extraction steps in the protocol, including the TRIzol step and the QIAamp® Viral RNA Mini Kit. The results showed that both steps are essential to maximize the recovery of viral RNA from wastewater, and therefore both steps should even be performed. A disadvantage of the original PEG precipitation protocol was that it was time-consuming, as it required 24 hours of precipitation time. However, optimization studies have shown that adequate levels of RNA recovery can be achieved by reducing the precipitation time to 0 hours. By reducing precipitation time, it increases the adaptability of the PEG method to high throughput environments, where rapid processing is critical. These modifications not only increase the efficiency of SARS-CoV-2 detection but also have broader implications for the use of WBE in wastewater monitoring for other viral infections.

Apart from the technological advances in virus concentration, this study highlights the wider public health potential of wastewater surveillance by conducting it in Cranfield University. The two-year wastewater analysis has consistently provided data on viral transmission on campuses through the use of optimized concentration techniques. Results showed that wastewater-based SARS-CoV-2 testing provided early warning signs of viral transmission, with increases in viral RNA concentrations often occurring before peaks in clinically reported cases. The early detection capability of WBE is useful in closed system like university campus. Rapid intervention could be made based on the warning system and prevent further spreading of the infectious virus.

One of the key advantages of WBE is its ability to detect viral shedding in both symptomatic and asymptomatic individuals when it comes to the SARS-CoV-2 identification. During the COVID-19 pandemic, asymptomatic transmission played an important role in the spread of the virus, and many people did not seek clinical testing unless they were symptomatic. However, the complete picture of the dynamics of virus transmission could be obtained by wastewater analysis. By gaining epidemiology data from wastewater, including the asymptomatic ones, authorities are allowed to identify hidden chains of transmission and take proactive measures.

Additionally, WBE may help fill gaps in clinical testing, particularly in situations with limited test availability or low compliance. A significant drop in reported clinical cases during 2023 was because the UK no longer provides free diagnostics for individuals. However, viral RNA was still found in the wastewater at levels almost identical to those at the end of 2022. The results indicate that the transmission was still occurring even though the number of clinical cases was decreasing. Hence, the importance of wastewater analysis as an ongoing monitoring is emphasized, which can detect viral persistence even as clinical testing rates decline.

This study also showed the two-year monitoring project offered an opportunity to assess effectiveness of public health interventions in Cranfield University. By monitoring changes in virus concentrations, it is believed that the spike in virus concentrations in sewage in early 2022 was linked to England's policy of formally removing all domestic statutory COVID limits. Also, by the end of 2023 the decline of SARS-CoV-2 concentrations in wastewater represented the effectiveness of containment tactics and the impact of universal vaccination. The wastewater surveillance, offering data of viral RNA changes for public health, could be harnessed to modify the response in pandemic and ensure the resources are used effectively.

In conclusion, this study demonstrates the efficacy of PEG precipitation as a method for concentrating SARS-CoV-2 from wastewater and highlights the broader public health value of WBE. By providing early warning signals, complementing clinical testing and offering continuous monitoring, WBE can play a critical role in addressing current and future public health challenges.

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## **Chapter 4: Development of a CRISPR/Cas12a-based fluorescent method for SARS-CoV-2 and variants**

### **Abstract**

In the SARS-CoV-2 pandemic, there was a clear and pressing need for the development of advanced diagnostic technologies for rapid and accurate detection of the virus and its variants. While conventional approaches, including RT-qPCR and sequencing technology, are feasible, they require substantial resources (e.g., facilities and well-trained personals), which makes them unsuitable for use in low-resource settings. This study explores the development of a CRISPR/Cas12a-based fluorescence assay for identifying SARS-CoV-2, with a particular focus on distinguishing between the Delta and Omicron variants. The design of specific guide RNAs allows the CRISPR/Cas system to target the specific sequence of SARS-CoV-2 and unique single-nucleotide polymorphisms characteristic of these variants. Integration with loop-mediated isothermal amplification (LAMP) technology further enhances the sensitivity of this innovative approach. The combination of precisely LAMP primers and guide RNAs facilitates the assay's ability to deliver rapid, sensitive, and cost-effective identification of the virus. Besides, the developed assay demonstrated high sensitivity and specificity, allowing for the differentiation of the original Wuhan sequence, the Delta variant, and the Omicron variant, and providing insights into the prevalence of variants. The developed assays are expected to support effective public health interventions and have the potential to be widely applicable in both clinical and environmental monitoring settings.

**Keywords:** CRISPR/Cas12a diagnostics; SARS-CoV-2; Variants detection; Delta; Omicron

## 4.1 Introduction

The global pandemic has highlighted the critical necessity for the development of rapid, sensitive, and specific diagnostic tools capable of detecting SARS-CoV-2, the virus responsible for the disease (Ghinai *et al.*, 2020). Conventional diagnostic techniques, such as reverse transcription polymerase chain reaction (RT-qPCR), have been instrumental in containing the outbreak, offering precise results (Dutta *et al.*, 2022). However, these methods often necessitate the use of sophisticated equipment, the acquisition of specialized training, and the availability of centralised laboratory settings, which can restrict their accessibility, particularly in resource-limited environments (Ahmed *et al.*, 2022). This limitation has accelerated the investigation of alternative diagnostic techniques that are not only effective but also adaptable to a range of contexts, including wastewater surveillance (Dinnes *et al.*, 2022).

The emergence of SARS-CoV-2 variants has presented substantial challenges for both the diagnosis of the virus and the implementation of public health management strategies. Variants of concern (VOCs) have demonstrated mutations that affect the virus's spike protein, which is the primary target of both neutralising antibodies and a significant number of diagnostic tests (Hirabara *et al.*, 2022). Such mutations can result in diagnostic escape, whereby conventional tests are unable to detect the virus or accurately identify the variants (Walensky, Walke and Fauci, 2021). Furthermore, the high mutation rate of SARS-CoV-2 requires continuous surveillance and the adaptation of diagnostic tools to ensure their continued efficacy in identifying new variants (Chen *et al.*, 2022).

In the initial phase of the SARS-CoV-2 pandemic, the majority of diagnostic endeavours concentrated on the identification of the virus itself, without differentiating between various strains (Tosta *et al.*, 2023). However, as the pandemic progressed and variants with distinct epidemiological and clinical profiles emerged, it became evident that variant-specific detection was necessary

(Fokam *et al.*, 2023). Conventional methods for variant identification, such as sequencing, are time-consuming, costly, and difficult for large-scale screening, particularly in settings where rapid results are required (Markov *et al.*, 2023). This has resulted in a gap in the diagnostic landscape, where there is a clear need for a rapid, reliable, and cost-effective method for variant identification.

Wastewater-based epidemiology (WBE) has emerged as a valuable tool for tracking the prevalence of SARS-CoV-2 within communities. It offers a non-invasive and cost-effective method for monitoring population-level infection trends, which is particularly beneficial in situations where there is a need for a rapid response (Amman *et al.*, 2022). Nevertheless, the successful implementation of WBE hinges on the capacity to detect and quantify viral RNA in complex environmental samples, where the target RNA may be present in low concentrations and is often degraded (Medema *et al.*, 2020). The detection of SARS-CoV-2 in wastewater is further complicated due to various inhibitors that can impair the efficiency of both the amplification and detection steps (Morvan *et al.*, 2022). In addition, the heterogeneity of wastewater samples, which contain a mixture of viral RNA from diverse sources and potentially distinct variants, necessitates a detection method that is not only sensitive but also capable of distinguishing between different strains of the virus (Cao *et al.*, 2022).

The CRISPR/Cas system, initially identified as a bacterial adaptive immune mechanism, comprises two principal components: the CRISPR-associated (Cas) proteins and the guide RNA (gRNA) (Nidhi *et al.*, 2021). In the context of viral detection, CRISPR/Cas12a has emerged as a particularly useful tool due to its distinctive mechanism of action (Aman, Mahas and Mahfouz, 2020). In contrast to the more widely documented Cas9, Cas12a exhibits both endonuclease activity for DNA cleavage and a collateral single-stranded DNA (ssDNA) cleavage activity that is initiated upon target recognition (Li *et al.*, 2022). The mechanism of a typical CRISPR/Cas12a system for nucleic acid detection is shown in the Figure 4.1.

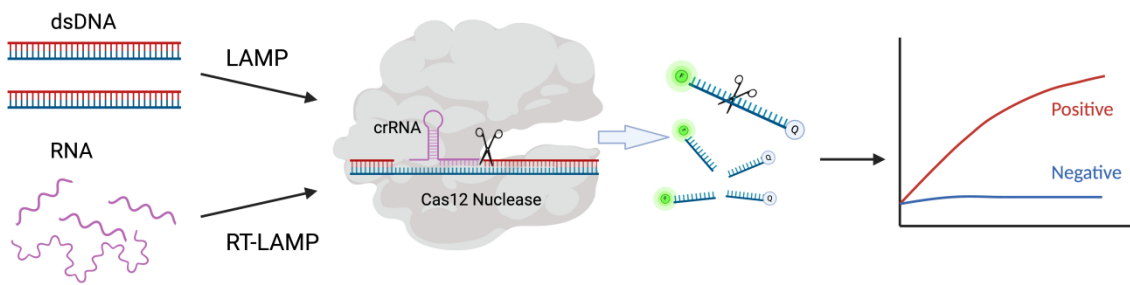


**Figure 4.1 The mechanism of CRISPR/Cas12a system for nucleic acid detection**

In a typical CRISPR/Cas12a-based assay, the target sequence is firstly amplified by an isothermal amplification approach, such as loop-mediated isothermal amplification (LAMP), to increase the initial concentration of the target (Broughton *et al.*, 2020). Then, the gRNA is designed to specifically bind to a target sequence within the viral genome. Upon recognition of the target sequence, the Cas12a protein is activated, leading to the cleavage of nearby single-stranded DNA (ssDNA) molecules. If the ssDNA molecule is labelled with a fluorescent dye and a quencher, this cleavage event generates a fluorescent signal that can be quantitatively measured to detect the target nucleic acid. The fluorescence-based readout offers several advantages of high sensitivity, and the potential for multiplexing, thereby rendering CRISPR/Cas12a a powerful tool for viral diagnostics (Chen *et al.*, 2022b).

The capacity of the CRISPR/Cas system to distinguish between sequences that differ by as little as a single nucleotide is of particular significance in the identification of single-nucleotide polymorphisms (SNPs), and SNPs can have significant implications for the virulence, transmissibility, and immune escape capabilities of viruses (Lia Gardenia Partakusuma *et al.*, 2023). VOCs, such as Delta and Omicron, have been associated with increased transmissibility and, in some cases, the resistance to neutralization by antibodies elicited by prior infection or vaccination (Combes *et al.*, 2022). Therefore, a diagnostic tool that

can not only detect SARS-CoV-2 but also differentiate between its variants based on SNPs is of immense value for both clinical diagnostics and wastewater epidemiological studies (Galani *et al.*, 2023). The specificity of the CRISPR/Cas system is largely determined by the design of the gRNA, and by modifying the gRNA sequence, it is possible to achieve selective binding to a target that harbours a specific SNP, while reducing non-specific binding to other sequences (Fasching *et al.*, 2022).

Despite the promise of CRISPR/Cas technology for clinical diagnostics, there is a lack of CRISPR/Cas-based methods specifically designed to detect the SARS-CoV-2 variants. In addition, the application of this technology in environmental surveillance, especially for monitoring pathogens in wastewater, remains at infant stage. If this technology can be used to track the spread of SARS-CoV-2 in the community in a more precise and timely manner, this would be a breakthrough in the field of WBE. In addition, the CRISPR/Cas assay will be able to distinguish between different mutated viruses and provide information about the prevailing strains in the community, which will help in outbreak control.

In view of the above challenges, this chapter aims to develop a CRISPR/Cas12a-based fluorescence assay for identifying SARS-CoV-2 in wastewater samples and detecting Delta and Omicron variants. This study aims to address the current limitations of applying CRISPR/Cas technology to environmental monitoring by developing an assay that is not only highly sensitive and specific, but also capable of variant identification in complex sample matrices. The proposed method will entail the development of a CRISPR/Cas12a fluorescent assay for the identification of SARS-CoV-2. The subsequent development will focus on the utilization of the high specificity of the CRISPR/Cas12 assay for the SNPs detection, distinguishing Wuhan, Delta and Omicron variants based on their characteristic mutations. Subsequent LAMP amplification optimization was performed with the aim of achieving a lower detection limit to enable the assay to be applied to WBE.

## 4.2 Materials and Methods

### 4.2.1 Target RNA generation

#### 4.2.1.1 Artificial gene sequences preparation

Artificial gene sequences for the N-gene of SARS-CoV-2(Wuhan, Delta, and Omicron variants) were purchased from Eurofins Scientific. These sequences are double-stranded DNA (dsDNA) and are supplied in a lyophilized form. To prepare a stock solution with a concentration of 1 nanogram (ng) per  $\mu\text{L}$ , the lyophilized DNA was reconstituted with an equal volume of nuclease-free  $\text{dH}_2\text{O}$  (e.g., 200 ng of DNA with 200  $\mu\text{L}$  of nuclease-free  $\text{dH}_2\text{O}$ ). The prepared solution was incubated at room temperature for 1 hour and then put into  $-4^\circ\text{C}$  fridge. For long-term storage, the DNA fragments were stored at  $-20^\circ\text{C}$  freezer. To transfer the concentration of genes from ng per  $\mu\text{L}$  to copies per  $\mu\text{L}$ , the equation below was used. Serial dilutions were then performed from the stock solution to achieve final concentrations at  $10^6$  copies  $\mu\text{L}^{-1}$ ,  $10^5$  copies  $\mu\text{L}^{-1}$ ,  $10^4$  copies  $\mu\text{L}^{-1}$ ,  $10^3$  copies  $\mu\text{L}^{-1}$ ,  $10^2$  copies  $\mu\text{L}^{-1}$ ,  $10^1$  copies  $\mu\text{L}^{-1}$ , and  $10^0$  copies  $\mu\text{L}^{-1}$ .

$$\text{Number of copies (molecules)} = \frac{x \text{ ng} \times 6.0221 \times 10^{23} \text{ molecules/mol}}{\left(1210 \times 660 \frac{\text{g}}{\text{mol}}\right) \times 1 \times 10^9 \text{ ng/g}}$$

#### 4.2.1.2 PCR method for N gene amplification

The PCR reaction mixture was prepared as follows: 10  $\mu\text{L}$  of Brilliant III Ultra-Fast QPCR Master Mix with Low ROX (Agilent Technologies), 4  $\mu\text{L}$  of Betaine (Sigma-Aldrich), 1  $\mu\text{L}$  of SYBR Green (Thermo Fisher Scientific, UK, UK), 2  $\mu\text{L}$  of nuclease-free  $\text{dH}_2\text{O}$ , 0.5  $\mu\text{L}$  of forward primer (N-Gene-F-T7), 0.5  $\mu\text{L}$  of reverse primer (N-Gene-R-T7), and 2  $\mu\text{L}$  of the artificial gene sequence (Wuhan, Delta, or Omicron). The information of forward primer and the reverse primer is given in the Table 4.1 In the case of internal negative controls, the artificial gene sequence should be replaced with 2  $\mu\text{L}$  of nuclease-free  $\text{dH}_2\text{O}$ .

**Table 4.1 Primers for N gene amplification before transcription**

| Primer Name | Sequence                                                 |
|-------------|----------------------------------------------------------|
| N-Gene-F-T7 | AATTCTAATACGACTCACTATAGGGCCAAATTGGCTACTA<br>CCGAAGAGCTAC |
| N-Gene-R-T7 | CACAGTTTGCTGTTTCTTCTGTCTCTGCGG                           |

The PCR amplification was performed using a QuantStudio 3 PCR Machine (Thermo Fisher Scientific, UK, UK). The specific PCR conditions are detailed in Table 4.2 below. Additionally, a melt curve analysis was conducted at the end of the PCR to assess the dissociation of the PCR product and probe by monitoring fluorescence from 65°C to 95°C at a rate of 1°C per second.

**Table 4.2 Conditions for N gene amplification before transcription**

| Step                 | Temperature | Duration   | Cycle |
|----------------------|-------------|------------|-------|
| Initial denaturation | 98          | 5 minutes  | 1     |
| Denaturation         | 98          | 15 seconds | 40    |
| Annealing            | 55          | 30 seconds | 40    |
| Extension            | 72          | 30 seconds | 40    |

#### **4.2.1.3 Electrophoresis and DNA separation**

To prepare a 2% agarose gel, 1 g of agarose (Thermo Fisher Scientific, UK) was weighed and mixed with 50 mL of 1xTAE buffer (Sigma-Aldrich). The solution was microwaved for 30 seconds to 1 minute, then gently swirled until the agarose was completely dissolved. After dissolution, 5 µL of Safe View Stain (Thermo Fisher Scientific, UK) was added and mixed thoroughly to ensure even distribution. The agarose solution was poured into the casting tray with the comb

inserted and allowed to solidify for approximately 40 minutes. The gel became opaque upon solidification.

The solidified gel was placed in the gel tank (Bio-Rad) with the wells oriented towards the negative electrode. The 1x TAE buffer was poured into the gel tank up to the maximum fill line, ensuring the gel was fully submerged. Each 20  $\mu$ L sample was prepared by adding 5  $\mu$ L of loading buffer (Thermo Fisher Scientific, UK), and each mixture was loaded into individual wells. 20  $\mu$ L of the DNA ladder (Thermo Fisher Scientific, UK) was loaded into the first well for reference. Electrophoresis was run at 90 volts (V) for 50 minutes. Upon completion, the gel was visualized using GeneSnap Software (SynGene, 2008). The target bands were identified, excised from the gel using a scalpel, and placed into microcentrifuge tubes for further separation.

To extract the target of interest, the Zymoclean Gel DNA Recovery Kit was employed. Three volumes of ADB were added to a microcentrifuge tube containing the agarose gel (e.g., 300  $\mu$ L ADB for 100  $\mu$ L agarose gel). The mixture was incubated at 55°C for 10-30 minutes until the agarose was fully melted. The melted agarose solution was then transferred to the spin column in the collection tube and centrifuged at 16,000 g for 1 minute at room temperature. The flow-through was discarded, and 200  $\mu$ L of Wash Buffer was added to the collection column. The column was centrifuged again at the same speed for 30 seconds. Subsequently, the column was transferred to an RNase-free microcentrifuge tube, and 20  $\mu$ L of the elution Buffer was added. The sample was centrifuged at 16,000 g for 1 minute at room temperature, and the ultrapure DNA was collected in the microcentrifuge tube and ready for transcription.

#### **4.2.1.4 RNA Transcription and purification**

To transcribe the artificial gene sequences (dsDNA) into RNA, the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) was employed. The reaction mix was prepared by combining 2  $\mu$ L of ATP (100 mM), 2  $\mu$ L of GTP

(100 mM), 2  $\mu$ L of UTP (100 mM), 2  $\mu$ L of CTP (100 mM), 2  $\mu$ L of T7 Polymerase mix, 2  $\mu$ L of 10x reaction buffer, 5  $\mu$ L of dH<sub>2</sub>O, and 3  $\mu$ L of the DNA template. The solution was thoroughly mixed by vortexing and then incubated at 37°C for 24 hours to achieve high RNA concentration.

The transcribed RNA was purified using the Monarch RNA Cleanup Kit (New England Biolabs). A starting volume of 50  $\mu$ L of the RNA sample was adjusted with nuclease-free water if necessary. To this sample, 100  $\mu$ L of RNA Binding Buffer and 150  $\mu$ L of >95% ethanol was added. The sample was mixed by pipetting or flicking rather than vortexing. The mixture was then loaded onto the column and centrifuged at room temperature for 1 minute at 16,000 g. The flow-through was discarded, and the column was transferred to a new collection tube. Subsequently, 500  $\mu$ L of RNA washing buffer was added, and centrifugation was repeated under the same conditions. The washing step and centrifugation were performed twice more. The column was then transferred to a new RNase-free microcentrifuge tube, and the RNA was eluted with 20  $\mu$ L of nuclease-free water by centrifugation at room temperature for 1 minute at 16,000 g. The RNA in supernatant could be used immediately or stored at -20°C for future use.

#### **4.2.1.5 DNA/RNA concentration measurement**

The DNA and RNA quantification was performed using the Qubit 4 Fluorometer (Thermo Fisher Scientific, UK). This instrument features a built-in reagent calculator to determine the necessary amounts of dye and buffer for sample and standard preparation. Specific assays were obtained from Thermo Fisher Scientific, UK. The High Sensitivity double-stranded DNA assay, which has a quantification range of 0.1 to 120 ng, was used for DNA quantification. For RNA measurement, the High Sensitivity RNA assay, with a quantification range of 5 to 1000 ng, was employed. The quantification process began with the calibration of two standards: one for low concentration and one for high concentration. After the machine generated the standard curve, the prepared

sample solutions were analyzed. The Qubit 4 Fluorometer provided the quantification results directly on its screen.

## 4.2.2 Optimization of the CRISPR/Cas assay for SARS-CoV-2 detection

### 4.2.2.1 LAMP assay for N gene amplification

To amplify the target DNA/RNA, LAMP/RT-LAMP amplifications were conducted prior to the CRISPR/Cas assay. Six primers were designed for each LAMP assay, as detailed in Table 4.3, which includes two primer sets. One set amplified the N1 gene of SARS-CoV-2, while the other set targeted the N3 gene. The WarmStart® Fluorescent LAMP/RT-LAMP Kit (with UDG) was used for the amplification. Each reaction was prepared as follows: 12.5  $\mu$ L of 2X Master Mix, 2.5  $\mu$ L of primer mix, 2  $\mu$ L of target DNA/RNA, and 8  $\mu$ L of dH<sub>2</sub>O.

To prepare the primer mix solution, each primer was first diluted to 20  $\mu$ M. The primers F3, B3, FIP, BIP, LF, and LB were then mixed in a ratio of 1:1:8:8:2:2. After preparing the reagents, the LAMP assay was incubated at 65°C for 40 minutes using the QuantStudio 3 PCR Machine.

**Table 4.3 The list of LAMP primers for N gene amplification**

| <b>Primer name</b> | <b>Sequence</b>                           |
|--------------------|-------------------------------------------|
| N1-F3              | TGGCTACTACCGAAGAGCT                       |
| N1-B3              | TGCAGCATTGTTAGCAGGAT                      |
| N1-FIP             | TCTGGCCCAGTTCCTAGGTAGTGACGAATTCGTGGTGGTGA |
| N1-BIP             | AGACGGCATCATATGGGTTGCAGCGGGTGCCAATGTGATC  |
| N1-LF              | GGACTGAGATCTTTCATTTTACCGT                 |
| N1-LB              | ACTGAGGGAGCCTTGAATACA                     |
| N3-F3              | AACACAAGCTTTCGGCAG                        |

---

|        |                                          |
|--------|------------------------------------------|
| N3-B3  | GAAATTTGGATCTTTGTCATCC                   |
| N3-FIP | TGCGGCCAATGTTTGTAATCAGCCAAGGAAATTTGGGGAC |
| N3-BIP | GCATTGGCATGGAAGTCACTTGATGGCACCTGTGTAG    |
| N3-LF  | TTCCTTGTCTGATTAGTTC                      |
| N3-LB  | CCTCGGGAACGTGGT                          |

---

#### 4.2.2.2 The development of the CRISPR/Cas assay

To perform the CRISPR/Cas assay, the EnGen® Lba Cas12a (Cpf1) (New England Biolabs, UK) was used as the programmable DNA endonuclease, and the guideRNA-Reference1 (gRNA-R1) and guideRNA-Reference2 (gRNA-R2) were utilized as the reference gRNA (Broughton *et al.*, 2020). The Cpf1 product was supplied with 1X NEBuffer™ r2.1, containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, and 100 µg/ml recombinant albumin. For fluorescent detection, fluorescein amidites (FAM) was employed as the cleavage reporter, with detailed information provided in Table 4.4. Each reaction began with a 30-minute incubation of the following mixture: 1 µL of Cpf1 (1 µM), 1 µL of gRNA (1 µM), 1 µL of FAM reporter (10 µM), 2 µL of NEBuffer, and 5 µL of nuclease-free water. After incubation, the 10 µL CRISPR mixture was combined with 10 µL of LAMP amplicons and incubated at 37°C for 30 minutes using the QuantStudio 3 PCR Machine (Thermo Fisher), and the schematic figure of the complete assay is shown in the Figure 4.2.

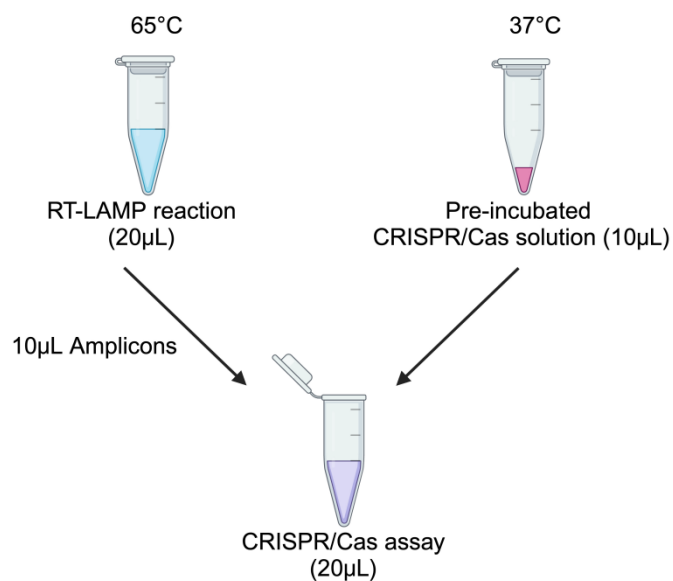


**Figure 4.2 The illustration of the CRISPR/Cas assay**

**Table 4.4 The list of components of the CRISPR/Cas assay**

| Component    | Sequence                                      |
|--------------|-----------------------------------------------|
| gRNA-R1      | UAAUUUCUACUAAGUGUAGAUCCCCCAGCGCUUCAGC<br>GUUC |
| gRNA-R2      | UAAUUUCUACUAAGUGUAGAUUGCAAUGUUGUCCUUG<br>AGGA |
| FAM reporter | /56-FAM/TTATTATT/3BHQ_1/                      |

#### 4.2.2.3 The design of the new gRNAs for N1 gene detection

The reference gRNA targeted the N3 gene of the SARS-CoV-2 (Wuhan sequence), and new gRNAs were designed and generated for N1 gene identification, as shown in the Figure 4.3. Based on the SARS-CoV-2 reference genome (GenBank accession no. MN908947), the nucleotide positions of N1 and N3 genes were 28,303 to 28,351 and 28,679 to 28,748 respectively.

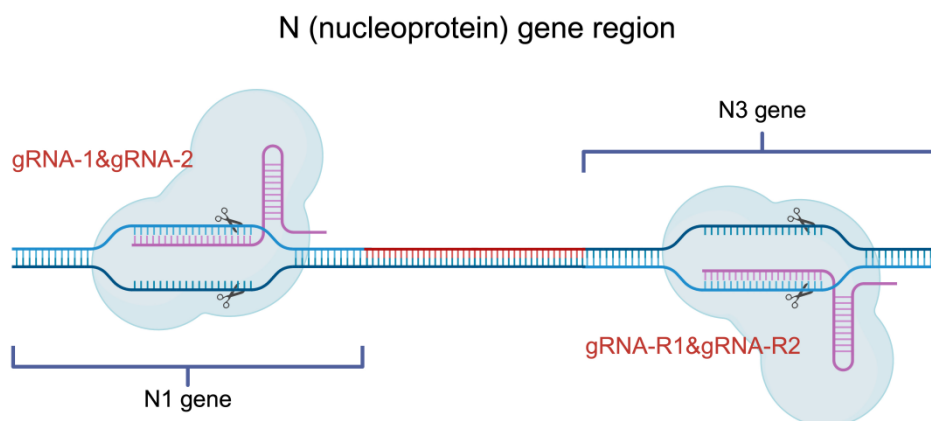


**Figure 4.3 The design of gRNAs for N1 gene**

The initial step for gRNA design involved locating the protospacer adjacent motif (PAM) sequence on the target gene, which is 5' TTTV in the CRISPR/Cas12a system. The target region was the 23-base DNA sequence downstream of the PAM, and the RNA sequence for identification was the corresponding 23-nucleotide sequence. To complete the gRNA, the crRNA sequence specific to the CRISPR/Cas12a system, UAAUUUCUACUAAGUGUAGAU, was added to the beginning of the 23-nucleotide sequence. After determining the gRNA, it was essential to validate its specificity to prevent off-target effects. Two gRNAs targeting the N1 gene were designed and generated, which were gRNA-1 and gRNA-2. These gRNAs were ordered from Eurofins and delivered in lyophilized form. All gRNAs were centrifuged and re-suspended in nuclease-free H<sub>2</sub>O and then be prepared in the stock solution of 100nM.

Figure 4.4 is an example showing what the DNA sequence for the gRNA-1 should look like with the PAM underlined, and the specific information of gRNA-1 and gRNA-2 is exhibited in the Table 4.5.

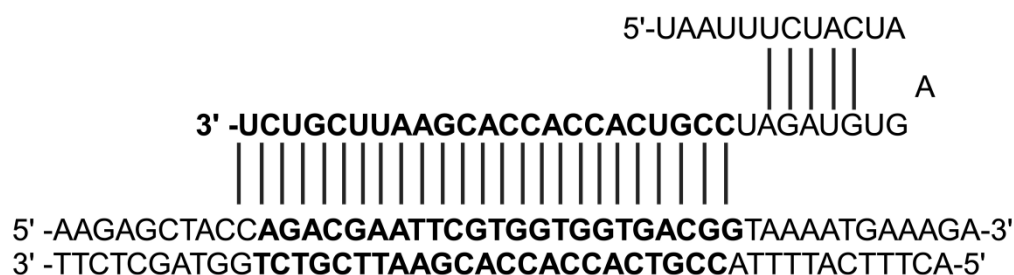


**Figure 4.4 The example of designing gRNA-1**

**Table 4.5 The information of gRNAs targeting N1 gene**

| Name   | Sequence                                        |
|--------|-------------------------------------------------|
| gRNA-1 | UAAUUUCUACUAAGUGUAGAUCGUCACCACCACGAAUUCG<br>UGU |
| gRNA-2 | UAAUUUCUACUAAGUGUAGAUCGUCACCACCACGAAUUCG<br>UGU |

#### 4.2.2.4 The validation of the gRNA-1 and gRNA-2

The designed gRNAs were tested in vitro using a CRISPR/Cas12 assay. This involved incorporating the crRNA with the Cas12a enzyme and the target DNA to confirm efficient and specific identification of the N1 gene. Validation began with the artificial Wuhan N gene sequence, followed by using transcribed RNA of the Wuhan N gene as the template. For the DNA validation test, 10  $\mu\text{L}$  of a  $10^8$  copies  $\mu\text{L}^{-1}$  stock solution was used as the template, mixed with 10  $\mu\text{L}$  of pre-incubated CRISPR/Cas solution. The final mixture was incubated at 37°C for 30 minutes using the QuantStudio 3 PCR Machine. For RNA validation, the RT-LAMP method described in section 2.2.1 of this chapter was used, with the N1

primer set for amplification. Following amplification, the CRISPR/Cas assay was performed as detailed in section 2.2.2 of this chapter.

#### **4.2.2.5 The optimization of the component of the CRISPR/Cas assay**

To improve the efficiency of the CRISPR/Cas assay, the optimal concentration of several key components was investigated, including the Cpf1 enzyme, the gRNA and the FAM reporter. Besides, the volume of the RT-LAMP amplicons to include in the final mixture was explored. The concentration of the Cpf1 enzyme was tested in the range of 20 to 140nM. The concentration of the gRNA was determined based on the optimal concentration of the Cpf1 enzyme, maintaining a studied ratio of Cpf1 to gRNA. As the ratio of Cpf1 to gRNA was 1:0.5, 1:1, 1:1.5 and 1:2, the concentration of gRNA was 25, 50, 75 and 100nM. The concentration of FAM reporter was investigated from 50 to 1000 nM. The volume of the RT-LAMP amplicons was studied from 20 to 10 $\mu$ L, since the volume of original RT-LAMP was 20 $\mu$ L, and the volume of the pre-mixed CRISPR/Cas solution was 10 $\mu$ L.

#### **4.2.2.6 The sensitivity test of the gRNA-1 and gRNA-2**

To evaluate the analytical sensitivity of gRNA-1 and gRNA-2, stock RNA solutions were used as the templates in a series of dilution tests. The template concentrations ranged from  $5 \times 10^5$  copies  $\mu\text{L}^{-1}$  to  $5 \times 10^0$  copies  $\mu\text{L}^{-1}$ , with each concentration being a tenfold dilution of the previous one. The sensitivity test started with the RT-LAMP amplification and followed by CRISPR/Cas assay. Additionally, controls were included to verify the integrity of the entire reaction process. These controls consisted of a non-template control using nuclease-free water as the input, which served to check for any contamination or non-specific amplification. The positive control was also included using gRNA-R1 to confirm the assay's functionality. The sensitivity test aimed to determine the lowest detectable concentration of the target RNA by gRNA-1 and gRNA-2.

#### **4.2.2.7 The specificity test of the gRNA-1 and gRNA-2**

For specificity evaluation, three other influenza viruses were tested with gRNA-1 and gRNA-2: influenza B (Flu B), influenza A virus subtype H7N9 (H7N9), and swine-origin influenza A virus (H1N1). Only the N genes of these viruses were used, with the detailed gene sequences provided in the Appendix. Each viral N gene was prepared in a DNA solution at a concentration of  $5 \times 10^7$  copies  $\mu\text{L}^{-1}$ , and each virus was tested in triplicate. This setup aimed to assess the specificity of gRNA-1 and gRNA-2 in distinguishing the SARS-CoV-2 N gene from those of other influenza viruses.

#### **4.2.3 Design and characterization of CRISPR/Cas assay for SARS-CoV-2 and its variants detection**

##### **4.2.3.1 The sequence analysis between Wuhan, Delta and Omicron**

Whole genome sequences of SARS-CoV-2 were sourced from repositories such as the National Center for Biotechnology Information (NCBI) and the Global Initiative on Sharing Avian Influenza Data (GISAID). The sequences obtained were complete genomes with high coverage, confirmed as the variants of interest. To identify single nucleotide polymorphisms (SNPs), these sequences were aligned against the original SARS-CoV-2 Wuhan reference sequence (NC\_045512.2, TaxID: 2697049) using the NCBI BlastN tool. Comparisons were made among 10 or more sequences for each variant to identify recurring SNPs, distinguishing them from unique mutations. The focus of this project is the N gene, which spans from position 28274 to 29533 in the Wuhan reference sequence. It is noted that the start and stop positions for specific variants may vary due to mutations or deletions, with the N gene generally comprising approximately 1200 base pairs (bp). The specific information of sequences, involving Wuhan, Delta and Omicron, is provided in the Appendix.

#### 4.2.3.2 The design of gRNAs for delta and omicron variants

Mutations on the N gene were identified through bioinformatic analysis by comparing the Wuhan reference sequence with VOCs. Once a mutation was identified, the next step was to determine whether a PAM sequence was located near the mutation (within 1-7 bases) or directly on it. Mutations relevant to the PAM sequence were considered potential sites for gRNA design. gRNAs were then designed based on these potential sites following the method described in section 2.2.4 of this chapter.

For the Delta variant, two gRNAs were designed, while one gRNA was developed for Omicron identification. These gRNAs were ordered from Eurofins, with each gRNA intended to specifically identify the presence of its corresponding VOCs without cross-reacting with other sequences. Detailed information about the designed gRNAs is provided in Table 4.6, and the sequence matching the target are highlighted with underline

**Table 4.6 The list of gRNAs for VOCs identification**

| Name            | Sequence                                                        |
|-----------------|-----------------------------------------------------------------|
| gRNA-Delta1(D1) | UAAUUUCUACUAAGUGUAGAU <u>AGGCCUCCUUGCCA</u><br><u>UGUUGAGU</u>  |
| gRNA-Delta2(D2) | UAAUUUCUACUAAGUGUAGAU <u>AUAAGCCUUCUUCUU</u><br><u>UUUGUCCU</u> |
| gRNA-Omicron(O) | UAAUUUCUACUAAGUGUAGAU <u>CUGCUGCCUGGAGUU</u><br><u>GAAUUUGU</u> |

#### 4.2.3.3 The validation of the gRNAs for VOCs discrimination

The designed gRNAs were validated using previously provided DNA stock solutions of the Wuhan, Delta, and Omicron variants. For the DNA validation test,

a high-concentration stock solution containing  $10^7$  copies  $\mu\text{L}^{-1}$  was prepared. To conduct the assay, 10  $\mu\text{L}$  of this stock solution was used as the template and mixed with 10  $\mu\text{L}$  of a pre-incubated CRISPR/Cas solution. The final mixture was incubated at 37°C for 30 minutes using the QuantStudio 3 PCR Machine. Each gRNA was tested separately with DNA from the three variants in triplicate to ensure accuracy and reproducibility. This validation aimed to confirm the specificity and efficiency of the gRNAs in detecting their respective variants.

#### 4.2.3.4 The LAMP primer design for variants detection

Since the LAMP amplification was necessary prior to the CRISPR/Cas assay, the LAMP primers were designed for each VOC. LAMP primers were designed using Primer Explorer V5 and specificity checks were carried out using Primer Blast (NCBI). The LAMP primers should be designed around the mutation, with inner primers (FIP and BIP) incorporating the mutation site to ensure it is included in the amplified product. The outer primers (F3 and B3) define the boundaries of the amplified region, and loop primers can enhance the reaction efficiency. The Figure 4.5 shows how these primers align relative to the mutation and PAM sequence, guiding their design to target the mutation effectively. Three primer sets for Omicron variant amplification and two primer sets for Delta variant amplification were designed respectively, and the specific information is given in the Appendix.

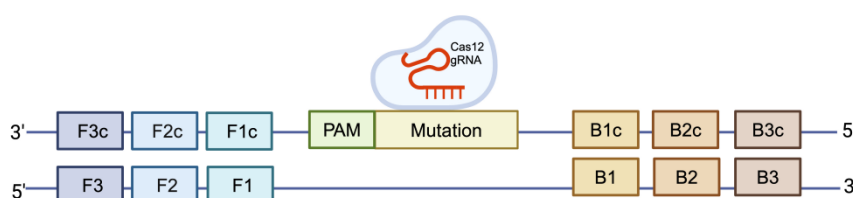


**Figure 4.5 Illustration of the mutation site and the location of the F3 and B3 primers used for LAMP amplification**

#### **4.2.3.5 The validation of designed LAMP primers with CRISPR/Cas assay for VOCs identification**

To validate the effectiveness of the LAMP primers in amplifying the N gene, transcribed RNA templates from the Wuhan, Delta, and Omicron variants were used at a concentration of  $10^5$  copies  $\mu\text{L}^{-1}$ . Each primer set was employed to amplify these three RNA templates. Following the amplification, a CRISPR/Cas assay was carried out to specifically identify the variants using gRNA-D2 for the Delta variant and gRNA-O for the Omicron variant. The validation tests were performed in triplicate to ensure reproducibility and accuracy. The RT-LAMP procedure was conducted as described in section 2.2.1 of this chapter, guaranteeing consistency in amplification conditions. Subsequently, the CRISPR/Cas assay was performed according to the protocol detailed in section 2.2.2 of this chapter, which includes preparing the CRISPR/Cas reaction mixture, incubation, and detection.

#### **4.2.3.6 The sensitivity test of the gRNA-D2 and gRNA-O**

To assess the analytical sensitivity of gRNA-D2 and gRNA-O, stock RNA solutions were utilized as templates in a series of dilution tests. Template concentrations ranged from  $5 \times 10^5$  copies  $\mu\text{L}^{-1}$  to  $5 \times 10^0$  copies  $\mu\text{L}^{-1}$ , with each concentration representing a tenfold reduction from the previous one. Each dilution was tested in triplicate to ensure precision and reliability.

The sensitivity evaluation began with RT-LAMP amplification, followed by the CRISPR/Cas assay. To ensure the integrity of the entire reaction process, controls were incorporated. These included a non-template control, using nuclease-free water as the input, to detect any contamination or non-specific amplification. Additionally, a positive control using gRNA-R1 was included to validate the functionality of the assay. The primary aim of the sensitivity test was to identify the lowest detectable concentration of target RNA by gRNA-D2 and gRNA-O.

## 4.3 Results and Discussion

### 4.3.1 The development and optimization of the CRISPR/Cas assay with new gRNAs for N gene detection

Wastewater analysis has become a critical tool in monitoring the spread of SARS-CoV-2, following a pattern similar to clinical testing but adapted for environmental samples. The nucleic acids released into wastewater from SARS-CoV-2 are RNA sequences/fragments, and specific genes of the SARS-CoV-2 virus are targeted in both wastewater analyzes and clinical assays to ensure accurate identification (Kasteren *et al.*, 2020). The major genes included in these assays are typically the N gene (encoding the nucleocapsid protein), the E gene (encoding the envelope protein), and the ORF1ab gene (encoding the large multimeric protein essential for viral replication) (Hu *et al.*, 2020). These genes are chosen because they are highly conserved in the SARS-CoV-2 strain and are reliable markers for the virus determination. In some cases, other genes, such as the S gene, which encodes a spike protein, may be included to confirm the presence of the virus and to study specific mutant strains (Reis *et al.*, 2024).

Previous microfluidic device for on-site wastewater surveillance in COVID-19 outbreak investigated the ORF1ab, S and N genes from wastewater, and it suggested that the ORF1ab gene in wastewater was detected less efficiently than the S and N genes (Pan *et al.*, 2024). The main reason for this result is that the N gene was the predominantly positive fragment in patients infected with SARS-CoV-2, ORF1ab gene presented a lower percentage of positives (Donia *et al.*, 2022). Based on observations of the dynamics of the ORF1ab and N genes in patients, the N gene has a longer duration of positivity than ORF1ab. Additionally, with the presence of new variants of SARS-CoV-2, detection of the N gene in wastewater is more stable than for the ORF1ab gene because there is less nucleotide variation in the N gene (Camila Daiane Silva *et al.*, 2022). The higher abundance of subgenomic N gene messenger RNAs also results in the N gene

being more sensitive than the ORF1ab and S genes. Given the critical role of the N gene in the accurate detection of SARS-CoV-2, designing a CRISPR/Cas assay targeting this gene for wastewater analysis presents a promising approach.

Regarding N gene detection, the US CDC 2019-Novel Coronavirus Real-time RT-PCR Diagnostic Panel was the first test to receive US Food & Drug Administration (FDA) - Emergency Use Authorization (EUA) for SARS-CoV-2 detection in the US, which was distributed and released in February 2020. Originally, this panel included three tests (N1, N2, and N3) that each targeted a different region of the N gene (Lu *et al.*, 2020). One of the first N gene segments that was addressed in diagnostic assays was the N1 region. It is usually found close to the 5' end of the N gene and is highly conserved. The purpose of assays focused on the N1 region is to identify this particular sequence, which is essential for verifying the existence of the virus (Wang *et al.*, 2022). In order to provide a second confirmation of the virus and reduce the likelihood of false negative findings, assays that target the N2 region, an additional section found in the middle of the N gene, are commonly used in combination with N1. The last section of the N gene, known as the N3 region, is usually found downstream. Not all diagnostic tests include the N3 target, but when they do, it serves as an extra confirmation marker for wastewater analysis. (Navarro *et al.*, 2021). The incorporation of the N3 region facilitates the assurance that even minor alterations to the viral genome do not affect the detection process. The first study employing CRISPR/Cas assay for SARS-CoV-2 identification was for clinical settings, and the designed gRNAs was for N3 gene targeting (Broughton *et al.*, 2020). Therefore, this study aimed to design new gRNAs for N1 gene identification, and two gRNAs were designed and generated, naming gRNA-1 and gRNA-2 respectively.

To validate the feasibility of gRNA-1 and gRNA-2 for CRISPR/Cas assay, the dsDNA of the N gene of SARS-CoV-2 was used as the template at first, and the results are shown in the Figure 4.6 (a). The CRISPR/Cas12a system uses gRNAs that were specifically designed to be complementary to a target sequence within the nucleic acid of interest, guiding the Cas12a protein to the target DNA sequence. Once the Cas12a protein was guided by the gRNA-1 and gRNA-2 to the complementary target N1 gene, it bound to this DNA, activating its collateral cleavage activity and generating the fluorescence signal to be detected.

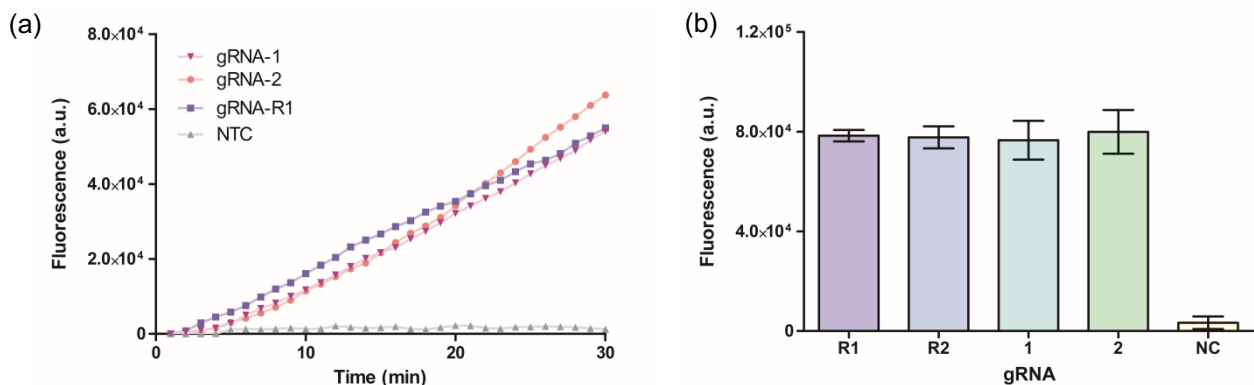


**Figure 4.6 The CRISPR/Cas12 assay for N gene detection with newly designed gRNA-1 and gRNA-2; (a) DNA template ( $10^8$  copies  $\mu\text{L}^{-1}$ ) validation (b) RNA template ( $10^5$  copies  $\mu\text{L}^{-1}$ ) validation; Results represent the mean  $\pm$  SD of three independent replicates.**

The graph captures the fluorescent signals indicative of the activity of the CRISPR/Cas12a system as a function of time, with the fluorescence intensity of the three gRNAs, measured in arbitrary units (a.u.), increasing over a 30-minute period. The results depicted in the figure demonstrate that both gRNA-1 and gRNA-2 successfully recognize and bind to the N1 gene, as evidenced by the increasing fluorescence signals. The performance of these newly designed gRNAs is comparable to that of gRNA-R1, a positive control known for its effective targeting of the N3 region (Broughton *et al.*, 2020). Notably, the

fluorescence signals produced by gRNA-1 and gRNA-2 are similar in magnitude to that of gRNA-R1, with gRNA-2 generating a slightly stronger signal over the same detection period. The inclusion of a no-template control (NTC) further validates the specificity of the assay. The NTC, which lacks the target DNA sequence, exhibits minimal fluorescence, indicating that the observed signals for gRNA-1, gRNA-2, and gRNA-R1 are indeed a result of specific target recognition of the Cas12a enzyme.

The Figure 4.6 (b) illustrates the comparative performance of several gRNAs in detecting SARS-CoV-2 RNA through a CRISPR/Cas12a-based assay following amplification via RT-LAMP. Given that SARS-CoV-2 is an RNA virus, the detection target within this diagnostic approach must first be converted into complementary DNA (cDNA). This conversion is achieved through RT-LAMP, an efficient method for amplifying specific RNA sequences into DNA, which can then be recognized and cleaved by the CRISPR/Cas12a system.

In this experiment, different sets of LAMP primers were employed to amplify distinct N1 and N3 regions of the SARS-CoV-2 N gene (Broughton *et al.*, 2020; Pan *et al.*, 2024). Following amplification, the generated DNA was transferred to the CRISPR/Cas12a assay using newly designed gRNA-1, gRNA-2, and positive controls gRNA-R1, and gRNA-R2. The bar chart in the figure displays the fluorescence signal intensities generated by the CRISPR/Cas12a system, which correlate with the efficiency of target recognition and subsequent collateral cleavage of the reporter molecule. The fluorescence signals are indicative of successful target binding and Cas12a activation, with higher fluorescence reflecting more efficient detection. As shown, all four gRNAs, involving gRNA-1, gRNA-2, gRNA-R1, and gRNA-R2, produced robust fluorescence signals, suggesting that gRNA-1 and gRNA-2 are comparable in effectiveness to the positive controls. This demonstrates that the RT-LAMP amplified cDNA, derived from the SARS-CoV-2 RNA, was successfully detected by the CRISPR/Cas12a assay.

In conclusion, the work demonstrates the successful design and validation of new gRNAs (gRNA-1 and gRNA-2) for use in a CRISPR/Cas12a-based assay targeting the N1 gene of SARS-CoV-2. The newly developed gRNAs showed robust performance comparable to that of the positive control, gRNA-R1, in recognizing and binding the target sequence, as evidenced by the strong fluorescence signals generated. The integration of RT-LAMP for initial RNA amplification further validated the assay's effectiveness in detecting SARS-CoV-2 RNA, confirming that the CRISPR/Cas12a system can reliably identify the amplified cDNA.

The efficiency of the CRISPR/Cas12a assay for the detection of the SARS-CoV-2 N gene was optimized by systematically varying key components, including the concentration of the Cas12a enzyme, gRNA, and the FAM reporter molecule. Also, the volume of the RT-LAMP amplicon adding to the CRISPR/Cas assay plays an important role in analytical performance. The results of these optimizations are depicted in Figure 4.7, each demonstrating the effect of different concentrations/volume on the fluorescence signal.

The concentration of Lba Cas12a enzyme was varied from 20 nM to 150 nM to determine its effect on the assay performance. The results are shown in Figure 4.7 (a), where the fluorescence signal increases significantly with increasing Cas12a concentration, reaching an optimal level at 50 nM. At this concentration, the fluorescence signal peaks, indicating that 50 nM Cas12a provides an optimal balance between sensitivity and background noise. Beyond 50 nM, further increases in Cas12a concentration did not enhance the signal, but instead resulted in a slight decrease in fluorescence, which may be due to saturation of the system or increased non-specific activity (Nguyen, Smith and Jain, 2020).

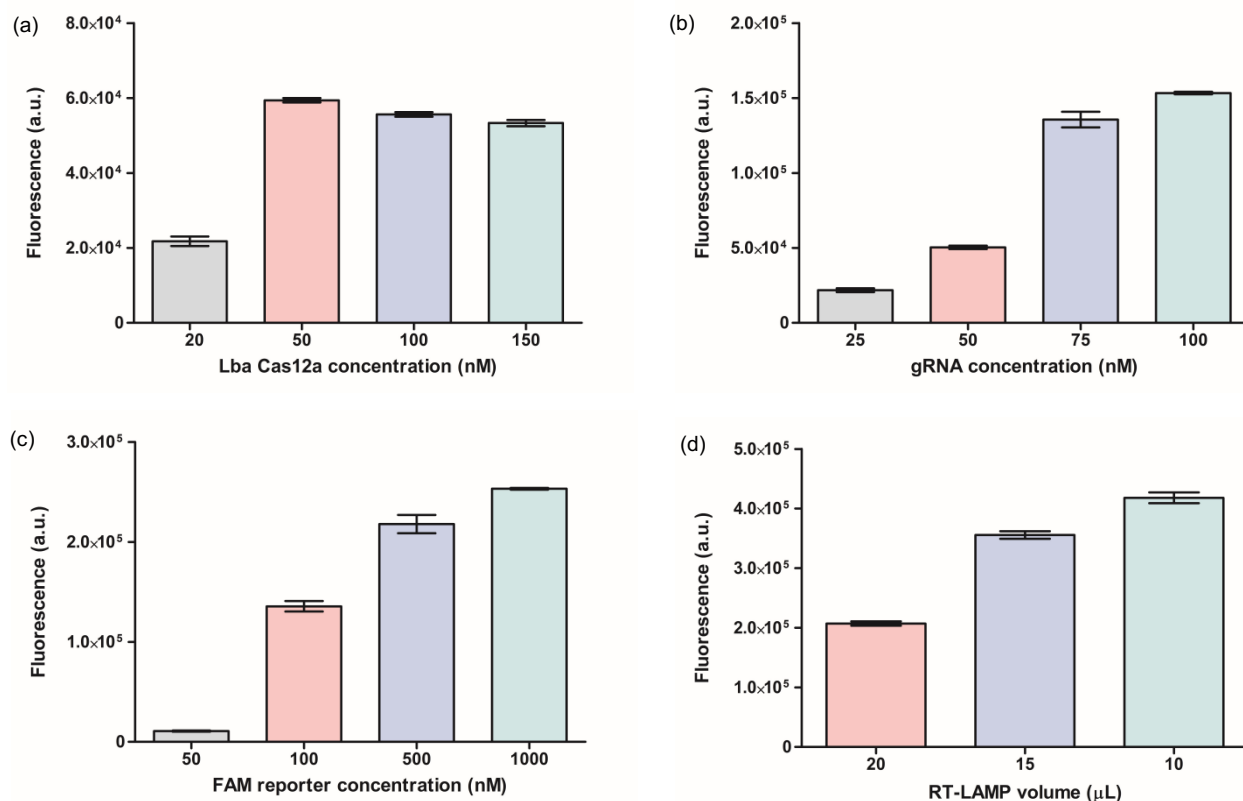


**Figure 4.7 The optimization of CRISPR/Cas assay; (a) Cas12a concentration (b) gRNA concentration (c) FAM reporter concentration (d) RT-LAMP volume; Results represent the mean  $\pm$  SD of three independent replicates.**

As shown in Figure 4.7 (b), the concentration of gRNA also plays a key role in the detection, with experiments focusing on gRNA concentrations ranging from 25 nM to 100 nM. The fluorescence intensity increased dramatically with increasing gRNA concentration, especially between 50 nM and 75 nM. At 75 nM and 100 nM, there was no significant enhancement of the fluorescence signal, suggesting that gRNA is sufficiently effective in recognizing the target and activating Cas12a when the concentration reaches 75 nM. Considering the cost of the assay, the optimal concentration of gRNA to ensure adequate target recognition and Cas12a activation was 75nM.

Similarly, the optimization of the FAM reporter concentration underscores the importance of this component in signal generation. The concentration of the FAM reporter molecule, was optimized by testing concentrations between 50 nM and 1000 nM, as shown in Figure 4.7 (c). The fluorescence signal continued to increase as the concentration of the reporter was increased, with the greatest increase occurring from 100 nM to 500 nM. The results indicate that increasing the concentration of the FAM reporter to 1000 nM resulted in enhanced signals, but because the 500 nM concentration was more cost-effective, the 500 nM concentration was chosen for the following assay. If the CRISPR/Cas assay is used for on-site testing, higher concentrations of FAM reporters will be considered to ensure the highest sensitivity of the assay and visualisation of the results (Bai *et al.*, 2019). Currently, the assay results are interpreted by the PCR instrument and a concentration of 500 nM produces enough reporters upon Cas12a activation.

The volume of the RT-LAMP with CRISPR/Cas assay is a critical factor in the assay process, as it directly influences the efficiency, sensitivity, and specificity of the assay. This refers to the quantity of the RT-LAMP amplification product that is employed in the mixture with the CRISPR/Cas system. A weak or undetectable signal may be attributed to the insufficient availability of target DNA for the CRISPR/Cas complex to effectively detect and cleave (Atçeken *et al.*, 2022). This is because the RT-LAMP reaction produces DNA that is the target DNA of the CRISPR/Cas system. For the CRISPR/Cas system to function optimally, a certain concentration of Cas12 and gRNA is required. If the volume of the RT-LAMP product is excessive in comparison to the CRISPR/Cas reaction mixture, the concentration of these essential components may be insufficient, thereby impairing their capacity to effectively detect and cleave the target DNA. Furthermore, an optimal volume ratio facilitates the prevention of the CRISPR/Cas system being overburdened by an excess of DNA, which can result

in non-specific cleavage and, consequently, the minimization of background noise.

The Figure 4.7 (d) demonstrates the effect of varying the RT-LAMP product volume on the efficiency of the CRISPR/Cas12a-based assay, as measured by fluorescence intensity. Three different volumes of RT-LAMP product—20  $\mu$ L, 15  $\mu$ L, and 10  $\mu$ L—were tested to identify the optimal volume ratio for maximum fluorescence signal, and this is because the original volume of RT-LAMP and CRISPR/Cas assay was 20 $\mu$ L and 10 $\mu$ L respectively. The results indicate a clear inverse relationship between the volume of RT-LAMP product used and the resulting fluorescence signal. Specifically, the highest fluorescence intensity was observed when 10  $\mu$ L of the RT-LAMP product was used in the CRISPR/Cas12a reaction. The use of 20  $\mu$ L of RT-LAMP product yielded the lowest fluorescence intensity, suggesting that this volume might dilute the CRISPR/Cas12a components too much, thereby reducing the efficiency of the detection reaction.

In summary, the optimizations facilitated the creation of a highly sensitive and specific CRISPR/Cas12a-based diagnostic tool for the detection of the SARS-CoV-2 N gene. The optimal concentrations identified provide a framework for future assay development, ensuring that the system operates at maximum efficiency while minimizing potential issues associated with non-specific activity or signal saturation.

#### **4.3.2 The sensitivity and specificity assessment of the CRISPR/Cas assay for SARS-CoV-2**

Sensitivity and specificity of diagnostic assays are critical parameters that determine the reliability and utility of these tools in clinical and environmental settings. For the CRISPR/Cas12a-based assay developed to detect SARS-CoV-2, sensitivity refers to the ability of the assay to correctly detect the presence of the virus even at low concentrations, while specificity refers to its ability to discriminate SARS-CoV-2 from other similar pathogens and to avoid false

positives. The Figure 4.6 illustrate the results of experiments designed to assess both the sensitivity and specificity of the assay using gRNA-1 and gRNA-2 targeting the N1 region of the SARS-CoV-2 genome.

Sensitivity was evaluated by testing the CRISPR/Cas12a assay with a range of viral RNA concentrations, spanning several orders of magnitude, from  $5 \times 10^5$  copies  $\mu\text{L}$  to  $5 \times 10^0$  copies  $\mu\text{L}^{-1}$ . The sensitivity assessment started with the RT-LAMP amplification and was followed by the CRISPR/Cas12a assay using two gRNAs respectively. The fluorescence intensity, measured in a.u., was monitored over a 30-minute period for both gRNA-1 and gRNA-2 (Figure 4.8 (a) and (b)).

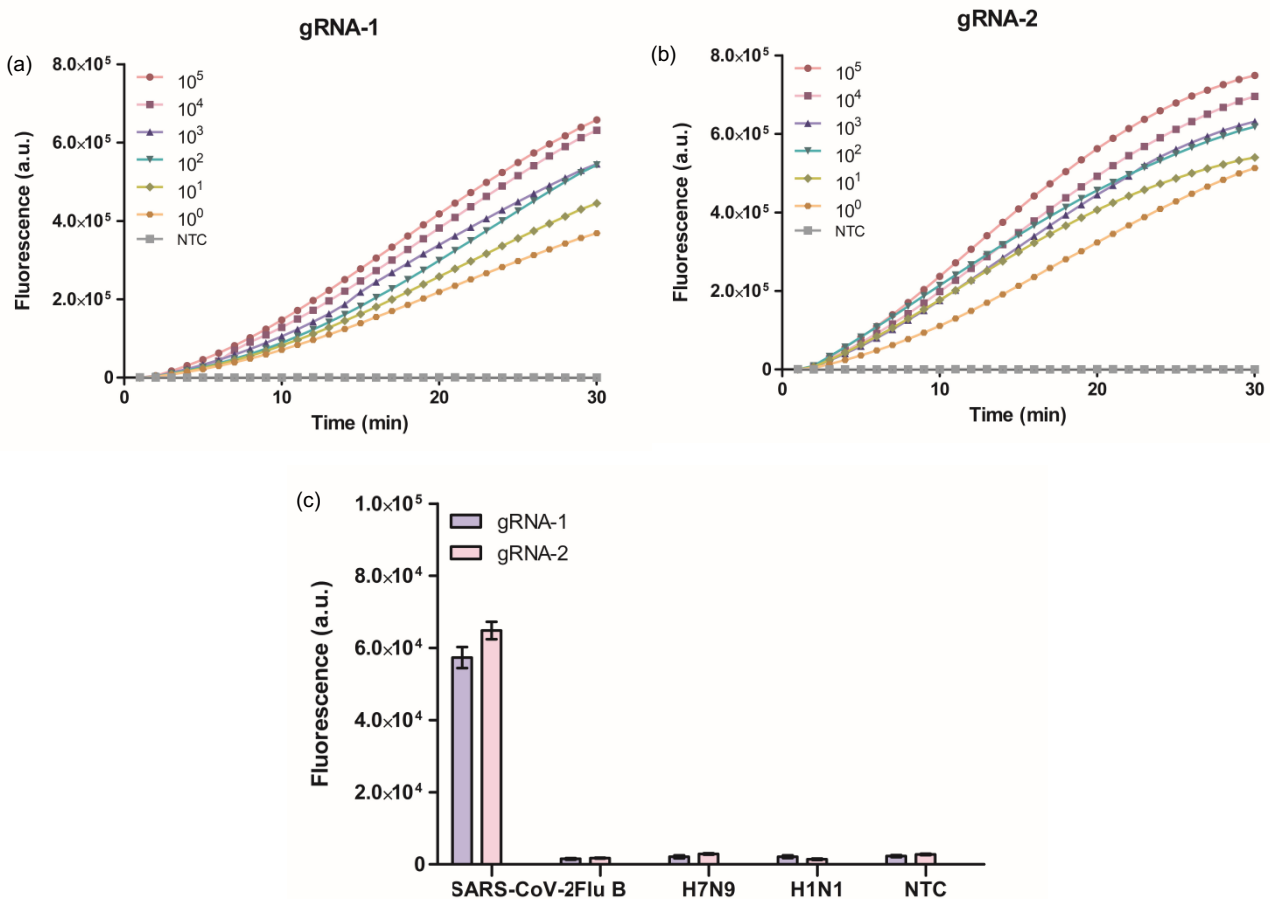


**Figure 4.8 The sensitivity and specificity assessment of the developed CRISPR/Cas assay (a) Sensitivity test of gRNA-1 with RNA (b)**

### **Sensitivity test of gRNA-2 with RNA (c) Specificity test of gRNA-1 and gRNA-2 with respiratory viruses**

A robust, concentration-dependent increase in fluorescence signal was observed for both gRNA-1 and gRNA-2 across all tested concentrations. The fluorescence intensity exhibited a direct correlation with viral RNA concentration, thereby demonstrating the assay's capacity to detect SARS-CoV-2 across a broad dynamic range. Even at the lowest concentration, both gRNAs produced a discernible fluorescence signal above the NTC, indicating the high sensitivity of the assay, which is consistent with the reference (Broughton *et al.*, 2020). The fluorescent signals produced by gRNA-2 were generally slightly stronger than those produced by gRNA-1, especially at lower concentrations, suggesting that gRNA-2 may have a slightly higher affinity or efficiency in recognising target sequences within the N1 region. The capacity to identify a limited number of viral RNA copies demonstrates the assay's potential for utilization in contexts where high sensitivity is of paramount importance, such as the tracing of virus dissemination in environmental wastewater samples. The slight advantage observed with gRNA-2 indicates that further optimization of gRNA sequences could further enhance the assay's performance.

The specificity of the CRISPR/Cas12a assay was evaluated by testing it against a panel of non-SARS-CoV-2 viral DNA samples, including Influenza B (Flu B), H7N9, and H1N1, in addition to the NTC. The fluorescence signals generated by gRNA-1 and gRNA-2 were quantified to ascertain the ability of the assay to discriminate between SARS-CoV-2 and other prevalent respiratory viruses (as illustrated in Figure 4.8 (c)). Upon testing against SARS-CoV-2 DNA, both gRNA-1 and gRNA-2 yielded robust fluorescent signals, aligning with the outcomes of previous tests. The results demonstrated that both gRNAs produced minimal fluorescence signals against influenza B, H7N9, H1N1, and NTC, indicating that the developed CRISPR/Cas12a assay did not cross-react with these non-target viral DNAs. Furthermore, the non-SARS-CoV-2 target exhibited

negligible fluorescence levels comparable to NTC, which highlighted the assay's specificity.

The specificity assessment corroborates the reliability of the CRISPR/Cas12a assay in differentiating SARS-CoV-2 from other respiratory viruses, thereby reducing the probability of false positives. This high specificity is of paramount importance in environmental monitoring scenarios where a mixture of viral RNAs might be present. The results substantiate that the gRNAs designed for this assay are highly selective for the SARS-CoV-2 N1 region, thereby further validating the assay's utility in accurately identifying the virus even in the presence of potentially confounding viral genomes.

#### **4.3.3 N-Gene bioinformatic analysis and the development of gRNAs for VOCs identification**

Following the development of a CRISPR/Cas assay for the detection of SARS-CoV-2, the second objective of this study was to create a CRISPR/Cas assay for the identification of VOCs. VOCs are specific strains of a virus that have undergone mutations, resulting in significant changes to their genetic code that may affect virus behavior in ways that have public health implications (Carabelli *et al.*, 2023). In the United Kingdom, several SARS-CoV-2 variants were classified as VOCs between 2020 and 2023 due to their increased transmissibility, potential to evade immunity, or association with more severe disease, which include Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) (Carabelli *et al.*, 2023). Among these, the Delta and Omicron variants received the greatest degree of attention and were selected for identification in this study.

The complete genomic sequences for each variant were obtained from the GISAID database. A BLASTN search (National Center for Biotechnology Information) was conducted to compare the sequences in question to the reference genome (Wuhan strain). The genome of SARS-CoV-2 offers numerous

potential targets due to its size. However, not all genes are equally suitable for this purpose, given the virus's natural evolutionary history and the basic rules of designing gRNAs. 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 SNP, as presented in the Figure 4.9.

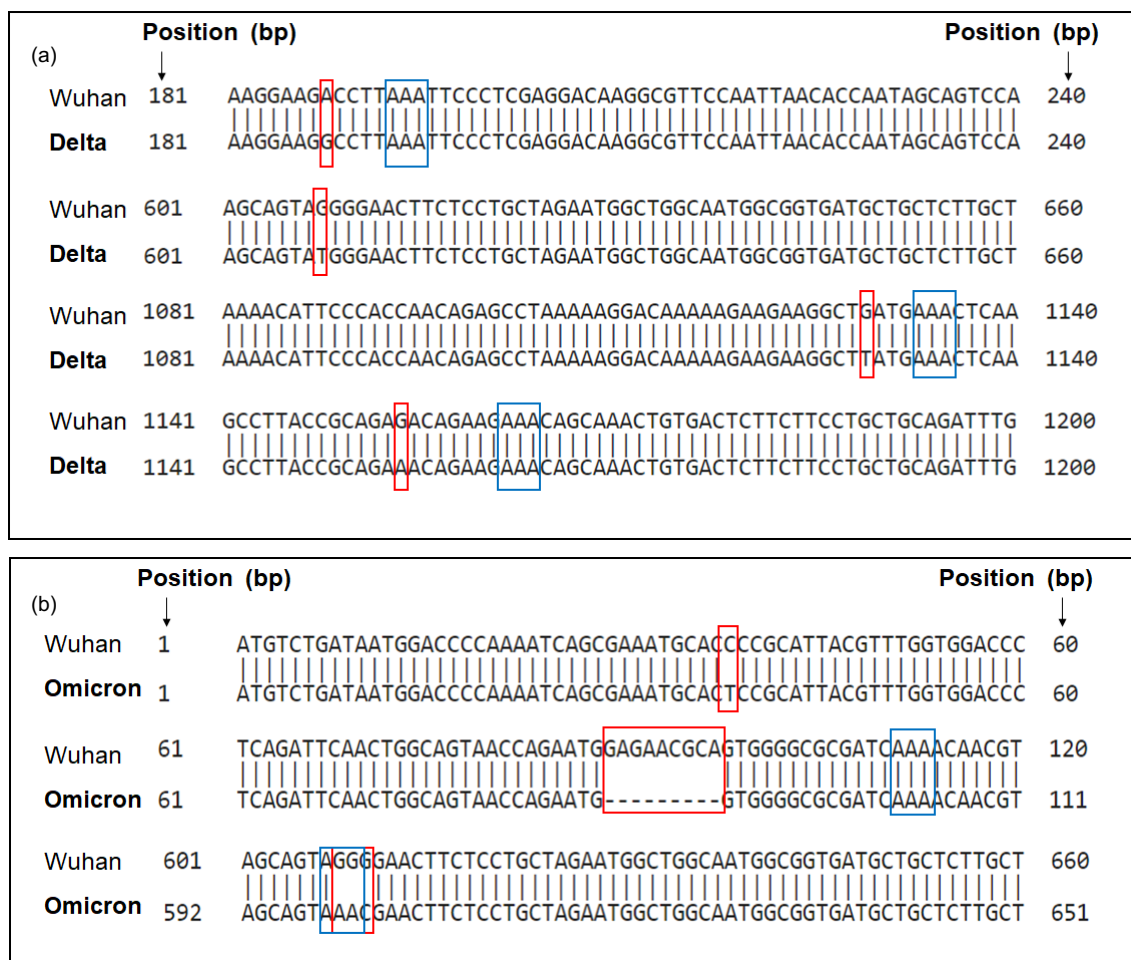


**Figure 4.9 The sequence alignment comparison results**

**(a) Wuhan and Delta (b) Wuhan and Omicron**

Figure 4.9 (a) illustrates a sequence alignment comparison between the original Wuhan strain of SARS-CoV-2 and the Delta variant. The alignment

serves to highlight specific nucleotide differences between the two variants at various positions within the genome. The aforementioned differences are indicated by red boxes, while the potential PAM sequences in the neighbourhood of the SNPs are illustrated by blue boxes. The SNP at base 608, with T replacing G, is not suitable for designing gRNA due to the absence of PAM sequences in the vicinity of the mutation. The PAM sequence is an indispensable element of the CRISPR/Cas complex, which is required for the recognition and binding of target DNA (Paul and Montoya, 2020). The Cas12a protein performs a scan of the DNA sequence in order to identify the presence of the PAM sequence. Once the PAM sequence has been identified, the DNA is unwound in the vicinity of the PAM, thereby creating a binding site for the gRNA to interact with the complementary target sequence (Jeon *et al.*, 2018). In the absence of the appropriate PAM sequence, the Cas protein is unable to bind to the DNA, and the gRNA is incapable of directing the complex to the intended location, resulting in the system becoming non-functional.

The PAM sequence acts as a key determinant for target recognition, influencing both where and how effectively the CRISPR/Cas complex can edit the genome. In the context of Cas12a system utilization, the PAM sequence is identified as TTTX, so the searching PAM sequence can be either TTT or AAA. In the event that the PAM is TTTX, it will be situated in front of the SNP site. Conversely, the AAAX will require positioning at the downstream end of the SNP site, since the gRNA needs to target the complementary sequence.

In the context of designing gRNAs for SNP detection utilising the CRISPR/Cas system, it is of significant importance to consider the distance between the SNP site and the PAM sequence, as this directly impacts the system's capacity to identify and detect the SNP in a specific and efficient manner. Among other three SNP candidates, the SNP site at base 1154 was excluded as well due to this reason. It is optimal for the SNP site to be situated in close proximity to the PAM sequence, as this enables the gRNA to direct the Cas

enzyme to a precise location on the DNA. The closer the SNP is to the PAM, the greater the probability that the gRNA will be able to effectively differentiate between the wild-type and mutant sequences (Gleditzsch *et al.*, 2019). Generally, the ideal SNP should be located within the seed region of the gRNA, and that is typically first 5-10 nucleotides proximal to the PAM (Swarts, van der Oost and Jinek, 2017). Mismatches happened in this region tend to prevent binding and trans-cleavage activity of the Cas12a and gRNA complexes (Kleinstiver *et al.*, 2016). Although the SNP at 1154 is still situated within the seed region of the gRNA, it is separated from the PAM sequence by a distance of seven bases. In comparison to two other candidates, the distances between the SNP and the PAM are three and four bases, respectively, the seven-base distance is not considered optimal.

Therefore, the SNPs at base 188 and base 1129 were selected as the potential SNP sites for gRNAs designing, and two gRNAs targeting Delta sequence at these regions were developed based on previously mentioned approach. The first one is gRNA-Delta1 (gRNA-D1), targeting the SNP at 188, while the second one binds the SNP at 1129, with the name of gRNA-Delta2 (gRNA-D2), as shown in the Figure 4.9. When the CRISPR/Cas system is employed for detection purposes, the presence of the Delta sequence is able to trigger the generation of fluorescent signals, and the presence of the Wuhan and Omicron sequences has no effect on the cleavage activity of the Cas12a enzyme.

A similar concept was employed in the development of gRNA for the identification of Omicron, as presented in the Figure 4.9 (b). The comparison results showed that there are two sequence deletions and one SNP difference between the Omicron and the Wuhan sequence. The initial SNP at base 38 cannot be regarded as a prospective position for gRNA development, as there is no suitable PAM sequence around the site. PAM sequence AAA is located at the upstream of the SNP site, while the TTT is at the downstream relative to the SNP site. The second mutation site is a large deletion at approximately base 100,

which could be considered an ideal site for the design of gRNA due to the significant sequence difference. Nevertheless, a single AAA sequence is situated in a distal position, with a distance of 13 bases between them, which is a risk for the design of gRNA.

The last mutation could be regarded as an optimal site for the gRNA development, and there are two main reasons. The initial consideration pertains to the three-base mutation itself, as the greater the number of base mutations that occur within the region complementary to the gRNA, the more pronounced the divergence between the target sequence and the wild-type sequence (Kaminski *et al.*, 2021). The larger difference increases the probability that the CRISPR/Cas system will identify the mutant sequence as distinct from the wild-type, thereby reducing the likelihood of off-target effects or false positives. The second reason lies in the PAM sequence, the mutant sequence contains the AAA, which is exactly the complementary sequence to the original PAM, while the Wuhan sequence does not include it. These findings have significant implications for the design and specificity of the gRNA in a CRISPR/Cas12 system, since exclusive targeting based on PAM recognition has the potential to increase specificity and reduce off-target effects (Liang *et al.*, 2022). Therefore, one gRNA was developed for Omicron determination according to the mutation at base 599-601, naming gRNA-Omicron (gRNA-O), as presented in the Figure 4.10.

|           |                               |
|-----------|-------------------------------|
| Wild type | TTTA-AGGTCCTTCCTTGCCATGTTGAGT |
| Delta     | TTTA-AGGCCTTCCTTGCCATGTTGAGT  |
| gRNA-D1   | .....AGGCCUUCUUGCCAUGUUGAGU   |

|           |                              |
|-----------|------------------------------|
| Wild type | TTTC-ATCAGCCTTCTTCTTTTGTCT   |
| Delta     | TTTC-ATAAGCCTTCTTCTTTTGTCT   |
| gRNA-D2   | .....AUAAGCCUUCUUCUUUUUGUCCU |

|           |                              |
|-----------|------------------------------|
| Wild type | CCTA-CTGCTGCCTGGAGTTGAATTCT  |
| Omicron   | TTTA-CTGCTGCCTGGAGTTGAATTCT  |
| gRNA      | .....CUGCUGCCUGGAGUUGAAUUUGU |

**Figure 4.10 The illustration of the gRNAs for VOCs identification**

#### **4.3.4 The employment of CRISPR/Cas assay for VOCs identification**

The validation of the newly developed gRNAs designed for the detection of SARS-CoV-2 VOCs, namely Delta and Omicron, was conducted using artificial gene sequences. The Figure 4.11 illustrates the fluorescence intensity over time for three gRNAs: gRNA-D1, gRNA-D2, and gRNA-O, each tested against DNA sequences representing the Wuhan strain, the Delta variant, and the Omicron variant, with concentrations set at  $10^7$  copies  $\mu\text{L}^{-1}$ . Furthermore, a positive control utilising gRNA-R1 with the Wuhan sequence and a negative control employing nuclease-free water were included to ensure the proper functioning and specificity of the CRISPR/Cas assay.

Figure 4.11 (a) presented the efficacy of gRNA-D1 in targeting the Wuhan, Delta, and Omicron sequences. The results demonstrate minimal fluorescence for all three variants, indicating that gRNA-D1 was unable to effectively target or cleave the DNA sequences of any of these variants. The fluorescence intensity remained low and stable across the 30-minute observation period, comparable to that of the negative control. In contrast, the positive control demonstrated a notable increase in fluorescence over time, confirming the assay's functionality

and the appropriate operation of the CRISPR/Cas system. This suggests that gRNA-D1 lacks specificity or affinity for the tested sequences, and the possible explanation may be the secondary structure of it.

The functionality of Cas12a is highly sensitive to the formation of hairpin structures at the downstream of the CRISPR array, and these structures have the potential to interfere with the efficient binding and activity of the Cas12a enzyme (Liao *et al.*, 2019a). Furthermore, gRNA processing is adversely affected when the repeat sequence forms base pairs with regions outside the gRNA, which disrupts the intended structure and function of the gRNA (Creutzburg *et al.*, 2020). Additionally, the global RNA structure of the CRISPR array can provide a detrimental impact on both gRNA processing and overall performance. This is because it may impede the correct folding and functionality of the gRNA, which is essential for effective target recognition and cleavage (Liao *et al.*, 2019b).

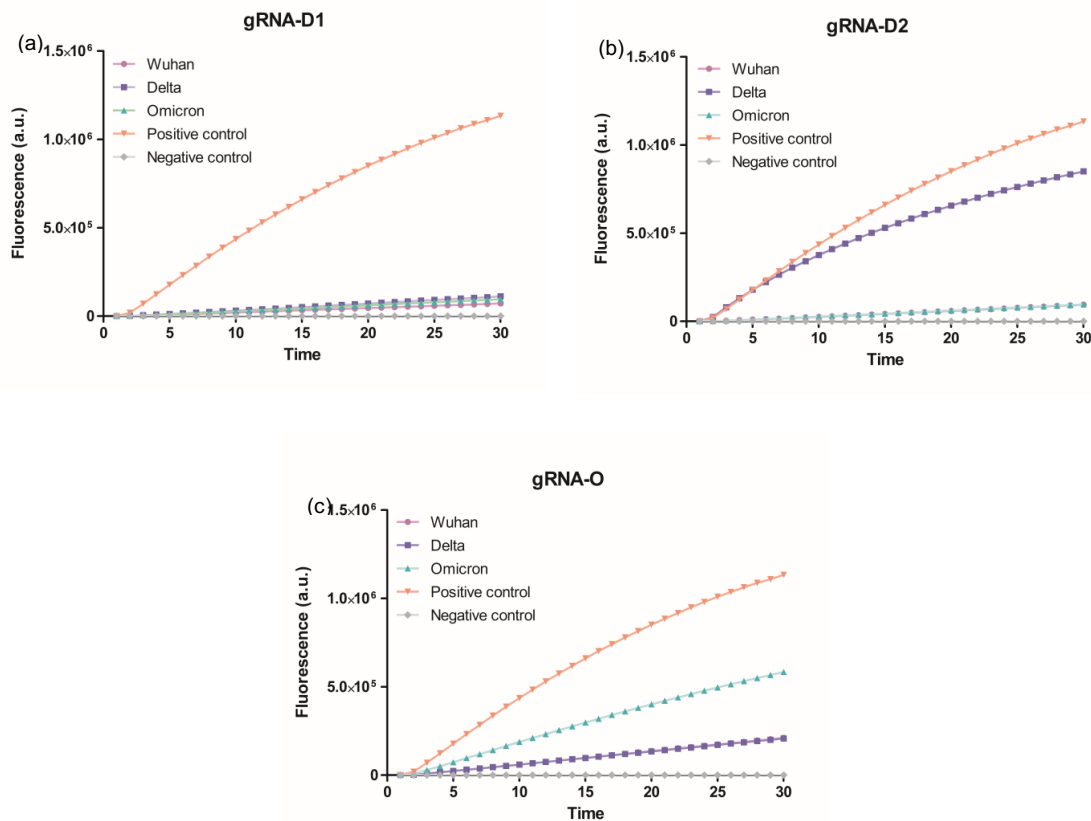


**Figure 4.11 The viability results of gRNAs for VOCs identification (a)  
gRNA-D1 (b) gRNA-D2 (c) gRNA-O**

In contrast to gRNA-D1, gRNA-D2 was observed to successfully recognise and bind to the Delta sequence, as evidenced by a marked increase in fluorescence intensity. This suggests that gRNA-D2 has a greater affinity for the Delta sequence than for the Wuhan and Omicron variants, which exhibited low fluorescence levels comparable to the negative control. Furthermore, the positive control demonstrated a consistent increase in fluorescence, thereby validating the assay. These findings suggest that gRNA-D2 is an effective method for distinguishing the Delta sequence from other types.

In the Figure 4.11 (c), gRNA-O was evaluated for its ability to detect the Omicron variant. The results demonstrate a clear increase in fluorescence intensity when gRNA-O was paired with the Omicron sequence, indicating successful targeting and cleavage by the CRISPR/Cas system. It is noteworthy that gRNA-O also demonstrated a moderate level of fluorescence with the Delta variant and the Wuhan sequence, although this was less pronounced than with the Omicron variant. This observation suggests the presence of some degree of cross-reactivity, and the possible explanation may relate to the wrong PAM recognition.

Prior research has indicated that the CRISPR/Cas12a system displays adaptability in recognizing alternative PAM sequences due to its distinctive mechanism of DNA cleavage. In contrast to Cas9, Cas12a initiates cleavage of target DNA at the distal end of a 5' T-rich PAM sequence, resulting in a 5-nucleotide staggered cut, and this allows for some tolerance to variations in the PAM sequence (Zetsche *et al.*, 2015; Nishimasu *et al.*, 2017). The PAM sequence 'TTN' has been successfully employed for Cas12a-mediated DNA cleavage and gene editing, with the enzyme retaining its activity under these conditions (Kong *et al.*, 2023; Yamano *et al.*, 2017).

In this case, a 5'-AAG-3' sequence can be observed downstream of the targeting site with a complementary sequence of 5'-TTC-3', which can be regarded as a similar PAM site. Consequently, a slight match between gRNA-O and the Wuhan sequence with the Omicron sequence may occur, resulting in the observation of fluorescence. Nevertheless, a notable discrepancy is evident when comparing these two sequences to the Omicron sequence, and it is postulated that following amplification, this difference may become more significant.

Following the DNA sequence validation test, gRNA-D2 and gRNA-O were selected for the subsequent identification of VOCs. In order to facilitate this process, primers for the LAMP method were designed for the amplification of the mutated sites, and the specific approach is presented in the method section. A total of five sets of primers were designed, three for Delta mutation amplification and two for Omicron mutation amplification. Of these, only one set of primers specifically targets the Delta SNP site, and the other selectively amplifies the Omicron mutation, which were selected for the following test. These primer sets were used to amplify the RNA ( $10^5$  copies  $\mu\text{L}^{-1}$ ) of Wuhan, Delta and Omicron at first. Subsequently, the CRISPR/Cas assay was conducted with gRNA-D2 or gRNA-O, as illustrated in Figure 4.12. gRNA-D2 was employed for the Delta LAMP group amplification results, while gRNA-O was utilized for the Omicron LAMP group amplification results.

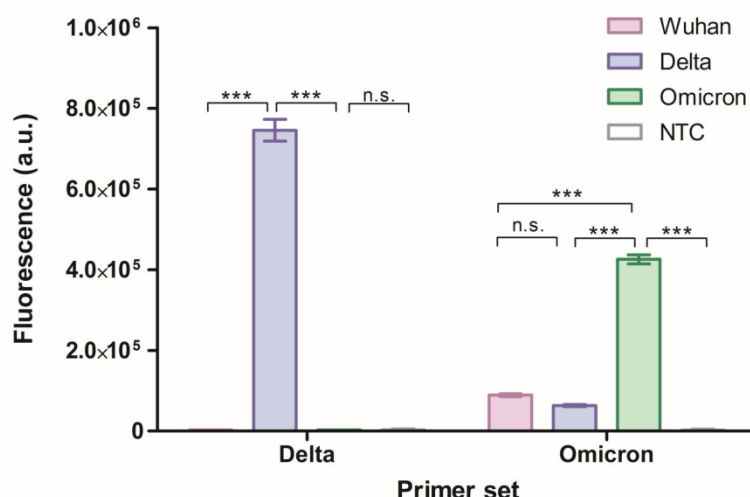


**Figure 4.12 The CRISPR/Cas assay for VOCs identification after RT-LAMP**

As illustrated in Figure 4.12, the fluorescence intensity was measured for each sequence, and the results represent the mean  $\pm$  SD of three independent replicates. The Delta-specific primer set, when used with the gRNA-D2, yielded a markedly elevated fluorescence signal for the Delta variant, whereas minimal fluorescence was observed for the Wuhan, Omicron variants and negative group. This suggests that the gRNA-D2 and Delta primer set combination is highly specific in detecting the Delta variant. Similarly, the Omicron-specific primer set with gRNA-O yielded a robust fluorescence signal for the Omicron variant, accompanied by subtle signals for the Wuhan and Delta variants. The reason for this slight positive result was previously outlined; however, despite this, a notable difference between the Omicron and wild types was still observed following amplification, as evidenced by the ANOVA and Tukey's Honest Significant Difference (HSD) tests, with a p-value of less than 0.001. The results from this CRISPR/Cas assay highlight the effectiveness of the LAMP-based amplification strategy combined with specifically designed gRNAs for the detection and differentiation of SARS-CoV-2 variants of concern. The observed fluorescence

patterns indicate that the selected gRNAs, gRNA-D2 and gRNA-O, are highly specific to the Delta and Omicron variants, respectively.

In order to explore the limit of detection (LOD) of these newly designed gRNAs, the sensitivity assessment was performed. The assessment is similar to the one for gRNA-1 and gRNA-2, using the specific LAMP primers to amplify the gradient concentration of RNA at first (starting from  $5 \times 10^5$  copies  $\mu\text{L}^{-1}$ ) and performing the CRISPR/Cas assay with amplicons. The Delta LAMP primer set and gRNA-D2 were used for RNA of Delta identification, while the Omicron LAMP primer set and gRNA-O were used for RNA of Omicron identification, and results are presented in the Figure 4.13.

According to the Figure 4.13, the data clearly demonstrates a concentration-dependent increase in fluorescence. It was observed that an increase in the concentration of the RNA template resulted in a corresponding enhancement in the intensity of the fluorescence signal. Nevertheless, in Figure 4.13 (a) when the template concentration was reduced to  $5 \times 10^1$  copies  $\mu\text{L}^{-1}$ , the fluorescent signal was markedly diminished, a phenomenon that was similarly observed in the lower concentration group. In other words, the CRISPR/Cas assay identifies Delta variant with a LOD of 500 copies  $\mu\text{L}^{-1}$ , which is significantly higher than the CRISPR/Cas assay identifying Omicron variant at a LOD of 5 copies  $\mu\text{L}^{-1}$ , as shown in Figure 4.13 (b).

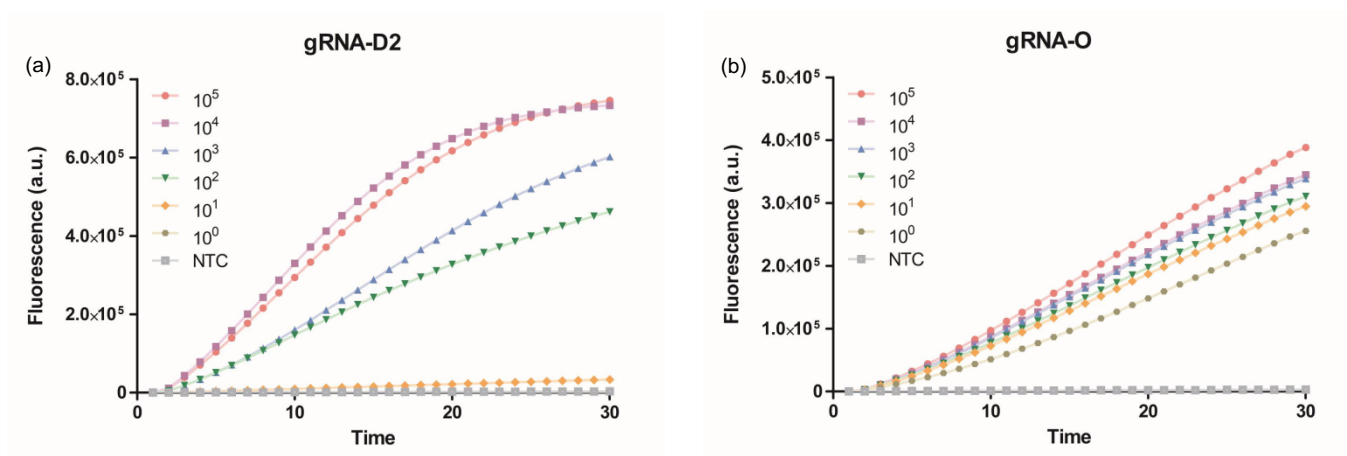


**Figure 4.13 The sensitivity assessment of gRNAs for VOCs identification**

When it comes to the LOD of CRISPR/Cas assay, although the sensitivity of the gRNA plays a crucial role in detecting the presence of target RNA sequences, it is the efficiency of the LAMP amplification that primarily determines the LOD (Lu *et al.*, 2022). If the LAMP primers are less efficient, the target RNA may not be amplified to detectable levels, thereby decreasing the LOD of the entire assay. The CRISPR/Cas targeting sequence occurs subsequent to the amplification process, and in the event that the target sequence is not sufficiently amplified by the LAMP process, the CRISPR/Cas system, regardless of the sensitivity of the gRNA, may be unable to detect it. In general, the LOD of gRNA for target dsDNA is approximately  $10^5$  copies  $\mu\text{L}^{-1}$ . In this experiment, the use of primers to amplify RNA at a concentration of 50 copies  $\mu\text{L}^{-1}$  resulted in a final amplicon concentration of less than  $10^5$  copies  $\mu\text{L}^{-1}$ , suggesting the low efficiency of the LAMP primers.

The efficiency of LAMP primers depends on the design of primers, and the selected Delta primer set consists only five primers, without the LB primer. A typical LAMP primer set comprises two inner primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (LF and LB). The inner primer involves two sequences, which are F1c/B1c and F2/B2, while the outer primers are shorter

primers that bind outside the F2 and B2 region. Loop primers facilitate the exponential amplification process by binding to the loop structure formed during LAMP amplification. However, it is possible that loop primers may be excluded when several key factors in primer development are considered.

The length of the primers is a crucial factor in the success of the reaction, and the optimal length for the inner and outer primers is 18-22 nucleotides and 18-24 nucleotides, respectively (Notomi *et al.*, 2015). Also, the content of Guanine (G) and cytosine (C) should be paid attention to when designing primer, with a GC content between 40% and 60% being optimal. Additionally, it is important to avoid runs of three or more identical nucleotides (e.g., CCC) to prevent non-specific binding. Melting temperature ( $T_m$ ) need to be taken into consideration as well, as inner primers should have a  $T_m$  of 60-65°C, while outer primers should have a  $T_m$  of 55-60°C. It is essential to ensure that the  $T_m$  between the inner and outer primers is not excessive, ideally within a range of 5°C. Based on all these rules, the Delta primer set was developed with the absence of the LB primer, resulting in the lower amplification efficiency.

## 4.4 Conclusions

This study developed and validated a CRISPR/Cas12a-based fluorescent assay for the detection of SARS-CoV-2 variants, specifically Delta and Omicron, in wastewater samples. This innovative assay uses the CRISPR/Cas system, which has the specific targeting capability, and the designed gRNAs could precisely bind to the unique SNP of each variant. The binding and recognition could prompt the collateral cleavage activity of Cas12a and therefore cut the reporter DNA sequences and result in a quantifiable fluorescent signal. The resulting fluorescent signal could be regarded as a direct measure of the presence of the virus.

The initial objective of this study was to establish the CRISPR/Cas assay system and optimize the assay's components, with the aim of maximizing the efficiency and reproducibility of the assay. Regarding the gRNA and Cas12a, the optimal concentration was determined to be 75nM and 50nM, respectively. This is on the basis that a higher concentration of gRNA may facilitate more effective recognition. The optimal concentration for the fluorescent reporter (FAM) was determined to be 500nM, which was found to be optimal for use with a fluorescent readout. It is possible to increase the concentration further for observation without the use of a machine and only by naked eyes. These conditions were identified as providing an optimal balance between sensitivity and signal-to-noise ratio, thereby ensuring clear and reliable detection of the target RNA.

A further significant aspect of this research was the design and validation of gRNAs capable of distinguishing the Delta and Omicron variants from the original virus. The gRNAs were designed to target specific mutations associated with each variant, and the assays demonstrated varying degrees of success in distinguishing these variants. The gRNA designed for Omicron identification demonstrated high specificity, and the introduction of LAMP six primers resulted in notable sensitivity, with a limit of detection reaching 5 copies  $\mu\text{L}^{-1}$ . The

detection of the Delta variant was less efficient, as the designed gRNA could not distinguish it from the other two strains, but the background signal was significant. By introducing LAMP amplification, the fluorescence signal of delta can be clearly distinguished from other signals. However, because of the limitation of LAMP primer design, it is not possible to design a complete set of six primers for this detection target. The amplification of only five primers resulted in a lower sensitivity of the assay against Delta virus, with a limit of detection around 500 copies  $\mu\text{L}^{-1}$ .

Although the results are promising, there are significant challenges associated with this research, particularly regarding the design of gRNAs. It seems reasonable to posit that the structure of these molecules affects both the efficiency with which they bind to Cas proteins and the specificity with which they target sequences. This, in turn, affects the accuracy of the assay. Concurrently, the recognition of PAM sequences is not exclusively confined to TTTX, and there are other instances where PAM sequences can activate the targeting function of the CRISPR/Cas system (e.g. TTX). To circumvent off-target effects, it is imperative that gRNAs are meticulously designed to guarantee the dependability of the assay. It is recommended that future research concentrate on enhancing the design of gRNAs through the utilization of sophisticated prediction algorithms and the investigation of advanced molecular techniques with the objective of augmenting binding efficiency and minimizing the possibility of mismatches.

Furthermore, the incorporation of this CRISPR/Cas12a assay into conventional wastewater detection programs necessitates careful consideration. The technical requirements for this assay are currently less onerous than those for conventional RT-qPCR. However, some laboratory infrastructure is still required to deploy the assay in a laboratory setting for the detection of SARS-CoV-2 and its variants. The development of a more straightforward approach that can be employed for field detection would facilitate the integration of wastewater analysis into routine monitoring, particularly in resource-limited settings. Given

the high sensitivity and specificity of the method and the absence of a stringent temperature control system, there is considerable potential for the method to be developed for point-of-use diagnostic applications.

Additionally, the findings indicate the possibility of extending the assay as a platform for other SARS-CoV-2 variants and additional pathogens. This study constructed a complete CRISPR/Cas system for the detection of novel coronaviruses, and the same approach can be used to design new primers and gRNAs for the detection of new pathogens. Furthermore, a rapid and cost-effective method for the identification of emerging variants can be readily developed, offering an alternative to sequencing for the straightforward and economical characterization of variants. By expanding the range of detectable targets, the assay has the potential to become the foundation of a more responsive and adaptable public health surveillance system. Furthermore, continued advances in CRISPR technology and its integration with other diagnostic platforms will facilitate the advent of a new era of rapid, accurate, and comprehensive pathogen surveillance.

In conclusion, the CRISPR/Cas12a-based fluorescent assay is a significant advancement in the field of virus diagnostics and environmental monitoring. The assay is capable of accurately detecting and differentiating key SARS-CoV-2 variants in samples, thus constituting an important tool for public health management in response to the ongoing pandemic. The assay's principal advantages are its high sensitivity, specificity and rapid turnaround time, which render it suitable for extensive utilization in the monitoring and control of the virus's dissemination. As global efforts to address the challenges posed by the COVID-19 pandemic, particularly the emergence of new variants, continue, the use of such techniques will facilitate the implementation of effective and timely interventions.

## 4.5 References

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## **Chapter 5: Development of a paper microfluidic device for rapid and on-site detection of SARS-CoV-2 from wastewater**

### **Abstract**

In the case of the COVID-19 pandemic, existing technologies rely on laboratory-based methods to accomplish the analysis of the virus and its variants in wastewater. This study focuses on the development and optimization of a paper-based microfluidic device for field detection of SARS-CoV-2 and its variants in wastewater, proposing a low-cost, portable and resource-efficient solution suitable for field applications. A syringe-based filtration method coupled with polyethylene glycol (PEG) precipitation was used to efficiently concentrate viral particles, followed by optimized viral RNA extraction using safer alternatives like AVL buffer. The design of the paper microfluidics prioritised RNA recovery by adjusting key parameters such as glass fibre diameter, microfluidic channel width and length, resulting in improved fluid retention and nucleic acid recovery efficiency. Loop-mediated isothermal amplification (LAMP) assays were integrated into the device for preliminary SARS-CoV-2 detection. The fluorescent LAMP showed high sensitivity, while the colourimetric LAMP provided a more intuitive interpretation of the results through a distinct pink to yellow colour change. Subsequently, the CRISPR/Cas12 colourimetric assay was incorporated into the highly specific and multiplexed detection of variants. Optimized probes and the Cas12a enzyme promoted clear differentiation between Delta and Omicron variants using different fluorescent signals, improving field applicability. The device effectively detects RNA concentrations as low as  $10^2$  copies  $\mu\text{L}^{-1}$  for Delta and  $10^1$  copies  $\mu\text{L}^{-1}$  for Omicron, providing reliable results even in complex wastewater matrices. The combination of optimized paper microfluidics and CRISPR/Cas detection shows great potential for field detection, and this

innovative approach advances wastewater analysis by enabling rapid, accurate, and decentralized diagnostics, contributing to timely outbreak management.

**Keywords:** Paper microfluidic device; CRISPR/Cas colourimetric assay; Field detection; Wastewater analysis; Variants identification

## 5.1 Introduction

As early as the late 1930s, the presence of the infectious poliovirus was detected by testing sewage from outbreak sites (Paul, Trask and Culotta, 1939). Viruses have shown to be one of the most promising of the many biomarkers and compounds that can be tested in sewage to evaluate health and well-being within population, and pathogen analysis from wastewater plays a critical role in environmental surveillance, public health, and early outbreak detection (Diamond *et al.*, 2022). During the COVID-19 outbreak, the data collected from wastewater was employed as supplementary information to clinical reports, offering detailed information on the disease burden and transmission (both asymptomatic and non-symptomatic), as well as which SARS-CoV-2 variants were endemic in the community (Amman *et al.*, 2022; Shah *et al.*, 2022). A common research approach is to transport collected samples to the laboratory and then use reverse transcription quantitative polymerase chain reaction (RT-qPCR) or next-generation sequencing (NGS) technologies to accomplish the identification of SARS-CoV-2 and the discovery of mutant strains (Karthikeyan *et al.*, 2022).

These methods are highly sensitive and specific, but their dependence on well-equipped laboratories, trained personnel and sophisticated instruments makes it challenging to promote wastewater analysis in resource-limited areas. However, detection for viruses in sewage is a more cost-effective way to understand the extent of infection in a community than clinical screening (Xiao *et al.*, 2022). Also, conventional sample processing techniques often involve multi-step protocols for concentration, extraction, and analysis, which are time-intensive and resource-heavy (Feng *et al.*, 2021). Nevertheless, delays in wastewater reporting can affect the value and early warning potential of this approach.

In order to further expand the application of detecting pathogens in wastewater, there is a need to gradually shift detection from relying on laboratory

resources to field testing, shortening the time of the entire process and providing reliable results in resource-limited settings. However, on-site detection of pathogens in wastewater presents additional complexities, especially in resource-poor environments. The presence of contaminants and the heterogeneity of wastewater matrices complicate accurate and reliable testing (Xiao and Zhang, 2023). Also, the generally lower concentration of viruses in wastewater compared to clinical samples poses another problem in terms of how to concentrate them in the field.

Microfluidic devices have emerged as transformative tools in diagnostics, leveraging the manipulation of small fluid volumes within intricate networks of channels (Niculescu *et al.*, 2021). These devices offer several advantages, including high sensitivity, rapid analysis, and minimal reagent consumption. They have been widely adopted for chemical analysis, disease diagnostics, and environmental monitoring (Gale *et al.*, 2018). Paper-based microfluidic devices represent a significant advancement in this field, combining affordability, portability, and biodegradability with the analytical capabilities of conventional microfluidics (Nishat *et al.*, 2021). Fabricated using techniques such as wax printing and screen printing, these devices rely on capillary action to transport fluids, eliminating the need for external pumps or power sources. Paper substrates enable the integration of diverse analytical methods, including colourimetric, electrochemical, and fluorescence-based assays (Zhang *et al.*, 2023; He and Liu, 2013). This versatility makes paper microfluidics particularly suitable for point-of-need (PON) diagnostics in both medical and environmental applications. For example, paper-based devices have been employed for the detection of infectious diseases such as malaria, HIV, and more recently, SARS-CoV-2 in wastewater surveillance (Reboud *et al.*, 2019; Tharakan *et al.*, 2022; Cao *et al.*, 2022).

Origami paper microfluidic devices build on the principles of conventional paper microfluidics, incorporating three-dimensional structures achieved through

folding techniques (Liu and Crooks, 2011). This innovative technology allows for more complex fluid pathways and multi-step analyzes on a small, portable paper microfluidic device. The folding process, inspired by origami, facilitates the creation of layered channels and stratified compartments for the orderly addition of reagents and controlled mixing of fluids (Sun *et al.*, 2022). These attributes make origami paper microfluidics particularly advantageous for on-site applications where space, resources, and time are constrained.

Origami paper microfluidic devices used for field testing are classified into the following three main types based on the detection principle. Firstly, the detection method based on the colourimetric principle is applied to the biomedicine detection of protein (Choi *et al.*, 2015), glucose (Sechi *et al.*, 2013) and HIV (Chen *et al.*, 2021). However, since this method is easily subject to external interference, it is generally applied to qualitative or semi-quantitative origami detection devices. The second category combines a fluorescent colour development reaction with an origami microfluidic device, which enables the rapid detection of chemical substances, such as phycocyanins (Li *et al.*, 2017) and cortisol (Weng *et al.*, 2022). The last type combines the electrochemical detection technology, which is highly resistant to interference and sensitive, with origami microfluidics, thus simplifying the electrochemical device and enabling field detection (Sun *et al.*, 2022). The unique geometry of origami paper microfluidics supports various sensing modalities, therefore is widely applied for cancer diagnosis, human-health monitoring and drug monitoring.

Wastewater-based epidemiology (WBE) faces significant challenges in point-of-diagnostics applications, but paper microfluidic devices present a promising solution. Their key advantages—low cost, lightweight construction, and biodegradability—make them well-suited for low-resource settings. Moreover, their portability and ease of use make origami-style devices ideal for field applications. Unlike conventional laboratory methods, these devices eliminate the need for extensive infrastructure or technical expertise. Additionally, rapid assay

times and compatibility with low-volume samples enhance their effectiveness in time-sensitive situations, such as outbreak management and environmental monitoring.

This study aims to develop a paper-based microfluidic device integrating a CRISPR/Cas12a colourimetric assay for on-site detection of SARS-CoV-2 variants, including the original strain, Delta, and Omicron, from wastewater. A PEG-based precipitation method, a filtration-based approach, and an efficient virus lysis protocol were investigated to enrich virus particles from large wastewater volumes, addressing the challenge of low virus concentrations in field samples. Next work will focus on designing and optimizing origami microfluidics to purify viral RNA from collected samples, which will then be used for field assays. Finally, three detection methods—fluorescent LAMP, colourimetric LAMP, and colourimetric CRISPR/Cas—will be evaluated, with the CRISPR/Cas assay enabling multiplex detection of SARS-CoV-2 variants.

## 5.2 Materials and Methods

### 5.2.1 Wastewater on-site concentration approach

#### 5.2.1.1 Filtration approach investigation

The scheme of on-site enrichment is shown in the Figure 5.1, and the initial two-filtration processes were investigated. Before filtration, the 50 ml of raw wastewater (collected on 04/01/2023) was combined with 15 ml of a solution comprising 40% polyethylene glycol 8000 (PEG 8000) and 8% sodium chloride (NaCl) (Sigma-Aldrich).

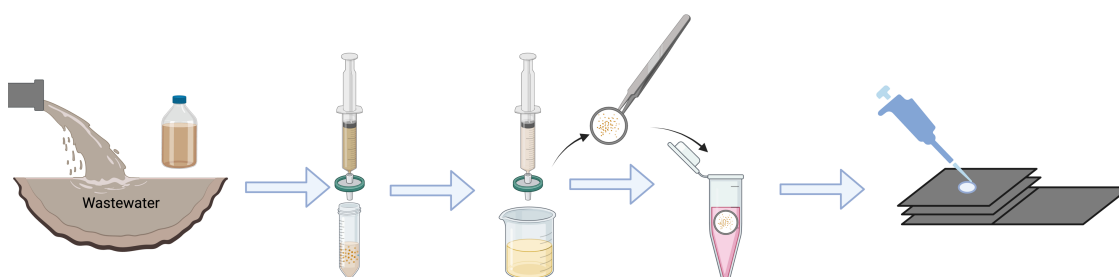


**Figure 5.1 The scheme of on-site enrichment**

A syringe was employed to extract a total of 200ml of mixed wastewater, with the filtrate subsequently collected through filtration using a 0.45  $\mu\text{m}$ /0.20 $\mu\text{m}$  filter membrane (Sigma, UK). Subsequently, a second filtration was conducted using a polypropylene filter holder containing a membrane filter. The filter membranes used for the second time were 25 nm mixed cellulose esters (MCE) filter membrane, and 50 nm MCE filter membrane, both were purchased from Sigma. Subsequently, the filtration membrane was transferred to a 2 ml centrifuge tube, which had been previously filled with 1ml of viral AVL lysis buffer from QIAGEN. The samples were then incubated at room temperature for 10 minutes to allow for complete viral lysis. The **Table 5.1** presented the filter membranes used in the four groups studies.

After the lysis, the QIAamp® Viral RNA Mini Kit was used to extract the RNA, as described in **Chapter 3, section 2.4**. After RNA extraction and purification, the SARS-CoV-2 RNA was detected using real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) as described in **Chapter 3, section 2.5**.

**Table 5.1 The investigation of different filtration membrane**

| <b>Group Name</b> | <b>First filtration</b> | <b>Second filtration</b> |
|-------------------|-------------------------|--------------------------|
| Group A           | 0.45 µm Filter          | 25 nm MCE membrane       |
| Group B           | 0.45 µm Filter          | 50 nm MCE membrane       |
| Group C           | 0.20 µm Filter          | 25 nm MCE membrane       |
| Group D           | 0.20 µm Filter          | 50 nm MCE membrane       |

#### **5.2.1.2 Effects of lysis buffer**

To evaluate which lysis method most effectively lysed SARS-CoV-2 for on-site detection, two different buffers were examined. The initial buffer, AVL viral lysis buffer from QIAGEN, is included in their viral RNA extraction kit. The alternative method utilized Trizol with chloroform, previously described for wastewater concentration in **Chapter 3**. To assess lysis efficiency, 400 ml of raw wastewater (collected on 20/06/2023) was mixed with 130 ml of a solution containing 40% PEG 8000 and 8% NaCl. This mixture was then equally divided into two samples for parallel processing. Each sample underwent dual filtration stages; following the second filtration, the membranes were transferred to mini-centrifuge tubes, each containing one of the two lysis buffers (1 ml). After incubation for 10 minutes, RNA was extracted using the QIAamp® Viral RNA Mini Kit, as outlined in **Chapter 3, Section 2.4**. Subsequently, the purified RNA was tested for SARS-CoV-2 using RT-qPCR, detailed in **Chapter 3, Section 2.5**. Five samples were processed in each lysis buffer group.

### 5.2.2 Design and fabrication of paper microfluidic device

The paper-based microfluidic device used in this study consists of five sections as shown in Figure 5.2. The initial two panels are the waste liquid area, which is used to absorb the waste liquid. The third panel has a circle made of glass fibre for absorbing the sample. The last two panels are microfluidic channels through which the prepared sample can pass and split into the reaction wells.

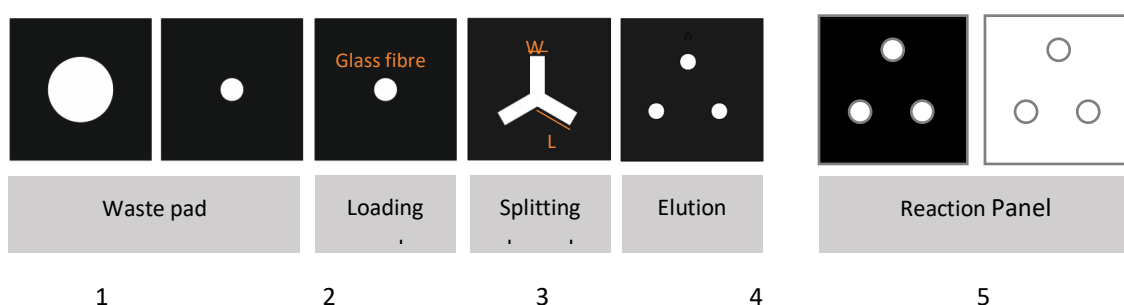


**Figure 5.2 The scheme of the paper microfluidic device**

The microfluidic device was fabricated using Whatman Grade 1 Qualitative Filter Paper, procured from Cytia in the United Kingdom. The Xerox ColorQube 8580 Colour Solid Ink Printer was employed to print the wax cover on the filter paper. The design of the microfluidic channels on the filter paper was accomplished using the Corel DRAW Standard 2021 software, which was employed to create the panels. The material of the reaction panels was cast acrylic purchased from Stockline Plastics (UK), while the wells were carved using an LS3020 desktop laser from HPC Laser (UK).

### 5.2.3 Operation of paper microfluidic device

Following lysis, the 50  $\mu\text{L}$  sample was loaded onto the glass fibre located at panel three. Because of the gravity, the excess liquid was initially washed to the waste pad at the first panel. The second step involved the washing of the loaded sample with 70% ethanol. This was conducted by loading 25  $\mu\text{L}$  of 70% ethanol

onto the glass fibre on two occasions. The loading of ethanol allowed the removal of non-nucleic acid components from the wastewater, which were transported to the waste pad. In contrast, the nucleic acid was retained on the glass fibre due to the electrostatic interactions. The RNA molecule is negatively charged because of the phosphate backbone, whereas the glass fibre filter surface is positively charged and can therefore interact with nucleic acids. Untreated glass fibres are typically negatively charged at neutral pH due to the deprotonation of surface silanol (Si–OH) groups. However, the glass fibres used in this study were coated with cationic polymers, such as quaternary ammonium compounds, thereby altering their surface charge. Following the second wash, the microfluidic device was folded in a different manner and positioned directly on the reaction plate. A narrow strip of filter paper was positioned within each reaction well. By overlapping and compressing the paper device and the reaction panel together, the narrow strip enabled to transfer the eluted sample from the paper device to the reaction well. To elute the RNA from the glass fibre, 30  $\mu\text{L}$  of nuclease-free water was added dropwise to the glass fibre using a pipette. The liquid was then directed through the microfluidic channel on the fourth panel and flowed to the paper spot on the fifth panel. By positioning the paper plate in close proximity to the reaction wells, the RNA can subsequently be transferred to the reaction wells via the filter strip. After adding the specific reagents to each reaction well, the plastic panel was sealed with the acetate film (MicroAmp Optical Adhesive Film, Thermo Scientific).

## **5.2.4 Optimization and evaluation of the paper microfluidic device**

### **5.2.4.1 Glass fibre diameter**

Glass fibre discs with diameters of 3.0 mm, 3.5 mm, 4.0 mm, 4.5 mm, and 5.0 mm were evaluated. A volume of 50  $\mu\text{L}$  containing  $4.1 \times 10^4$  copies  $\mu\text{L}^{-1}$  of prepared SARS-CoV-2 RNA stock solution, as outlined in **Chapter 4, Section 2.1**, was applied to each disc within the paper microfluidic device. Two washing

steps were conducted using 70% ethanol, followed by elution with 30  $\mu\text{L}$  of nuclease-free water. After elution, paper strips from each device were transferred to PCR tubes to undergo RT-qPCR, using the method described in **Chapter 3, Section 2.5**.

#### **5.2.4.2 Channel width**

The channels on the panel with width 1.5mm, 2.0mm, 2.5mm, 3.0mm, 3.5mm were assessed. A 50  $\mu\text{L}$  volume of prepared SARS-CoV-2 RNA stock solution, containing  $4.1 \times 10^4$  copies  $\mu\text{L}^{-1}$  as described in **Chapter 4, Section 2.1**, was applied to each disc in the paper microfluidic device. Two washing steps were conducted using 70% ethanol, followed by elution with 30  $\mu\text{L}$  of nuclease-free water. After elution, paper strips from each device were transferred to PCR tubes to undergo RT-qPCR, using the method described in **Chapter 3, Section 2.5**.

#### **5.2.4.3 Channel length**

The channels on the panel with length 5.0mm, 5.5mm, 6.0mm, 6.5mm, 7.0mm and 7.5mm were evaluated. A volume of 50  $\mu\text{L}$  containing  $4.1 \times 10^4$  copies  $\mu\text{L}^{-1}$  of synthesized SARS-CoV-2 RNA stock solution, as outlined in **Chapter 4, Section 2.1**, was applied to each disc within the paper microfluidic device. Two washing steps were conducted using 70% ethanol, followed by elution with 30  $\mu\text{L}$  of nuclease-free water. After elution, paper strips from each device were transferred to PCR tubes to undergo RT-qPCR, using the method described in **Chapter 3, Section 2.5**.

#### **5.2.4.4 Evaluation of the elution efficiency**

Following the optimization of the paper microfluidic device, its extraction efficiency was evaluated using four SARS-CoV-2 RNA stock solutions with concentrations ranging from  $1.2 \times 10^2$  to  $1.2 \times 10^5$  copies  $\mu\text{L}^{-1}$  (for stock solution preparation, refer to **Chapter 4, Section 2.1**). A 50  $\mu\text{L}$  aliquot of each

concentration was pipetted onto the glass fibre within the device. The procedure included two washes with 70% ethanol and an elution step using 30  $\mu\text{L}$  of nuclease-free water. Subsequently, the paper strips from each device were placed in PCR tubes for RT-qPCR analysis, as detailed in **Chapter 3, Section 2.5**. After the RT-qPCR, the RNA concentrations were quantified using a standard curve, and the RNA recovery efficiency was calculated using the following equation.

$$\text{RNA recovery efficiency}\% = \frac{\text{Detected RNA}}{\text{Original RNA}} \times 100\%$$

### **5.2.5 The integration of LAMP assay with the paper microfluidic device**

To validate the use of the paper microfluidic device for on-site application, the loop-mediated isothermal amplification (LAMP) assay was integrated with it for SARS-CoV-2 detection. The templates were spiked wastewater samples, concentrations ranging from  $10^5$  copies  $\mu\text{L}^{-1}$  to  $10^1$  copies  $\mu\text{L}^{-1}$ , and the NTC was the nuclease-free water. The 50  $\mu\text{L}$  of sample was applied to each disc within the paper microfluidic device. Two washing steps were conducted using 70% ethanol, followed by elution with 30  $\mu\text{L}$  of nuclease-free water to transfer eluted sample to the reaction well. After transferring the sample from the paper microfluidic device to the plastic panel, 20  $\mu\text{L}$  of pre-mixed LAMP reagent was pipetted into the reaction wells, followed by sealing the panel with acetate film and heating.

#### **5.2.5.1 Fluorescent LAMP assay**

For fluorescent detection that can be observed by the naked eye, the real-time LAMP reaction mixture consisted of 25  $\mu\text{L}$ , which included 12.5  $\mu\text{L}$  of WarmStart LAMP 2X Master Mix, 2.5  $\mu\text{L}$  of primers mixed in a 1:8:2 ratio, 1.0/1.5  $\mu\text{L}$  of fluorescent dye, 5.0/4.5  $\mu\text{L}$  of nuclease-free water, and 5  $\mu\text{L}$  of template

RNA. NTCs were conducted by substituting the template RNA with 2  $\mu\text{L}$  of nuclease-free water. The reactions were conducted at 65°C for 40 minutes using a heat block.

#### **5.2.5.2 Colourimetric LAMP assay**

For colourimetric detection that can be observed by the naked eye, the real-time LAMP reaction mixture consisted of 25  $\mu\text{L}$ , which included 12.5  $\mu\text{L}$  of WarmStart colourimetric LAMP 2X Master Mix, 2.5  $\mu\text{L}$  of primers mixed in a 1:8:2 ratio, 5  $\mu\text{L}$  of nuclease-free water, and 5  $\mu\text{L}$  of template RNA. NTCs were conducted by substituting the template RNA with 5  $\mu\text{L}$  of nuclease-free water. The reactions were conducted at 65°C for 40 minutes using a heat block.

#### **5.2.6 The visualization of the CRISPR/Cas assay**

The CRISPR/Cas assay developed for the detection of SARS-CoV-2 has been described in detail in Chapter 4. However, it should be noted that the detection process requires the use of a PCR machine in order to obtain a fluorescent readout. In order to facilitate on-site analysis, the component of the CRISPR/Cas assay has been modified to permit observation without the use of laboratory equipment.

##### **5.2.6.1 The probe investigation**

To determine the optimal probe and concentration for on-site detection using the CRISPR/Cas assay, this study focused on the comparative analysis of two fluorescent probes: carboxyfluorescein (FAM) and carboxyrhodamine (ROX), both utilizing Black Hole Quencher 1 (BHQ1) as a quenching agent. The purpose of the assay was to differentiate between the Delta and Omicron variants of a virus. Accordingly, each variant-specific assay incorporated one of the probes for targeted detection, with previous studies indicating detection limits of 500 copies  $\mu\text{L}^{-1}$  for the Delta variant and 5 copies  $\mu\text{L}^{-1}$  for the Omicron variant. The concentration ranges for the assay templates were set from  $5 \times 10^5$  to  $5 \times 10^2$

copies  $\mu\text{L}^{-1}$  for the Delta variant and from  $5 \times 10^3$  to  $5 \times 10^0$  copies  $\mu\text{L}^{-1}$  for the Omicron variant.

The assay started with a LAMP process, wherein 2  $\mu\text{L}$  of the template was combined with 10  $\mu\text{L}$  of WarmStart LAMP 2X Master Mix, 2  $\mu\text{L}$  of mixed primers in a 1:8:2 ratio, and 6  $\mu\text{L}$  of nuclease-free water. This mixture was then incubated on a heating block set at 65 °C for 40 minutes. After the amplification, 10  $\mu\text{L}$  of the products were transferred to new PCR tubes, each containing 0.75  $\mu\text{L}$  of Cas12a enzyme (5  $\mu\text{M}$ ), 1.25  $\mu\text{L}$  of gRNA (5  $\mu\text{M}$ ) (gRNA-D2 and gRNA-O mentioned in **Chapter 4**), 2  $\mu\text{L}$  of reporter (10  $\mu\text{M}$ ) (either FAM or ROX), 2  $\mu\text{L}$  of NEBuffer, and 4  $\mu\text{L}$  of nuclease-free water. These tubes were incubated at 37 °C for 30 minutes on a heating block. Subsequently, the reactions were exposed to UV light for visualization of the results. The specific information of the FAM and ROX probes are listed in the Table 5.2 below.

**Table 5.2 The information of ssDNA fluorescent reporters**

| Name | Sequence (5'-3')              |
|------|-------------------------------|
| FAM  | /5'-FAM/GTATCCAGTGCG/3'-BHQ1/ |
| ROX  | /5'-ROX/GTATCCAGTGCG/3'-BHQ2/ |

#### 5.2.6.2 The Cas enzyme investigation

This study aimed to evaluate and compare the efficacy of three distinct Cas12a enzymes for on-site detection of SARS-CoV-2, utilizing a high-concentration RNA template. The enzymes tested included the EnGen® Lba Cas12a (Lba1) from New England Biolabs, the A.s. Cas12a Nuclease (As), and the L.b. Cas12a Ultra Nuclease (Lba2), both sourced from Integrated DNA Technologies. An RNA template of the original SARS-CoV-2 virus at a concentration of  $5 \times 10^5$  copies  $\mu\text{L}^{-1}$  was used for the assays.

The experimental procedure began with a LAMP reaction. In this step, 2  $\mu\text{L}$  of the RNA template was mixed with 10  $\mu\text{L}$  of WarmStart LAMP 2X Master Mix, 2  $\mu\text{L}$  of primers at a 1:8:2 ratio, and 6  $\mu\text{L}$  of nuclease-free water. The mixture was then incubated in PCR tubes on a heating block set to 65 °C for 40 minutes. Following amplification, 10  $\mu\text{L}$  amplicons were transferred into new PCR tubes containing the CRISPR/Cas mixture.

Each tube was supplemented with 0.75  $\mu\text{L}$  of one of the Cas12a enzymes at 5  $\mu\text{M}$  concentration, 1.25  $\mu\text{L}$  of gRNA-1 (the one referred in **Chapter 4**) at 5  $\mu\text{M}$ , 5  $\mu\text{L}$  of ROX/FAM reporter at 10  $\mu\text{M}$ , 2  $\mu\text{L}$  of NEBuffer and 1  $\mu\text{L}$  of nuclease-free water. These tubes were incubated at 37 °C for 30 minutes on a heating block. Also, a parallel group was PCR machine to observe the fluorescence signal results. Each enzyme was tested in a separate set of reactions to compare their relative efficacies. Post-incubation, the reactions were subjected to UV light exposure to facilitate visualization of the results.

## **5.2.7 The integration of the CRISPR/Cas assay with the paper microfluidic device**

### **5.2.7.1 The specificity test of the CRISPR/Cas assay**

The whole assay was divided into two main parts, involving the amplification by LAMP and the detection via CRISPR/Cas. Different LAMP primers and different gRNAs were specifically designed to distinguish Delta and Omicron from original sequence. To ensure the LAMP amplicons of Delta and Omicron can only be detected by its unique gRNAs, the RNA templates of original SARS-CoV-2, Delta variant, and omicron variant at  $5 \times 10^5$  copies  $\mu\text{L}^{-1}$  were prepared. Each template was amplified using three sets of LAMP primers, involving the N3 set, the Delta primer set, and the Omicron primer set. After the LAMP reaction, three amplicons of each template were detected using three gRNAs, including the gRNA-R1, gRNA-D2 and gRNA-O.

The experimental procedure began with a LAMP reaction. In this step, 2  $\mu\text{L}$  of the RNA template was mixed with 10  $\mu\text{L}$  of WarmStart LAMP 2X Master Mix, 2  $\mu\text{L}$  of primers at a 1:8:2 ratio, and 6  $\mu\text{L}$  of nuclease-free water. The mixture was then incubated in PCR tubes on a heating block set to 65 °C for 40 minutes. Following amplification, the amplicons were transferred into new PCR tubes. Each tube was filled with 1  $\mu\text{L}$  of Cpf1 (1  $\mu\text{M}$ ), 1  $\mu\text{L}$  of gRNA (1  $\mu\text{M}$ ), 1  $\mu\text{L}$  of FAM/ROX reporter (10  $\mu\text{M}$ ), 2  $\mu\text{L}$  of NEBuffer, and 5  $\mu\text{L}$  of nuclease-free water. These tubes were incubated at 37°C for 30 minutes using the QuantStudio 3 PCR Machine (Thermo Fisher, UK).

#### **5.2.7.2 Detection of SARS-CoV-2 and its variants with paper microfluidic device**

RNA templates for the Delta and omicron variants were prepared at a concentration of  $5 \times 10^5$  copies  $\mu\text{L}^{-1}$  and the templates were spiked into the wastewater. Before spiking, it was confirmed that the wastewater used did not contain templates for SARS-CoV-2. For Delta detection, the concentrations of spiked sample were from  $5 \times 10^4$  copies  $\mu\text{L}^{-1}$  to  $5 \times 10^2$  copies  $\mu\text{L}^{-1}$ . For Omicron detection, the concentrations of spiked sample were from  $5 \times 10^4$  copies  $\mu\text{L}^{-1}$  to  $5 \times 10^1$  copies  $\mu\text{L}^{-1}$ .

Fifty microliters of each of the mixed sample solutions were taken and loaded on the paper microfluidic device, performing the operation given in **Section 2.3**, including the two times of washing and one-time elution. After transferring eluted sample to the reaction panel, the premixed LAMP reagents were added to each well. Each reaction required 10  $\mu\text{L}$  of WarmStart LAMP 2X Master Mix, 2  $\mu\text{L}$  of primers at a 1:8:2 ratio, and 6  $\mu\text{L}$  of nuclease-free water. The reaction panel was then sealed with the acetate film and put on the heating box setting at 65°C for 40 minutes. Afterwards, the reaction panel was removed and placed the back side up, and the premixed CRISPR/Cas solution was pumped into the reaction chamber using 20  $\mu\text{L}$  to a pipette tip. The premixed CRISPR/Cas solution

contained 3  $\mu\text{L}$  of Cas12a enzyme Lba2 (5  $\mu\text{M}$ ), 5  $\mu\text{L}$  of gRNA (5  $\mu\text{M}$ ) (gRNA-D2 and gRNA-O mentioned in **Chapter 4**), 8  $\mu\text{L}$  of reporter (10  $\mu\text{M}$ ) (either FAM or ROX), and 4  $\mu\text{L}$  of NEBuffer, with the total volume was 20  $\mu\text{L}$  for single reaction. The reaction panel was sealed with the acetate film again at the back side, and then it was put on the heating box setting at 37°C for half an hour. Finally, the results could be observed by putting the reaction panel under the portable UV light.

## 5.3 Results and Discussion

### 5.3.1 Wastewater on-site enrichment approach investigation

The developed on-site enrichment approach was designed to concentrate the viral particles from the complex wastewater matrix using minimal resource, therefore the syringe-based filtration method was selected (Cao *et al.*, 2022). As previously investigated in **Chapter 3**, the PEG-based precipitation method was adopted to help precipitate virus particles from large volumes of wastewater at the first stage. After the precipitation, the aim was to concentrate and separate the virus from the raw wastewater using the syringe-based filtration method. The first filtration step was to remove the large particles from the wastewater, which may hinder the following amplification and identification processes. So, the filtration with big sizes was selected for the first-round filtration in this study, including the 0.45  $\mu\text{m}$  filter and the 0.20  $\mu\text{m}$  filter. The second filtration step was to keep the virus on the filtration membrane, and discard the filtrate, so the pore size of the filter membrane needed to be smaller than the size of the virus, which was 60 to 140 nanometers (Bar-On *et al.*, 2020). Based on this criterion, the MCE membranes with pore size at 25nm and 50nm were selected.

MCE is made from a blend of cellulose nitrate and cellulose acetate, combining mechanical strength and flexibility. The uniform pore structure of this material ensures stable filtration efficiency and high retention capacity, making it suitable for concentrating low-abundance analytes such as viruses (Junter and Lebrun, 2017). In addition, the material is hydrophilic, allowing for effective wetting and filtration of large amounts of aqueous solutions, resulting in high throughput (Majaneva *et al.*, 2018). The thin structure of this membrane is also designed for efficient filtration and reduced clogging, and it is flexible and easy to handle without breaking.

The real-time PCR cycle threshold ( $C_t$ ) values for the four filtration groups are presented in Figure 5.3. Group A, which utilized a 0.45  $\mu\text{m}$  pre-filtration step followed by a 25 nm MCE membrane, exhibited the lowest mean  $C_t$  values. The lower  $C_t$  values, the higher RNA concentration. As four groups used the same raw wastewater as the template, the higher RNA concentration meant the higher RNA concentration efficiency. This outcome suggests that the viral particles were effectively concentrated using this combination of filtration Group A.

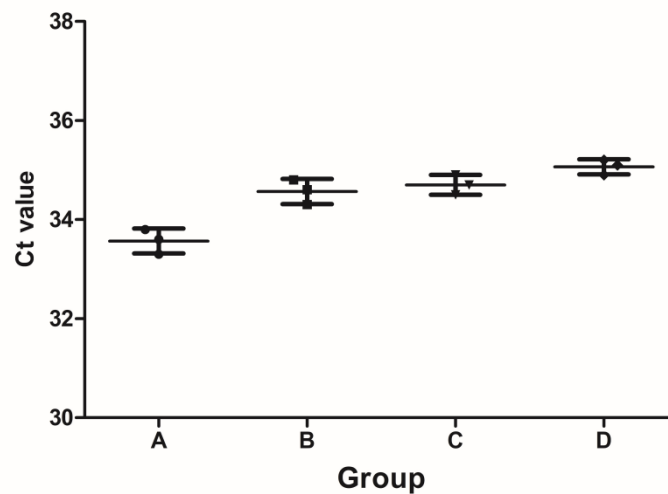


**Figure 5.3 The investigation of the filtration membrane**

**Group A (0.45  $\mu\text{m}$ ; 25 nm); Group B (0.45  $\mu\text{m}$ ; 50 nm); Group C (0.20  $\mu\text{m}$ ; 25 nm); Group D (0.20  $\mu\text{m}$ ; 50 nm)**

The Group B displayed higher  $C_t$  values than Group A, and the increase in  $C_t$  implies a reduced capture efficiency or slightly lower viral recovery when employing the 50 nm membrane. The reason may be the relatively larger pore size MCE membrane used in the second filtration to trap the virus particle. Although in theory the virus size was already larger than the pore size (50nm), there was possibly to have more viral particles trapped by the 25 nm MCE membrane.

$C_t$  values were also higher in Groups C and D compared to Group A, both of which underwent a more critical first filtration (0.20  $\mu\text{m}$ ) followed by filtration of 25

nm or 50 nm MCE membranes. These elevated  $C_t$  values suggest that the finer initial filtration step (0.20  $\mu\text{m}$ ) may have inadvertently reduced viral load. This is because the purpose of the first filtration is to remove large particulate impurities from a large volume of liquid, and the larger the pore size of the membranes used, the more wastewater impurities are removed. The presence of these impurities may interfere with subsequent filtration and the possibility of clogging of the micron-sized membranes cannot be ruled out, resulting in a less efficient second recovery and thus limiting the amount subsequently captured on the MCE membrane.

The differences in  $C_t$  values observed in the different filtration protocols emphasize the importance of selecting the optimal pore size at each step for the efficient concentration of viruses from environmental samples. The significantly lower  $C_t$  values associated with group A highlight the potential benefits of using a slightly coarser initial filtration (0.45  $\mu\text{m}$ ) and a tighter secondary membrane (25 nm) in favour of improved recovery of virus particles. These results are consistent with the premise that the initial filtration step does not unduly restrict the passage of viruses but instead removes as much as possible of the large particulate impurities that impede virus enrichment, and that subsequent fine filtration membranes maximise virus retention and enrichment.

For the on-site pathogens detection from environmental samples, the next step after the virus particle enrichment is the lysis, which is similar to the clinical point-of-diagnostics. In the use of the lateral flow assay (LFA) for SARS-CoV-2 detection, the process begins with obtaining a clinical sample, commonly a nasopharyngeal or nasal swab (Budd *et al.*, 2023). The swab is placed into a small volume of a specialized extraction or buffer solution. This step releases the target analyte—usually the viral antigen—into the solution, ensuring the sample is properly solubilized and ready for testing. Here, the target analyte was the nucleic acid instead of the viral antigen, so the different extraction buffer was used.

Two extraction buffers were investigated, and both were previously used in the Chapter 3, involving the lysis buffer (Group A) from QIAamp Viral RNA Mini Kit and the TRIzol/chloroform extraction combination (Group B), as displayed in the Figure 5.4. The mean  $C_t$  values for both groups were closely aligned, and statistical analysis indicated no significant difference (n.s.) between the two treatments (t-test). This demonstrates that the AVL buffer of the QIAamp kit and the TRIzol/chloroform method are equally effective in lysing SARS-CoV-2 particles and subsequently recovering RNA from wastewater samples. Although conventional extraction protocols often favour kits such as QIAamp for their simplified workflow, ease of operation and reliable results (Haramoto *et al.*, 2020). TRIzol/chloroform-based extraction methods offer a cost-effective and versatile alternative that has been widely reported in environmental nucleic acid sample recovery (Trujillo *et al.*, 2021). The conclusions of the various literatures regarding the efficiency of these two schemes for the recovery of viral RNA from wastewater are not consistent.

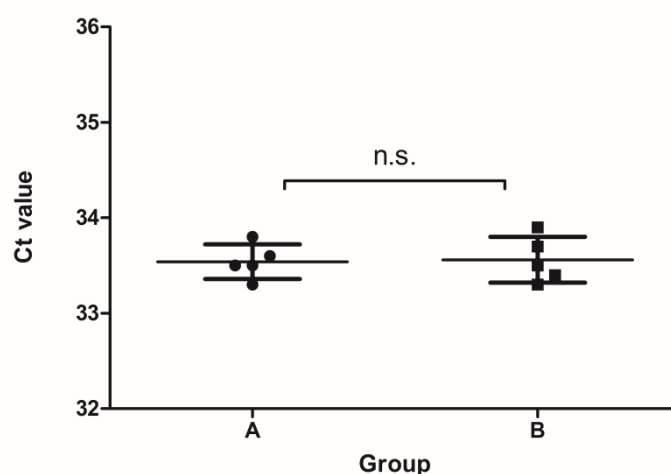


**Figure 5.4 The investigation of lysis buffer**

**Group A (AVE lysis buffer); Group B (TRIzol/chloroform)**

The lack of a significant difference in  $C_t$  values between the two approaches indicates that, under the conditions tested, both methods are capable of

effectively lysing viral particles and releasing RNA suitable for downstream RT-qPCR analysis. However, the AVL buffer was used as the preferred choice for the next experimental scenario, considering security issues. TRIzol is a widely used reagent for the isolation of RNA, but the hazardous chemicals it contains, which are phenol and guanidinium thiocyanate, require careful handling (Rio *et al.*, 2010). Phenol is corrosive and can cause severe chemical burns on contact with skin and mucous membranes (Agency for Toxic Substances and Disease Registry, 2014). It is also readily absorbed through the skin and may cause systemic poisoning. Guanidine thiocyanate is also harmful and can irritate the skin, eyes and respiratory tract on contact, and it is also present in the AVL lysis buffer from the QIAamp Viral RNA Mini Kit (Shen, 2019). Considering that the application scenario for virus lysis is a resource-limited field, a safer lysis buffer was chosen for subsequent operations. However, the TRIzol/chloroform method may serve as a flexible backup or adjunct to kit-based protocols.

### **5.3.2 The optimization of the paper microfluidic device**

The paper-based microfluidic device used in this study was originally inspired by the design reported by Yang *et al.* (2018). In their work, the platform had multiple channels dedicated to the detection of four different pathogens, with an additional channel serving as a negative control. Drawing on this basic concept, a simplified three-channel device was generated to the field detection of SARS-CoV-2 variants in wastewater samples. Each channel was optimized to identify different targets: one channel was used to detect the presence of SARS-CoV-2 in general, and the other two channels were used to distinguish between Delta and Omicron variants. By modifying the original multi-channel configuration to focus on a single pathogen with multiple variants, this updated device maintains the principle of multiplex detection while simplifying the detection methodology to meet current epidemiological challenges.

Investigating the glass fibre size and channel size in paper-based microfluidic devices is crucial for optimizing fluid flow, analyte transport, and overall assay performance. The glass fibre is the first medium on the device to load the lysed sample and serves to interact with the nucleic acids of the virus, retaining them on the surface. At the same time, the rest of the impurities are rinsed from the glass fibre onto the waste pad via two-time washing steps. It must therefore be sized to ensure that nucleic acids are maximally retained, and impurities are maximally removed. Finally, the RNA was transferred to the reaction well through the microfluidic channels driven by capillary action, and the capillary flow rate, fluid volume and sample distribution were significantly affected by the size of the channel (Noviana *et al.*, 2021). If the channel is too wide, it will reduce the capillary pressure, decreasing the flow rates. In addition, wider and longer channels imply a larger flow area, which may also result in more loss of RNA recovery, as a small amount of RNA may be retained in the flowing channels (Nguyen *et al.*, 2018). However, the channel width and length determine how much fluid can be transported at a given time. Larger channels can carry a greater volume of sample, potentially improving signal intensity if more analyte passes through to the detection zone (Nishat *et al.*, 2021). Therefore, it is significant to investigate the parameters of glass fibre and the microfluidic device, optimizing the device and ensuring the maximum RNA recovery efficiency.

The results in Figure 5.5 (a) show the relationship between the diameter of the glass fibre components and the RT-qPCR assay results. A total of five glass fibre sizes were evaluated, ranging in diameter from 3.0 mm to 5.0 mm. The smallest diameter tested (3.0 mm) produced relatively high  $C_t$  values, indicating low RNA recovery. As the diameter was increased to 3.5 mm, the  $C_t$  values decreased slightly, but did not result in optimal RNA retention. Notably, the  $C_t$  value reached its lowest point at a diameter of 4.0 mm, reflecting the most efficient capture and subsequent transfer of RNA through the microfluidic

channel. Outside of this optimal size, further increases in glass fibre diameter (4.5 mm and 5.0 mm) resulted in a slight increase in  $C_t$  values, suggesting that continuing to increase the area of the glass fibres instead resulted in a decrease in RNA recovery efficiency.

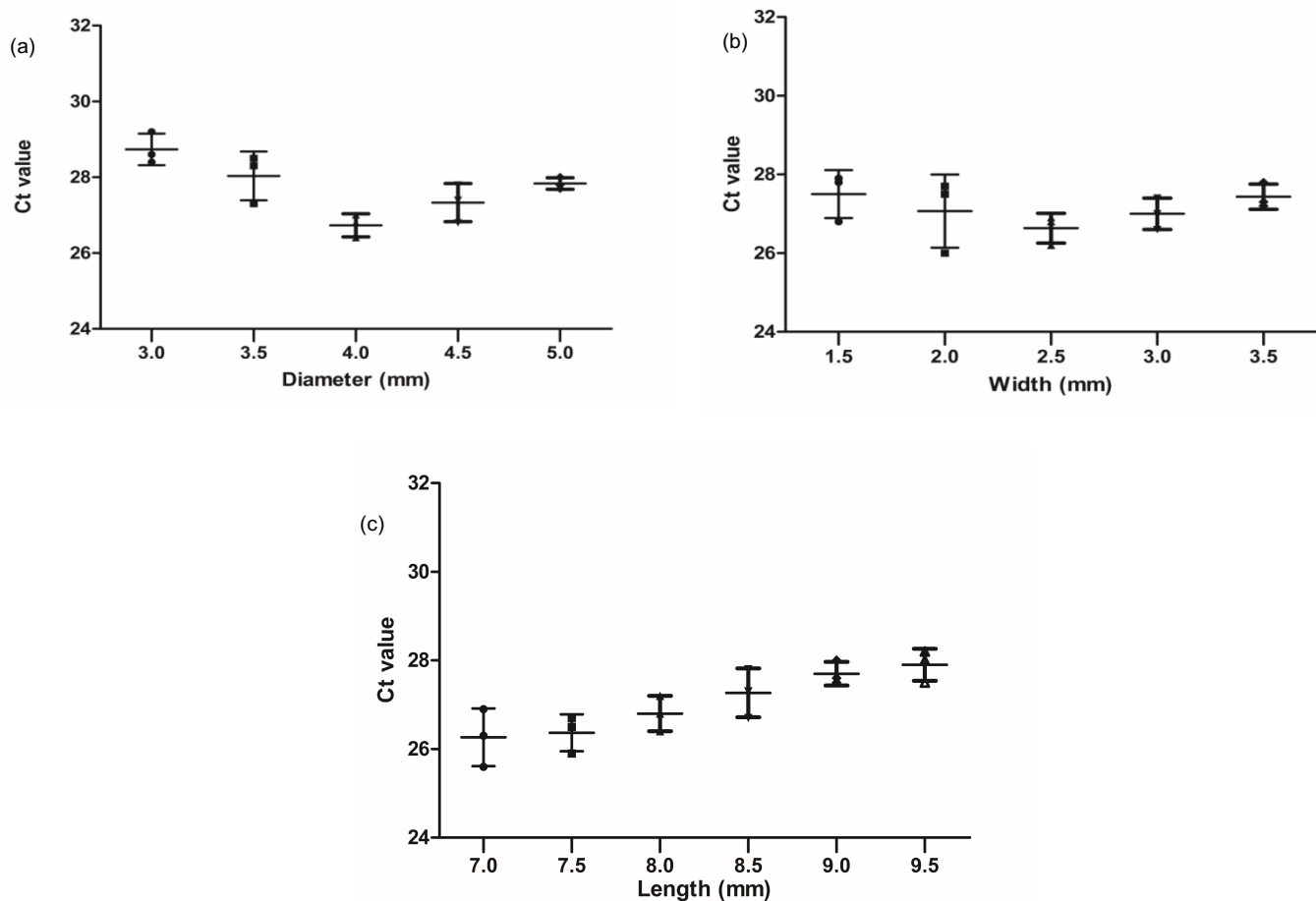


**Figure 5.5 The optimization of the paper microfluidic device.**

**(a)Glass fibre diameter; (b) Channel width; (c) Channel length**

A glass fibre diameter of 4.0 mm had the lowest detected  $C_t$  value and the highest detected RNA concentration, indicating that this size provided a balanced environment for effective RNA retention. When fibre diameter is too small, the reduced surface area or pore space may limit the ability of RNA to bind to the fibres. Conversely, if the fibre diameter is too large, it may result in loss of nucleic

acids during the washing step or lead to wider lateral dispersion, both of which dilute the final analyte concentration transferred downstream.

The results of how the width and length of the channel affects the RNA recovery efficiency of the paper microfluidic device are shown in Figure 5.5 (b) and (c). The channel widths tested ranged from 1.5 mm to 3.5 mm. Overall, the results show a non-linear relationship between channel width and RNA recovery efficiency. At narrower widths (1.5 mm and 2.0 mm), the  $C_t$  values were relatively high, indicating that the concentration of RNA recovered was not optimal. In contrast, as channel widths were increased to 2.5 mm and 3.0 mm,  $C_t$  values decreased, indicating improved RNA retention and more efficient transfer of nucleic acids to the detection zone. On the widest channel tested (3.5 mm), the  $C_t$  value increased again, indicating a possible decrease in RNA recovery. Based on these results, the channel width was determined to be 2.5mm.

These findings are consistent with those of previous studies on the importance of microfluidic geometry in optimizing analyte transport and retention in paper-based diagnostic equipment. In this study, intermediate channel widths (2.5 mm and 3.0 mm) appeared to strike a balance between fast fluid flow and efficient analyte recovery. At these sizes, the channels provided sufficient fluid volume to carry enough nucleic acids through the device while still maintaining good flow and reducing excessive lateral dispersion and analyte loss.

Channel lengths from 7.0 mm to 9.5 mm were evaluated as shown in Figure 5.5 (c). At shorter channel lengths (7.0 mm and 7.5 mm), the  $C_t$  values are relatively low, indicating more efficient RNA recovery. As the channel length increased above 8.0 mm, the  $C_t$  values gradually increased. Since more consistent results were observed in the 7.5 mm group and there was no significant difference in  $C_t$  values compared to the 7.0 mm channel, the optimal channel length was 7.5 mm. These results highlight the importance of tailoring channel sizes to maximize RNA recovery efficiency according to the specific

scenario. Such optimization ensures that the device can reliably detect target pathogens in challenging matrices such as wastewater, ultimately increasing the utility of point-of-use diagnostics in environmental monitoring and public health surveillance.

After optimizing the paper-based microfluidic device, it was also crucial to assess its RNA extraction efficiency and ensure it is suitable for field assay. The evaluation used the four SARS-CoV-2 RNA stock solutions with concentrations ranging from  $10^2$  to  $10^5$  copies  $\mu\text{L}^{-1}$  as the template. The paper microfluidic device was used to load the sample with known concentration, performing the washing and elution. At the end of the whole process, the eluted sample was transferred to a reaction panel fitted with small filter paper strips. Eventually, the small filter paper strips were then moved into PCR tubes and the recovered concentrations were quantified by RT-qPCR. The results are shown in the Figure 5.6, as there were three channels on each device, each concentration group presented three recoveries of individual channel.

At the highest concentration tested ( $10^5$  copies  $\mu\text{L}^{-1}$ ), recoveries were relatively similar for all three channels, around 15-25%. When the initial RNA concentration was reduced to  $10^4$  copies  $\mu\text{L}^{-1}$ , the average recovery for all channels increased slightly, around 25%. Notably, at an initial concentration of  $10^3$  copies  $\mu\text{L}^{-1}$ , the performance of one channel improved significantly, with recoveries approaching 35%, while the other two channels had more moderate recoveries around 20%. The large difference may be due to an error in the operation, which resulted in the liquid not flowing uniformly into the reaction wells during the elution process. There is also an error in transferring the small filter paper strip to the PCR tube. At the lowest input concentration ( $10^2$  copies  $\mu\text{L}^{-1}$ ), the device was able to maintain a detectable recovery, but the recovery was slightly lower ( $\sim 15\%$ ), reflecting the challenges inherent in capturing and eluting sparse analytes.

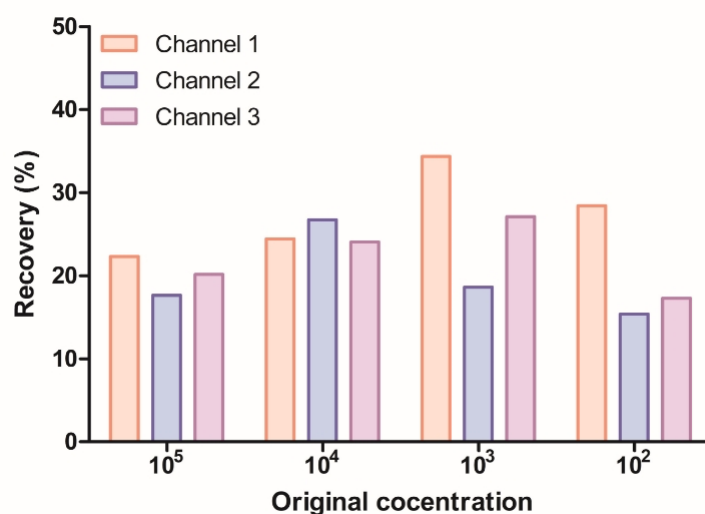


**Figure 5.6 The evaluation of the paper microfluidic device for RNA recovery**

These findings demonstrate that the optimized paper-based microfluidic device is capable of recovering SARS-CoV-2 RNA over a wide range of input concentrations. Considering that the raw sample had to go through three flow paths to reach the final reaction well, the ideal recovery should be around 33% without any losses. The results show that the recovery using the device was maintained at roughly 20%, a value that suggests that the overall recovery efficiency using the paper device was maintained at roughly 60% when compared to the theoretical recovery of 33%. Although there were differences between individual channels, this may be due to subtle differences in channel geometry, liquid distribution, or manufacturing processes.

At higher concentrations, the performance of the channels was relatively consistent, suggesting that the device was capable of reliably capturing large amounts of RNA. When the concentration decreased, the differences that appeared between the channels highlight the need for further refinement to improve consistency. At lower concentrations, the overall recovery was expected to decrease due to the reduced number of target molecules available for binding

and elution. Nevertheless, the ability of the device to detect and quantify RNA at low concentrations remains a positive indicator of its sensitivity.

Overall, these data emphasize the potential suitability of the device for field testing where the target viral load is highly variable. Future improvements may focus on standardizing the channel size, improving material homogeneity or introducing an additional concentration step to minimize variability and improve RNA recovery efficiency across the range of concentrations tested.

### **5.3.3 The integration of LAMP assay with paper microfluidic device**

This chapter outlines and optimizes a range of options for on-site wastewater analysis, including sample concentration, pathogen lysis, RNA purification and isolation. The final results of these steps can be used for on-site diagnostic testing and subsequent interpretation of results. A simpler alternative method, the LAMP assay, was used as an initial screening tool before the CRISPR/Cas-based method was employed for on-site testing. The reason for this approach is the procedural difference between the two technologies. Unlike the CRISPR/Cas assay, which requires a two-step reaction and therefore more time and complexity, the LAMP assay can be completed in a single step. Although some studies have proposed one-step CRISPR/Cas approaches, they are not compatible with the paper microfluidic device (Ding *et al.*, 2020; Li *et al.*, 2022). This feature makes LAMP particularly suitable for rapid, preliminary on-site testing to confirm the presence of SARS-CoV-2 in wastewater samples.

However, while LAMP has the advantage of speed and simplicity, it is not as discriminating as the CRISPR/Cas method, which is single-base specific. Therefore, once the LAMP assay results indicate the presence of a virus, the CRISPR/Cas assay can be used as a follow-up test to identify the specific variant of SARS-CoV-2. This two-tiered strategy of LAMP screening followed by targeted CRISPR/Cas analysis maximizes the efficiency and resolution of on-site testing, thus supporting more informed public health decision-making.

Two different LAMP approaches were studied, comprising the fluorescent LAMP assay and the colormetric LAMP assay, and the results were given in the Figure 5.7 and Figure 5.8. The Figure 5.7 (a) and (b) were the results of LAMP fluorescent assay, but the concentration of the fluorescent dye was 4.0  $\mu\text{M}$  and 6.0  $\mu\text{M}$ . According to previously study, the limit of detection of the LAMP assay was  $10^1$  copies  $\mu\text{L}^{-1}$ , so the spiked SARS-CoV-2 wastewater samples concentration from  $10^1$  copies  $\mu\text{L}^{-1}$  to  $10^5$  copies  $\mu\text{L}^{-1}$  were used as the templates (Broughton *et al.*, 2020). The results were displayed from left to right as negative control, minimum concentration of template ( $10^1$  copies  $\mu\text{L}^{-1}$ ), to maximum concentration of template ( $10^5$  copies  $\mu\text{L}^{-1}$ ), and the photos were taken at the beginning and the end of the reaction (40 minutes).

As shown in the Figure 5.7 (a) and (b), at the beginning of the assay, there was no signal could be observed in all the reaction panels, but some fluorescent signal could be observed after the 40-minute assay. When the concentration of FAM dye was 4.0  $\mu\text{M}$ , significant fluorescent dye could be observed in the templates of  $10^5$  copies  $\mu\text{L}^{-1}$  and  $10^4$  copies  $\mu\text{L}^{-1}$ . However, faint fluorescence may be present in the  $10^3$  copies  $\mu\text{L}^{-1}$  and  $10^2$  copies  $\mu\text{L}^{-1}$  templates, but it was indistinguishable from the negative control. Based on these findings, the concentration of the FAM dye was increased to 6.0  $\mu\text{M}$ , and improvements could be seen from the Figure 5.7 (b). By the end of the assay, all the template generated apparent signals, which were green light, but the negative control also generated weak background signal. Even the lowest tested concentration of  $10^1$  copies  $\mu\text{L}^{-1}$  produced a discernible green fluorescence by the 40-minute endpoint, indicating that enhancement of dye concentration can facilitate more consistent detection near the LOD. Although higher dye concentrations enabled clearer distinction of positive samples, a weak background fluorescence was also observed in the negative control. Despite this background, the contrast between positive and negative results remained sufficient to allow accurate interpretation.

Thus, careful optimization of dye concentration proved critical for maximizing assay sensitivity and interpretability.

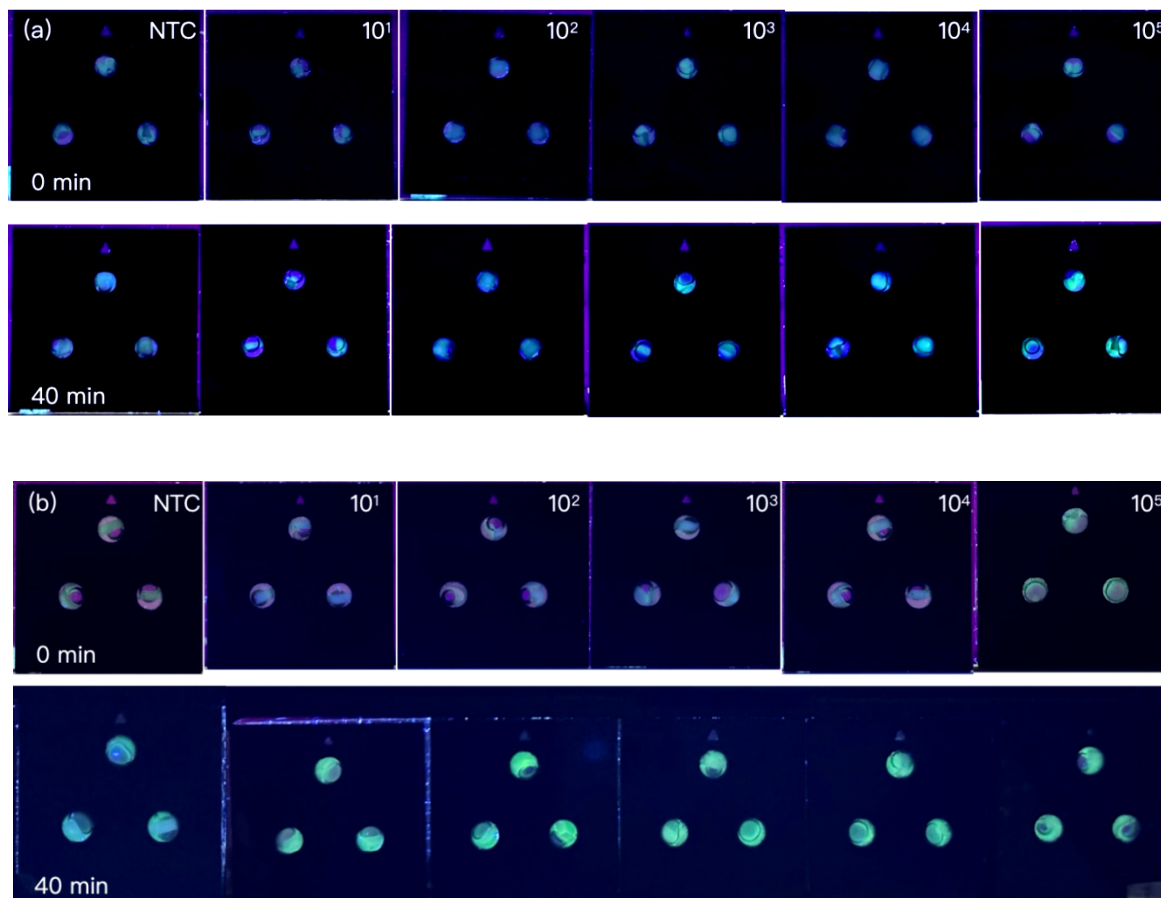


**Figure 5.7 The fluorescent LAMP assay with paper microfluidic device**

**(a) FAM concentration at 4.0  $\mu\text{M}$  (b) FAM concentration at 6.0  $\mu\text{M}$**

The observed fluorescence signals at the end of the reaction indicate that the LAMP assay can be easily integrated into a paper-based platform. By relying on fluorescent intensity, the detection of SARS-CoV-2 RNA becomes straightforward, enabling use in a variety of field conditions. However, one limitation is that fluorescence-based results currently require UV illumination and low-light conditions, which may constrain practical application in the field. In addition, the weak background signal observed in negative controls could complicate result interpretation. To address these challenges, a colourimetric LAMP assay was also investigated, offering a potentially simpler, more direct readout for on-site testing.

The mechanism of colourimetric LAMP assays relies on monitoring specific byproducts of the amplification reaction that lead to a visible change in color. Unlike fluorescent assays, which depend on specialized dyes and light sources, colourimetric methods use indicators that can shift color as the reaction proceeds, offering a simple, equipment-free readout (Wittaya Jomoui *et al.*, 2022). This study used a common pH-sensitive dye, which was phenol red. During the LAMP assay, when the large amounts of DNA are synthesized under isothermal conditions, another byproduct, protons, is generated by consuming dNTPs (Thi *et al.*, 2020). As the concentration of proton increases, the pH of the reaction mixture decreases. At the beginning of the assay, the presence of the phenol makes the liquid pink, as the mixture is not at a neutral pH (around 8.0-8.5). By the end of the assay, since the pH declines, the indicator presents to a distinctly different yellow colour, indicating that the amplification has occurred.

As presented in the Figure 5.8, at the beginning all samples, involving the NTC and those containing SARS-CoV-2 template concentrations from  $10^1$  to  $10^5$  copies  $\mu\text{L}^{-1}$ , appeared uniformly pink in the reaction well. After 40 minutes of heating and amplification, a significant difference emerged between the NTC and the spiked samples. While the NTC retained its pink color, each positive sample underwent a noticeable colour change, shifting from pink to various intensities of yellow. This shift from pink to yellow is a reliable indicator of the success of the target amplification, and the results only need to be read with the naked eye against a relatively clean background, without the use of specialized instrumentation. These results highlight the suitability of the colourimetric LAMP assay as a practical point-of-need diagnostic tool that can be coupled with designed paper microfluidic devices. Its clear visual endpoints reduce reliance on laboratory infrastructure, enabling users with limited resources or in field settings to screen samples rapidly.

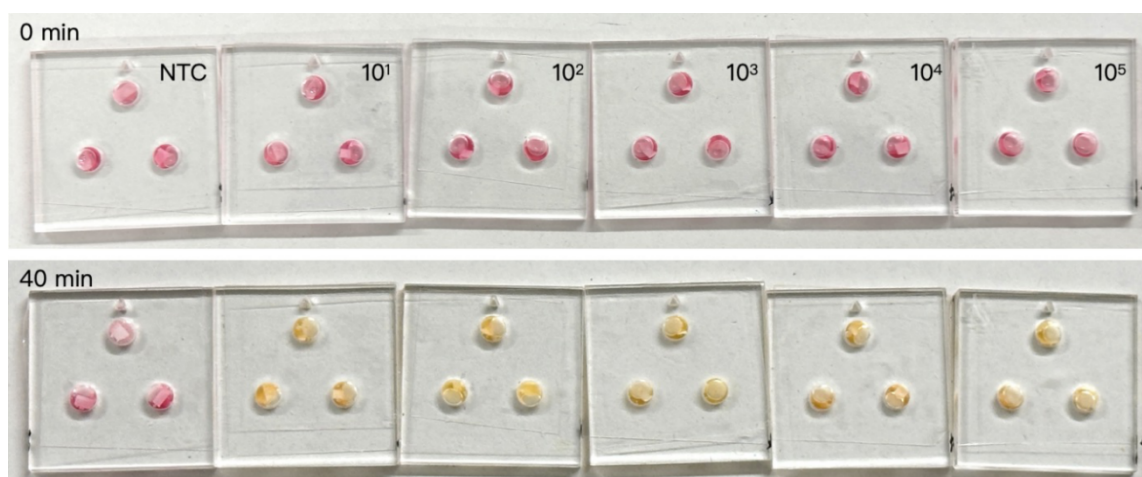


**Figure 5.8 The colourimetric LAMP assay with paper microfluidic device**

Despite its advantages, the colourimetric LAMP assay can be prone to generate false-positive results, which pose a significant challenge for field diagnostics. Unlike laboratory settings, where contamination can be minimized and conditions strictly controlled, on-site testing often involves more variable and less sterile environments. Non-specific amplification may occur due to poor primer design, impurities in the reagents, or the presence of inhibitory substances in the sample matrix, even if the operation is performed in a laboratory setting (Kim *et al.*, 2023). This has occasionally occurred in past experiments, as shown by the colour change that also occurs in the NTC group. When this occurs, it is not possible to accurately determine whether the colour change that occurs in the sample group is a false positive. As a result, users relying solely on colourimetric indicators might incorrectly interpret a yellow tint as evidence of target amplification, even when no target RNA is present. The false positive results occurred in fluorescent LAMP assay as well, but not as common as the colourimetric one (Aoki *et al.*, 2021). In some study, quenched fluorescent primers were designed to reduce the incidence of false positives originating from mis-priming events and improve LAMP assay reporting, since the primers became more specific (Hardinge and Murray, 2019).

Comparing the fluorescent and colourimetric LAMP assays reveals distinct advantages and drawbacks that influence their suitability for various testing scenarios. The fluorescent assay, which relies on dyes such as FAM, provides a highly sensitive and specific readout when optimized properly. However, the fluorescent assay's reliance on specialized equipment and the need for a low-light environment can limit its field applicability. In addition, weak background signal can be observed in NTC, increasing the risk of misdiagnosis and undermining trust in the test results. In resource-limited or on-site testing situations, these requirements may be impractical, reducing the assay's potential impact where rapid and accessible diagnostics are most needed.

In contrast, the colourimetric LAMP assay requires no special equipment and is much easier to use and promote. By employing a pH-sensitive, eye-visible colour change, it provides a simple and rapid endpoint. This method is more attractive for on-site diagnostics because it provides an immediate interpretation without the need for external instrumentation. However, colourimetric assays occasionally produce false-positive results, which may be due to non-specific amplification, contamination, or environmental factors affecting the pH of the assay. Such inaccurate results can have serious consequences, especially in public health surveillance or outbreak management.

#### **5.3.4 The visualization of the CRISPR/Cas assay**

Previous LAMP-based approaches have shown promise in detecting SARS-CoV-2 RNA in wastewater samples using paper microfluidic devices. However, several challenges remain before these devices can be reliably deployed for field analyzes. To address these issues and improve both accuracy and specificity, the CRISPR/Cas assay was incorporated into the microfluidic platform for more robust result interpretation. The first step in this process involved developing a

visualization method for the CRISPR/Cas assay that would enable field detection, thus eliminating the dependency on specialized fluorescence instrumentation.

The common techniques for CRISPR/Cas12 detection readout in resource-limited settings are handheld UV light and the LFA. By holding a LED-based blue-light source or a portable UV flashlight, the cleavage of the fluorescent reporter leads to a visible signal that can be seen by the naked eye in a darkened environment, which is similar with the fluorescent assay. When combining LFA with a CRISPR/Cas assay, a fluorophore or modified reporter gene cleaved because of the presence of the assay target produces a coloured line on the paper strip (Mukama *et al.*, 2020). This is due to the fact that the paper strip is pre-loaded with gold nanoparticles or latex beads on which antibodies or streptavidin molecules can interact with the reporter gene label (Broughton *et al.*, 2020). When a coloured line is generated on the strip, the presence of the target can be assumed, and the line can be read by the naked eye within a few minutes.

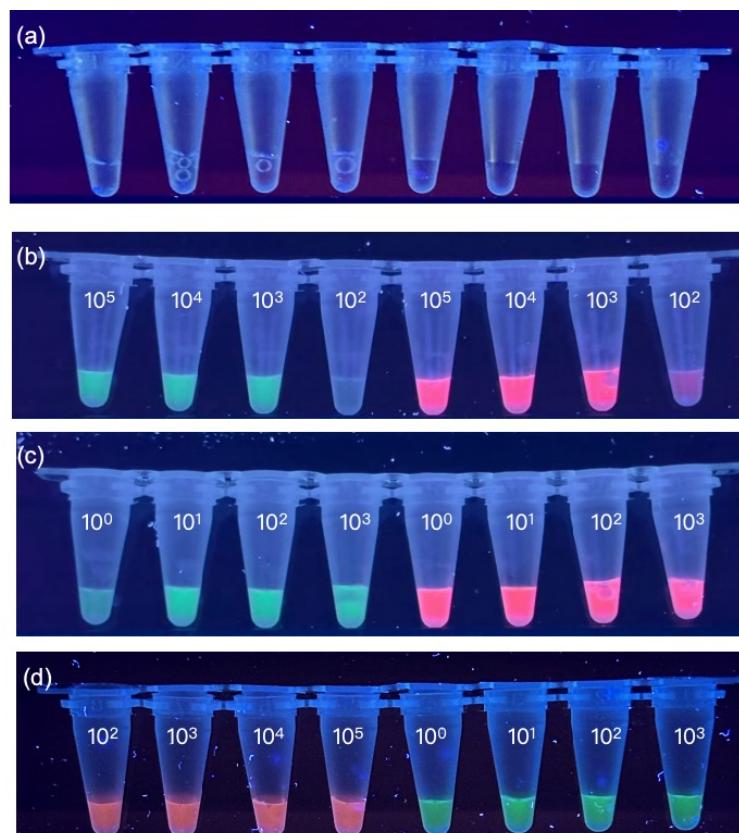


**Figure 5.9 The investigation of reporters in CRISPR/Cas assay**

**(a) The beginning point of the CRISPR/Cas assay**

**(b) The results of Delta detection**

**(c) The results of Omicron detection**

**(d) The results of improved Delta and Omicron detection**

In this case, the former approach was employed as the handheld UV light was available in the lab. In order to translate the established CRISPR/Cas assay for resource-limited use, some crucial components were investigated and adjusted, and the first was the probe. The performance of 16 different single-stranded DNA-fluorophore-quencher types for CRISPR/Cas12a-based visual colourimetric assays was assessed by Xie *et al* (2021). The findings showed that nine reporters were appropriate for direct visual colourimetric detection, with ROX-labeled reporters exhibiting particularly good performance. Significant colour changes were also seen in the FAM-labeled reporter's performance, but one drawback was the increased background noise, which might compromise the accuracy of detection. These two reporters were selected and compared for gRNA-D2 and gRNA-O detection.

Figure 5.9 illustrates the comparative performance of FAM- and ROX-based reporters in a CRISPR/Cas12a assay designed for rapid detection and differentiation of SARS-CoV-2 Delta and Omicron variants. Initially, as shown in Figure 5.9(a), all reaction tubes appeared colourless under UV illumination, indicating that before CRISPR/Cas-mediated cleavage, neither the FAM nor the ROX fluorophores were activated. After 30 minutes of incubation, clear fluorescence signals presented in samples where the Cas12a system successfully cleaved the target-specific reporter probes. The FAM-labeled probe emitted green fluorescence, while the ROX-labeled probe emitted red fluorescence. The intensity and clarity of these color changes were directly tied to both the probe used and the template concentration.

For the Delta variant (Figure 5.9(b)), both FAM (green) and ROX (red) showed distinct fluorescence at high template concentrations ( $\geq 10^3$  copies  $\mu\text{L}^{-1}$ ). However, at lower concentrations ( $10^2$  copies  $\mu\text{L}^{-1}$ ), the FAM signal diminished significantly, becoming nearly indiscernible. In contrast, the ROX probe still generated a weak yet clearly visible red fluorescence at these low concentrations. Although not as intense as at higher concentrations, the persistent ROX signal at the low end of the detection range suggested better sensitivity and reliability compared to FAM.

A similar pattern was observed for the Omicron variant (Figure 5.9(c)). At high-concentration templates, both FAM and ROX probes yielded robust fluorescence, demonstrating that, under optimal conditions, both reporters were capable of providing a clear positive signal. Nevertheless, as the template concentration decreased, the relative performance of the two fluorophores again diverged. Even at low levels of Omicron target, the ROX probe consistently produced more pronounced fluorescence than the FAM probe, highlighting its superior sensitivity.

These findings were consistent with previously published results (Xie *et al.*, 2021), reinforcing the conclusion that ROX offers more reliable and sensitive detection in low-concentration scenarios. From a purely analytical standpoint, ROX appears to be the optimal choice if only one reporter could be selected for sensitive detection. However, the overarching goal of this study is to integrate Delta and Omicron variant detection into a single testing device for simultaneous screening. To achieve clear multiplexing, it is advantageous to assign distinct fluorescent reporters to each variant. This approach ensures that a single positive test producing different colors can be easily interpreted as the presence of multiple variants, or the presence of one variant over the other, without ambiguity. Given the performance characteristics observed, it is logical to use the more sensitive ROX reporter for detecting the Delta variant, as Delta appears more

challenging to detect at lower concentrations. In contrast, the slightly less sensitive but still reliable FAM reporter can be designated for the Omicron variant.

Figure 5.9(d) presents the CRISPR/Cas12a-based Delta and Omicron variant detection assay after increasing the concentration of both the ROX and FAM reporters from 1  $\mu$ M to 2.5  $\mu$ M. This enhancement in reporter concentration was introduced to improve the visibility and distinction of fluorescence signals, especially at lower template concentrations where previous tests showed weaker color changes. Such a strategy supports the goal of multiplexed variant detection on a single platform. Assigning each variant a unique fluorescent signal helps ensure a straightforward, color-based interpretation of the results.

Another important component in the CRISPR/Cas system is the Cas12 enzyme, because the fundamental sensitivity limit of this assay is governed by kinetic rates of the Cas12 enzyme itself (Huyke *et al.*, 2022). Two subtypes of CRISPR-Cas12 effector proteins were comprehensively investigated for nucleic acid diagnostics, and they were Cas12a ortholog of Lba Cas12a (from *Lachnospiraceae* sp.) and A.s. Cas12a (from *Acidaminococcus* sp.) (Zetsche *et al.*, 2015; Gao *et al.*, 2017). LbaCas12a has been reported to be generally robust and relatively efficient for genome editing of a wide range of targets and to be superior to AsCas12a (Wang *et al.*, 2018).

Figure 5.10 compares the performance of three Cas12a effectors—Lba Cas12a from NEB (Lba1), Lba Cas12a from IDT (Lba2), and As Cas12a from IDT (As)—in a CRISPR/Cas-based assay for detecting Delta and Omicron SARS-CoV-2 variants. The assays were performed in duplicate and included positive controls (indicated as “+”) and negative controls (indicated as “–”) for both target variants. Figure 5.10(a) shows the reaction tubes at the beginning of the assay, while Figure 5.10(b) shows the results after 30 minutes of incubation at 37°C.

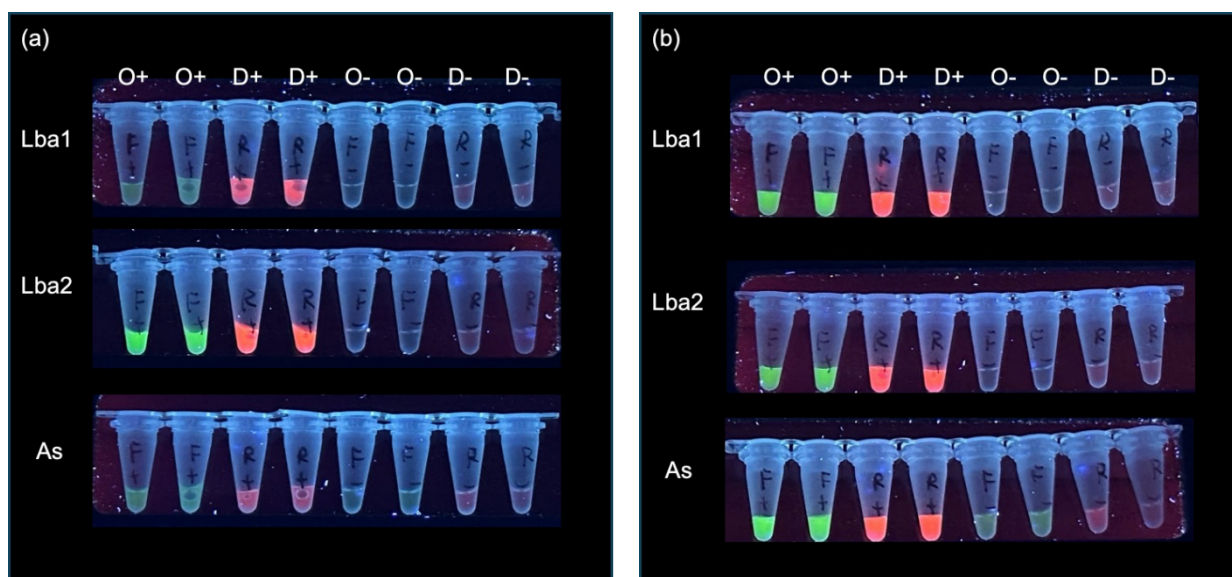


**Figure 5.10 The investigation of Cas12a enzyme**

Noticeable fluorescence was detected in the positive control at the beginning of the assay (Figure 5.10(a)), even before the system reached to its optimal reaction conditions. This early fluorescence can be attributed to the elevated temperature of the LAMP amplicons ( $\sim 65^{\circ}\text{C}$ ) when mixed with CRISPR/Cas reagents. Since the CRISPR/Cas system has an optimal reaction temperature of 37 degrees Celsius and activates rapidly upon recognition of the target, even a brief overlap between the higher temperature and the target availability may immediately result in cleavage of the fluorescent reporter. Among the tested enzymes, Lba2 exhibited the strongest initial activity, producing a pronounced fluorescence signal in the positive tubes at the initial time point. In contrast, As Cas12a demonstrated some unexpected background fluorescence in the negative controls, suggesting off-target activity or non-specific cleavage (Kleinstiver *et al.*, 2016).

After 30 minutes of reaction time (Figure 5.10(b)), strong and distinct fluorescence signals were observed in all positive controls across the three Cas12a proteins, indicating robust and consistent target recognition and cleavage. Both Delta and Omicron variants could be clearly identified, emitting strong red (ROX) and green (FAM) fluorescence. This confirms that, given

sufficient reaction time, all three enzymes are capable of detecting their intended targets with high efficiency. However, differences in specificity remained evident. While Lba1 and Lba2 continued to show negligible background in negative controls, but Cas12a maintained a high level of non-specific signal. This persistent background suggests that As Cas12a may have reduced specificity in this particular setup, potentially complicating result interpretation and requiring careful optimization.

LbaCas12a variants have been widely recognized for their robust genome-editing capabilities and relatively high on-target efficiency (Kleinstiver *et al.*, 2019). These findings align with previous reports, both Lba1 and Lba2 produced strong target signals with minimal background fluorescence, thereby providing clear and reliable results. Lba2, in particular, demonstrated exceptional sensitivity, exhibiting immediate fluorescent signals in positive samples even before the reaction settled at the optimal 37°C temperature. Such heightened responsiveness can be advantageous for rapid testing scenarios, where rapid detection and minimal assay time are critical.

### **5.3.5 The integration of CRISPR/Cas assay with paper microfluidic device**

The development of a CRISPR/Cas12a-based visual colourimetric assay to differentiate between Delta and Omicron variants has been completed, and the next step is to integrate the assay with the designed paper-based microfluidic device. The previous process of integrating the paper microfluidic device with the LAMP assay was relatively simple because the LAMP assay requires only one step to perform the reaction at a constant temperature. When it comes to the CRISPR/Cas assay, it involves two separate reactions that successively should be incubated at different temperatures. According to this, a different reaction panel was designed to fit the paper microfluidic device, which included two sets of reaction chambers, connecting the two sets of chambers with a channel.

A schematic of the two-group reaction chamber is presented in the Appendix. Although it was studied, it was not used to complete the two-step CRISPR/Cas reaction. The primary issue was that the channels within the chamber made it larger. At the LAMP incubation temperature of 65°C, certain reaction components evaporated, migrated into these channels, and then condensed, resulting in a loss of necessary reagents. This loss prevented the first amplification step from occurring, ultimately reducing the number of amplicons available for the CRISPR/Cas system to recognize.

To address these issues, the two-step reaction was conducted in the same chamber but with a reduced volume. The original reaction plate featured three circular reaction wells arranged in an equilateral triangle. By enlarging each well's diameter to 5 mm, the increased volume could accommodate the necessary liquids for both steps of the reaction. Figure 5.11 shows the operational schematic of the reaction panel.

First, the reaction panel is divided into two sides, A and B. After adding the eluted sample to the plate, the side facing the operator is labeled as side A. Once the LAMP mixture is added, the plate is sealed with film and placed in a heating box at 65°C for 30 minutes with side A facing up. Next, the plate is flipped so that side B faces up, and the pre-mixed CRISPR/Cas solution is introduced into the reaction wells using a pipette. The pipette tip (20 µL) can pierce the sealing film, allowing for direct addition of the CRISPR/Cas mixture. After this step, the plate is sealed again and placed B-side up on a heating table. Upon completion of the 30-minute reaction, results can be visualized using a portable UV lamp.

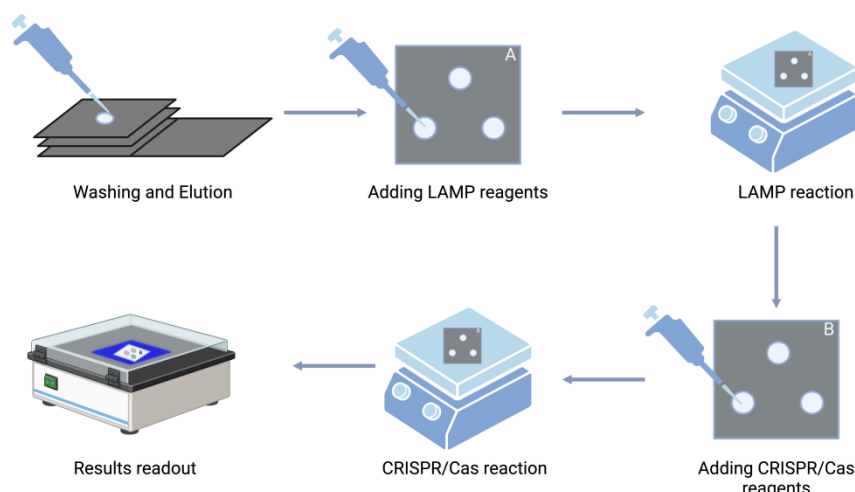
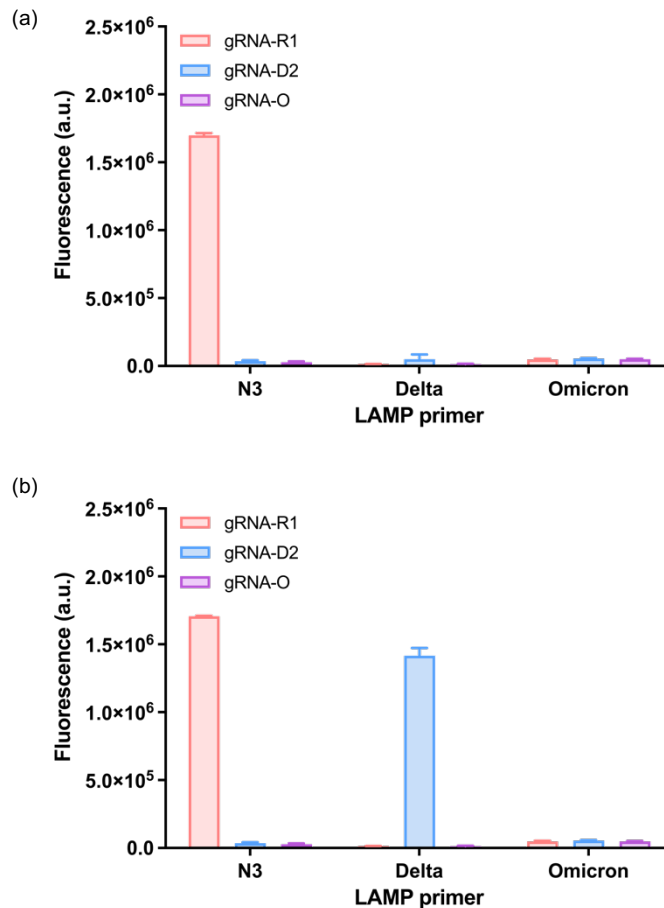


**Figure 5.11 The operation of the reaction panel**

One potential challenge is the introduction of the CRISPR/Cas mixture through the seal. Improper piercing or handling can lead to liquid spillage and possible contamination. Since assays for SARS-CoV-2, Delta, and Omicron variants are conducted on the same panel, it is crucial to prevent cross-contamination between wells. Ensuring proper handling and sealing helps maintain the specificity of each assay and avoids unintended recognition of amplicons by other CRISPR/Cas reaction wells. It is also important to ensure the specificity of each assay to prevent false-positive results in the event of oozing fluid into other reaction wells.

The specificity results of each assay are presented in the Figure 5.12. Three templates were amplified with three LAMP primer sets (N3, Delta set and Omicron set), including the original SARS-CoV-2 sequence (a), the Delta variant (b), and the Omicron variant (c). Following amplification, the samples were tested with three distinct gRNAs: gRNA-R1, gRNA-D2, and gRNA-O. Among them, gRNA-R1 is for the identification of SARS-CoV-2, and a positive reaction indicates the presence of the virus, regardless of the presence of the mutant strain. gRNA-D2 and gRNA-O are specific for the Delta variant and the Omicron variant identification and produce strong fluorescent signals only when the corresponding strains are present.

Figure 5.12 (a) shows the results of a specific assay using the original SARS-CoV-2 as a template. After amplification and detection, only the group amplified with the N3 primer set and analyzed with gRNA-R1 showed a strong fluorescent signal. This is because the original sequence does not contain the delta and omicron mutations and therefore the delta and omicron specific assays do not give positive results. The absence of fluorescent signal after amplification using the N3 primer set and analysis using gRNA-D2 and gRNA-O also indicates that the assay is highly specific. False positive results will not occur if an error occurs during the procedure that causes the reaction solution for broad spectrum SARS-CoV-2 to flow into the reaction wells for Delta or Omicron.



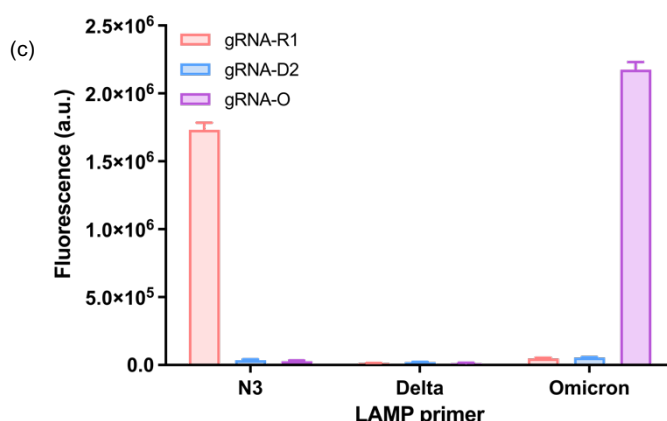


**Figure 5.12 The specificity of CRISPR/Cas assays**

**(a)Original SARS-CoV-2 as template**

**(b)Delta variant as template**

**(c)Omicron variant as template**

Similar results are presented in Figure 5.12 (b) and (c), for identifying Delta and Omicron respectively. The specific assays against the variants all showed strong signals when the detected object was a variant, while the broad-spectrum test against SARS-CoV-2 also showed strong positive results. In addition, two other non-specific gRNAs failed to recognise the amplicons after specific amplification was used. For instance, in the Figure 5.12 (b), after the use of delta-specific primers to amplifying the target, only the gRNA-D2 could generate sufficient fluorescence, while the gRNA-R1 and gRNA-O had the negative results. This suggests that in the assay targeting Delta variants, even if the amplicons from the previous step accidentally flowed into the other reaction wells during the addition of the CRISPR/Cas solution, the results of the test would not be disturbed. The results regarding the specificity of the Omicron assay were similar, demonstrating the extremely high specificity of the established CRISPR/Cas method for recognising variants. When making it compatible with paper microfluidic devices, its extremely high specificity prevents interference with the accuracy of the results, even in the event of an operational error.

After confirming the specificity of each CRISPR/Cas assay, the paper microfluidic device was employed for spiked wastewater sample detection. Different concentrations of spiked Delta wastewater sample and Omicron wastewater sample were prepared. The paper microfluidic device was used to extract the nucleic acid from samples with complex matrix, and the eluted RNA was transferred to the reaction panel. The established CRISPR/Cas fluorescent assays were conducted on the reaction panel, and the results were interpreted with the UV light, as shown in Figure 5.13.

As shown in the figure, when the analyte was the Delta variant, there were two reaction wells with a significant fluorescence colour change, both red. The top circle was used to identify the SARS-CoV-2 virus, which fluoresces pink when detected by the CRISPR/Cas system, regardless of the variant being detected, as long as it is present in the sample. The well on the left in the second row was designed for the analysis of the Delta variant, and if the variant was present, this reaction compartment will also show pink after a two-step reaction. As the template concentration decreased, the colour change in the left well became weaker, but the fluorescent signal from the top well was always evident. This was because the Delta-specific assay was not as sensitive as the SARS-CoV-2 assay. In the negative control group, all three wells showed no colour change.

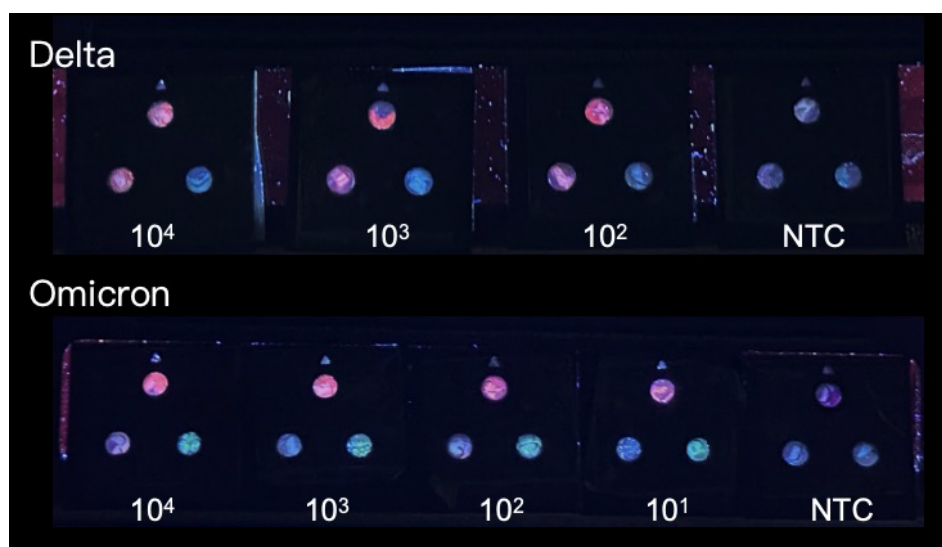


**Figure 5.13 The detection of Delta and Omicron variants with paper microfluidic device**

The Omicron-specific assay had comparable results, and the limit of detection was lower at  $10^1$  copies  $\mu\text{L}^{-1}$ . The reporter used in the Omicron-specific assay was FAM probe, which has lower sensitivity compared to the ROX, but it also performed well when adapted to FAM due to the better sensitivity of the developed CRISPR/Cas assay for Omicron analysis. The presence of the Omicron variant resulted in the green colour change in the lower right well of the figure. Although the green fluorescence signal was not as strong as the pink one in the figure, and the results were still more obvious to the naked eye, especially when compared to the negative control.

The specificity of the CRISPR/Cas assays, as validated in the laboratory, is effectively translated into real-world applications using paper microfluidic devices. These devices simplify the process of extracting nucleic acids from challenging samples such as wastewater, where the presence of numerous contaminants could potentially interfere with detection. By using a paper-based system, the process becomes more accessible and feasible for on-site operations without the need for sophisticated laboratory equipment. This approach not only reduces the

time and cost associated with sample processing but also enhances the ability to perform rapid testing in the field. The colourimetric assays performed on the reaction panel allow for visual interpretation of results under UV light, simplifying the detection process. The distinct color changes (pink for SARS-CoV-2 and green for Omicron) facilitated by the CRISPR/Cas system provide clear, easily interpretable indicators of the presence of specific variants.

## 5.4 Conclusions

This study presents the development and optimization of a paper-based microfluidic device for the on-site detection of SARS-CoV-2 in wastewater samples. The research addresses critical steps including sample concentration, RNA extraction, and the integration of nucleic acid amplification and detection assays, ultimately enabling rapid, accurate, and field-ready diagnostics for public health surveillance.

The detection of low-abundance viral targets depends on the efficient processing of wastewater samples. Syringe-based filtering and PEG precipitation worked well together to enrich SARS-CoV-2 RNA from intricate wastewater matrices. A 0.45  $\mu\text{m}$  pre-filter step followed by a 25 nm MCE membrane had the best RNA recovery efficiency among the examined filtration methods, as seen by the lowest  $C_t$  values in RT-qPCR experiments. By balancing the removal of contaminants with the capture of virus particles, this approach highlights the significance of pore size optimization for both filtering phases. The use of AVL buffer for viral lysis provided a safer and more efficient substitute for TRIzol/chloroform, allowing for RNA recovery while lowering the possible dangers related to dangerous chemicals.

The goal of the paper microfluidic device's design was to maximise RNA recovery while maintaining ease of use and field compatibility. Significant gains in test performance were achieved by optimizing important parameters, such as the capillary pathway length, microfluidic channel width, and glass fibre diameter. Fluid retention, flow rate, and nucleic acid recovery were best balanced with a glass fibre diameter of 4.0 mm, a channel width of 2.5 mm, and a channel length of 7.5 mm.

Tests of RNA recovery efficiency at various input concentrations confirmed the device's resilience. Variability rose at lower RNA concentrations, suggesting

potential for better liquid distribution and channel homogeneity, even while greater concentrations produced steady recovery rates. However, the device's potential for wastewater surveillance is highlighted by its capacity to detect RNA at quantities as low as  $10^2$  copies  $\mu\text{L}^{-1}$ .

The effective integration of LAMP assays with the paper microfluidic technology allowed for the rapid and accurate detection of SARS-CoV-2 RNA. Both colourimetric and fluorescent LAMP tests were assessed. High sensitivity was established by fluorescent LAMP using FAM dye, although signal observation needed additional equipment and dark surroundings. Although poor background fluorescence in negative controls presented difficulties, dye concentration optimization increased assay clarity at low RNA concentrations. The colourimetric LAMP assay, on the other hand, offered a more straightforward, equipment-free substitute with findings that could be easily understood. Due to pH changes during amplification process, the colour changed from pink to yellow, making it easy to detect SARS-CoV-2 RNA. However, sporadic false positives limited its wider use for on-site analysis and brought attention to the necessity of cautious primer design and contamination management.

The CRISPR/Cas assay was included into the microfluidic device to improve specificity and variant discrimination. Changes made it possible to use UV light for visual readouts, doing away with the need for fluorescence detection in a lab. Comparative probe investigations showed that ROX-labelled reporters were more sensitive than FAM-labelled reporters, especially at lower RNA quantities. Additionally, the study confirmed that the CRISPR/Cas assay has a high degree of specificity for identifying SARS-CoV-2 and its Delta and Omicron variants. Different fluorescent reporters were assigned to each variation in order to accomplish multiplexed detection. The assays' robustness and dependability are shown by their capacity to differentiate between variations even when operational mistakes are present. A complete solution for on-site SARS-CoV-2 detection is provided by the combination of the paper microfluidic device with the LAMP and

CRISPR/Cas assays. The device is suitable for field applications because of its portability, ease of use, and low resource requirements, especially in environments with restricted resources. Efficiency and diagnostic resolution are maximized by the two-tiered diagnostic approach, which consists of initial LAMP amplification followed by CRISPR/Cas-based variant detection.

But there are still some challenges for real sample analysis. The first issue that needs to be addressed is the recovery of low-concentration samples, which requires further enhancement of the performance of the paper microfluidic device to ensure higher RNA recovery efficiency. In addition, the established CRISPR/Cas assay showed high sensitivity in the identification of Omicron variants, but the sensitivity of Delta variants needs to be improved. Reliability of field operations also depends on maintaining uniform reagent handling and avoiding cross-contamination, and perhaps the problem of two-step reaction spiking could be addressed through better device design. This study ultimately used spiked samples for testing and did not involve the testing of real samples. In the future, further validation experiments can be conducted to ensure the reliability and robustness of the established method under the condition of ensuring the presence of viral variants in the real samples.

In conclusion, this study shows that a paper-based microfluidic system can be used to rapidly and on-site identify SARS-CoV-2 and its variants from wastewater. An important development in environmental monitoring and public health surveillance is the device's combination of optimized RNA extraction, LAMP amplification, and CRISPR/Cas-based analysis. This technology has the potential to enhance epidemic control and slow the spread of SARS-CoV-2 and its variations by facilitating prompt and precise diagnoses. To aid in the worldwide fight against infectious diseases, future research will concentrate on improving the device's sensitivity, consistency, and applicability to more infections.

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## Chapter 6: Overall Discussion and Conclusions

### 6.1 Introduction

Wastewater-based epidemiology was originally proposed to track the illicit drugs in communities, as this field has advanced, it has been applied to pathogens, biomarkers, or antimicrobial resistance (AMR) genes detection (Choi *et al.*, 2018; Sims and Kasprzyk-Hordern, 2020). During the COVID-19 pandemic, it played an important role as a complementary approach to clinical diagnostics, providing early warning surveillance of SARS-CoV-2 circulation in communities (Bibby *et al.*, 2021). Large volumes of wastewater samples were collected from wastewater treatment plants and delivered to analytical laboratories where viral RNA was concentrated and extracted for quantification using quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays (Bivins *et al.*, 2020b). By collecting samples regularly, it is possible to monitor changes in the concentration of viral load from wastewater over time. The data obtained can be viewed as trends in infection in the community and an estimate of the infected population and compared with data obtained in the clinic (McMahan *et al.*, 2021). In addition, next-generation sequencing technology can be used to identify emerging variants and their distribution in the community, and early signals can help policymakers take public health action. (Karthikeyan *et al.*, 2022).

One of the most significant advantages of WBE is its early warning capability, given that SARS-CoV-2 can be shed in feces before symptoms appear—even by asymptomatic individuals (Bivins *et al.*, 2020a). Consequently, a sharp increase in viral load in wastewater can signal an imminent outbreak before clinical cases are officially confirmed (Zhu *et al.*, 2021; Trigo-Tasende *et al.*, 2023). Another benefit is cost-effectiveness: WBE can capture infections in a measured population without requiring large-scale individual screening, which is especially valuable in resource-limited settings or when public health systems are

overwhelmed (Shrestha *et al.*, 2021). However, current wastewater analysis methods rely heavily on laboratory-based assays. While these assays are sensitive and specific, they require specialized personnel, expensive equipment, sample transport, and lengthy turnaround times, all of which delay reporting. Addressing these challenges would greatly amplify the advantages of WBE, enabling broader applications and a more substantial contribution to public health.

This PhD research aimed to address these limitations by integrating advanced molecular diagnostics, such as CRISPR/Cas12a technology, with paper-based microfluidic devices to develop innovative tools for SARS-CoV-2 and its variants detection in wastewater. This chapter synthesizes the key findings of the thesis, highlights the contributions to advancing WBE and molecular diagnostics, and discusses their implications for public health. It also outlines the research limitations and proposes future directions for developing and applying field-deployable diagnostic platforms.

## **6.2 Key findings and contributions**

The thesis offers novel insights into the optimization of conventional approach for SARS-CoV-2 analysis in wastewater, long-term wastewater monitoring at Cranfield University, the development of CRISPR/Cas12a-based fluorescent assays, and the design of paper microfluidic devices for on-site detection.

## **A) The optimization of wastewater concentration approach and wastewater surveillance in Cranfield University**

This chapter focuses on the optimization of conventional wastewater analysis methods for detecting SARS-CoV-2 and offers insights into a two-year wastewater surveillance study conducted at local wastewater treatment plant (**Objective 1**). Through methodical experimentation and analysis, the study highlights the efficacy of WBE as a critical public health tool and details advancements in sample processing for enhanced RNA recovery.

Since virus concentration and extraction are critical initial steps in wastewater analysis, a significant finding of this chapter is the determination that polyethylene glycol (PEG) precipitation is superior to ultrafiltration for concentrating SARS-CoV-2 RNA from wastewater, as PEG consistently demonstrated higher viral RNA recovery efficiencies. Following comparing two methods, the following key outputs was optimizing the PEG precipitation protocol to establish a more efficient and time-saving approach for routine wastewater analysis. Unlike conventional protocols, the optimized method excluded the centrifugation step for large solids removal, thereby enhancing RNA recovery, likely due to the retention of viral particles adsorbed onto suspended solids. A dual-extraction process—combining TRIzol/chloroform and the QIAamp® Viral RNA Mini Kit—further maximized RNA recovery while effectively removing inhibitors. Additionally, reducing the precipitation time from 24 hours to immediate processing not only improved RNA recovery but also made the method significantly more time-efficient, particularly in high-throughput scenarios.

Following the development and optimization of the wastewater analysis method, it was employed for a two-year surveillance project on the Cranfield University campus. Weekly sampling revealed transmission dynamics, with a key finding that peaks in viral RNA concentration often preceded clinically reported cases. This finding highlighted WBE's value as an early warning system for

outbreaks. Moreover, WBE demonstrated its capability to detect asymptomatic and hidden cases, as evidenced by high viral loads in wastewater during periods of low clinical reporting, addressing gaps in testing and providing a more comprehensive view of community transmission. Long-term wastewater surveillance also proved essential beyond the height of the epidemic. In 2023, when free individual clinical testing was no longer widely available, WBE continued to detect persistent transmission on campus, and this data could be collected and used as the early warning system. Finally, aligning wastewater data with key public health interventions, such as vaccination campaigns and the relaxation of COVID-19 restrictions, demonstrated the ability of WBE to track the effectiveness of containment measures through changes in viral loads.

The findings in this chapter significantly advance the WBE field by addressing methodological challenges and demonstrating its application as an early warning system. The optimized PEG precipitation method improves the efficiency and practicality of wastewater analysis, enabling timely and effective public health responses. The two-year wastewater study not only highlights the importance of WBE during the COVID-19 pandemic, but also demonstrate the potential for a long-term surveillance or other infectious disease and support the public health management.

## **B) The development of CRISPR/Cas12a assay for SARS-CoV-2 and its variants identification**

This chapter presents the development and optimization of a CRISPR/Cas12a-based fluorescent assay for SARS-CoV-2 detection, particularly focusing on differentiating its Delta and Omicron variants (**Objective 2**). This innovative approach addresses the limitations of traditional diagnostic methods, particularly their inability to rapidly and cost-effectively identify specific variant sequences, and offers a sensitive, specific, and scalable solution for both clinical diagnostics and environmental monitoring.

The primary outcome of this chapter is the successful establishment of a CRISPR/Cas12a-based assay capable of distinguishing Delta and Omicron variants from the original SARS-CoV-2 strain. Using the single-nucleotide specificity of the CRISPR/Cas12a system, newly designed guide RNAs (gRNAs) efficiently targeted the N gene mutations characteristic of these variants. The assay's functionality relies on variant-specific fluorescence signals: the presence of a target variant generates a detectable signal, whereas other variants or unrelated viral sequences yield no such response. By integrating reverse transcription loop-mediated isothermal amplification (RT-LAMP) into the assay, sensitivity was significantly enhanced, enabling the detection of viral RNA at low concentrations. Variant-specific primers were carefully designed to amplify the mutated regions, achieving limits of detection (LOD) as low as 5 copies  $\mu\text{L}^{-1}$  for Omicron and 500 copies  $\mu\text{L}^{-1}$  for Delta variant. The reduced LOD for Delta variant was due to the incomplete six-primer LAMP set, highlighting the importance of primer design for optimal assay performance.

In addition to variant-specific detection, this chapter optimized the concentrations of assay key components, including the Cas12a enzyme, gRNAs, and fluorescent reporter molecules, to maximize sensitivity while minimizing background noise. The optimal concentrations were identified as 50 nM for Cas12a, 75 nM for gRNAs, and 500 nM for the fluorescent reporter in a 20  $\mu\text{L}$  reaction, striking a balance between efficiency and cost-effectiveness. The developed assay demonstrated high specificity, as the gRNAs successfully discriminated SARS-CoV-2 from other respiratory viruses, including influenza B (Flu B), influenza A virus subtype H7N9 (H7N9), and swine-origin influenza A virus (H1N1). Furthermore, the assay's LOD for the original SARS-CoV-2 strain was validated at 5 copies  $\mu\text{L}^{-1}$ .

This chapter contributes a novel, optimized CRISPR/Cas12a-based fluorescent assay with significant implications for both clinical and environmental applications. The assay achieves high sensitivity and adaptability by integrating

LAMP technology, making it suitable for WBE and clinical diagnostics. The use of isothermal amplification eliminates the need for sophisticated PCR machines, making the method particularly promising for on-site detection in resource-limited or field settings. The assay's low cost, simplicity, and specificity provide a foundation for further development, including adaptation for other emerging variants or pathogens.

### **C) The development of the paper-based microfluidic device for on-site variants detection**

This chapter outlines the development and optimization of a paper-based microfluidic device designed for on-site detection of SARS-CoV-2 and its variants in wastewater (**Objective 3**). The research addresses key challenges in WBE by introducing a cost-effective, portable, and resource-efficient diagnostic tool suitable for field applications.

To enable on-site detection, a key achievement was the development of an efficient sample concentration and RNA extraction methodology, which involved the integration of PEG precipitation and a syringe-based filtration approach. PEG precipitation enabled the pre-concentration of viral particles from large wastewater volumes, while optimal filtration was achieved through a two-step process. A 0.45 µm pre-filter was used to remove large debris, followed by a 25 nm mixed cellulose ester (MCE) membrane for viral retention. Two lysis buffers, AVL buffer from the QIAamp Viral RNA Mini Kit and TRIzol/chloroform were evaluated for RNA extraction. Both demonstrated comparable RNA recovery efficiencies, but the AVL buffer was chosen for its safety, compatibility with field applications, and reduced handling risks.

The design and optimization of the paper microfluidic device represented another significant contribution. The device consists of distinct components, including a waste pad, a glass fibre-based RNA retention pad, and microfluidic channels that connect the paper device to a reaction panel. Key parameters

affecting RNA retention and distribution were optimized, with a 4.0 mm glass fibre diameter and channel dimensions of 2.5 mm width and 7.5 mm length identified as ideal. These optimizations minimized losses and ensured consistent RNA recovery across a broad range of input concentrations ( $10^2$  to  $10^5$  copies  $\mu\text{L}^{-1}$ ).

For potential field testing, two-step diagnostic workflow was developed for field testing: initial screening with a LAMP assay, followed by a CRISPR/Cas-based assay for variant differentiation. This approach balances speed and diagnostic resolution. The fluorescent LAMP assay exhibited high sensitivity, achieving a LOD of 10 copies  $\mu\text{L}^{-1}$ . Optimizing the FAM dye concentration enhanced clarity at low target levels, although some background fluorescence persisted in negative controls. The colourimetric LAMP assay provided straightforward interpretation through a visible pink-to-yellow colour change, making it suitable for field use. Nevertheless, occasional false positives highlighted the need for precise primer design and robust contamination control.

The most significant outcome of this chapter is the integration of a CRISPR/Cas12 assay with the paper microfluidic device for on-site identification of SARS-CoV-2 variants in wastewater. Delta and Omicron variants were successfully distinguished in spiked wastewater samples using variant-specific gRNAs and fluorescent probes (ROX for Delta and FAM for Omicron). The assay achieved detection limits as low as 10 copies  $\mu\text{L}^{-1}$  for Omicron and  $10^2$  copies  $\mu\text{L}^{-1}$  for Delta. The ROX-labeled reporter demonstrated superior sensitivity and reliability at low concentrations, making it ideal for the Delta assay, which required greater sensitivity due to its higher LOD. The fluorescence-based CRISPR/Cas assay was further adapted to a colourimetric format, enabling visible fluorescence under a hand-held UV light and simplifying result interpretation without complex equipment.

This device addresses the limitations of conventional laboratory-based methods by integrating viral concentration, RNA extraction, amplification, and

detection into a single platform. Its portability and ease of use make it ideal for resource-limited settings and rapid outbreak responses. Beyond SARS-CoV-2 detection, the modular design and compatibility with CRISPR/Cas systems make this platform adaptable for monitoring other pathogens in environmental samples, enhancing its utility for global health surveillance and pandemic preparedness.

### **6.3 Research limitations**

One of the biggest challenges of this research is the variability of wastewater composition, which affects the efficiency of RNA recovery and the sensitivity of the assay. Wastewater contains a variety of contaminants, including organics, chemicals, and microbial populations, all of which may affect subsequent molecular detection (Randazzo *et al.*, 2020). When investigating standardized ways of recovering viral RNA by concentration from wastewater, the best approach is to spike the original virus or similar other viruses, recover them at a known concentration and then quantitatively analyze them (Farkas *et al.*, 2022). However, due to restricted laboratory conditions to access the biological safety Level 3 laboratory, this study was unable to use the standardized research process and directly used collected wastewater samples as the study object to compare the two methods evaluated by comparing the recovery of viral RNA. Also, the number of wastewater samples used in the comparison was not very large, with only two cases. Although the consistent conclusions were reached, and there was a significant difference in the concentration of RNA recovered by the two concentration methods.

This thesis does not incorporate the normalization of biomarkers in wastewater when comparing WBE data to clinical reports, a crucial step for ensuring data accuracy and comparability. In WBE for SARS-CoV-2, normalization process entails the adjustment of measured viral RNA concentrations to account for variations in wastewater characteristics, including

flow rate, population size, and sample composition (Hsu *et al.*, 2022). However, external factors such as rainfall, infiltration, and water usage patterns can potentially dilute wastewater, thereby altering viral RNA and biomarker levels (Feng *et al.*, 2021). Furthermore, the consideration of population fluctuations on campus, both daily and seasonally, is pivotal in ensuring the accuracy of the data by implementing population-specific normalization to reflect viral prevalence accurately. The employment of biomarkers, such as human-specific HF183 *Bacteroides* 16S rRNA, eukaryotic 18S rRNA, and Pepper mild mottle virus (PMMoV), is a common practice in the field to estimate human fecal input, thus enabling the adjustment of viral RNA concentrations relative to the contributing population (D'Aoust *et al.*, 2021). Integrating biomarker standardization within the present study will enhance the quality of the data, thereby yielding a clearer and more accurate depiction of infection trends across time and locations. Furthermore, it would enhance the alignment with clinical data, thereby reinforcing the validity of WBE as a reliable surveillance tool. The resulting study model could serve as a benchmark for other WBE projects, contributing to the standardization and advancement of the field.

Although the developed CRISPR/Cas12a assay demonstrated high sensitivity in detecting the original virus and Omicron variant, the LOD for the Delta variant remains relatively high. The underlying reasons for these results are extensively discussed in Chapter 4. However, there is still room for improvement to enhance the assay's sensitivity for the Delta variant. One potential solution involves designing gRNAs that target alternative genes of SARS-CoV-2. By focusing on regions with more consistent mutation patterns, there might be more potential sites for designing gRNAs as well as LAMP primers, achieving improved recognition and detection of the Delta variant. Additionally, exploring complementary isothermal amplification methods offers another avenue for optimization. For instance, recombinase polymerase amplification (RPA), which has been successfully combined with CRISPR/Cas12a for *Staphylococcus*

*aureus* analysis (Li *et al.*, 2022), could enhance amplification efficiency and lower the LOD.

Although the paper microfluidic device was designed to minimize reliance on laboratory infrastructure, specific components, such as CRISPR and LAMP reagents, remain reliant on cold-chain transport for preservation. For practical application, it is essential to explore the use of lyophilization technology to pre-process these reagents, enabling long-term storage at ambient temperatures without compromising functionality. Furthermore, the stability and efficacy of the reagents after extended storage periods must be thoroughly evaluated to ensure the reliability of the device under real-world conditions.

Another limitation is that the developed device was employed exclusively for spiked sample testing rather than real-sample diagnostics. While sufficient samples were collected for the study, identifying the presence of viral sequences in these samples requires confirmation using gold-standard techniques, such as next-generation sequencing. However, sequencing service was not available at Cranfield University, and the commercial service was time-consuming, restricting the ability to validate the assay directly against sequencing results. To enhance the robustness and credibility of the developed device, future studies should focus on testing real wastewater samples and comparing the diagnostic results with sequencing data.

## **6.4 Future work and perspectives**

### **A) Integration of biomarker normalization**

To address the limitations described previously, incorporating biomarker normalization into WBE is essential for aligning wastewater data with clinical reports and gaining a more comprehensive understanding of epidemiological trends. Biomarker normalization allows for adjustments to viral concentration data, thereby enhancing accuracy, comparability, and reliability. Future research

should initially focus on identifying and evaluating widely used human-specific biomarkers. These biomarkers can be utilized to normalize the effects of population variability, ensuring that the viral RNA concentrations reflect actual prevalence rather than fluctuations in population size or contributions. Advanced modelling approaches can then be employed to integrate and analyze the normalized data, providing deeper insights into the impact of population-specific adjustments. Additionally, future studies should prioritize a comprehensive literature review to identify the parameters required for flow normalization, such as wastewater flow rates, rainfall, and infiltration patterns. Developing robust frameworks for combining population and flow normalization markers will further enhance the accuracy of WBE data. This dual normalization approach—integrating population-specific and flow-related markers—has the potential to mitigate external influences and improve the precision of infection trend analysis.

## **B) Enhancement of the CRISPR/Cas12 assay**

Enhancing the sensitivity of the CRISPR/Cas12a assay, particularly for detecting the Delta variant, remains a critical area for future research and development. Improvements could significantly broaden the utility of the assay for variant-specific detection and point-of-care diagnostics. One promising improvement involves designing gRNAs and LAMP primers that target alternative conserved genes or genomic regions of SARS-CoV-2. Focusing on regions with higher mutation frequencies or those associated with key functional changes, such as increased transmissibility or immune evasion, could enhance the assay's ability to detect variants like Delta with better precision. Additionally, targeting genes with specific mutations that are known to influence viral behaviour or neutralizing antibody resistance would provide clinically relevant information, enabling the assay to contribute to more tailored therapeutic interventions.

Integrating complementary isothermal amplification techniques like RPA, represents another strategy to improve sensitivity, particularly the incubation

temperature of RPA reaction is the same as the CRISPR/Cas assay. Therefore, the dual-reaction assay could be performed in one-pot, improving simplicity and efficiency. The rapid advancements in the study of Cas12 enzymes also present opportunities for further refinement. A new engineered type of the Cas12 enzyme with enhanced specificity, reduced off-target effects, or improved activity under varied conditions could be incorporated into the assay, making it more robust and versatile. These enhancements would not only improve variant-specific detection but also establish the assay as a reliable tool for rapid diagnostics, contributing to both public health surveillance and clinical decision-making.

### **C) Advancement of the paper microfluidic device**

To eliminate the dependency on cold storage for CRISPR and LAMP reagents, which is an obstacle to effective field detection, the ideal paper microfluidic device would incorporate lyophilized reagents pre-loaded within the device. This design would enable convenient on-site use by simply adding ambient temperature dH<sub>2</sub>O to rehydrate the reagents, making the device ready for immediate operation. Such a feature would greatly enhance its practicality for decentralized diagnostics, particularly in resource-limited settings. Advancing this technology should prioritize optimizing the microfluidic device design to facilitate the lyophilization process while maintaining the integrity and functionality of the reagents. This includes the development of spaces that can safely accommodate lyophilized reagents and prevent degradation during storage and transport, and the construction of the device should also ensure that the reagents can be efficiently rehydrated within the device after activation.

Equally critical is the long-term stability of the reagents under ambient conditions. Future studies should systematically evaluate the stability and activity of lyophilized CRISPR and LAMP reagents when stored for extended periods across varying environmental conditions. This may involve incorporating stabilizing agents to preserve reagent functionality during storage. Testing under

simulated conditions will be essential to confirm the device's reliability and effectiveness after prolonged storage.

#### **D) Validation of the CRISPR/Cas12 assay and paper microfluidic device with real samples**

To enhance the practical utility and robustness of the CRISPR/Cas12 assay and the paper microfluidic device developed in this study, it is essential to validate their performance using real wastewater samples. Furthermore, testings at real-world settings will provide critical insights into their functionality and adaptability outside the laboratory environment. The initial step in this validation process should involve analyzing real wastewater samples with known SARS-CoV-2 variants. These samples can be characterized through sequencing technologies to confirm the presence of specific variants and determine their original concentrations. Such a controlled setup will allow for an accurate assessment of the assay's ability to detect and differentiate variants under real sample conditions. To further evaluate the LOD, these samples can be systematically diluted to lower concentrations, simulating scenarios with varying viral loads. This will help identify the assay's sensitivity and establish its efficacy in detecting low viral concentrations, a critical factor for early outbreak detection.

Additionally, testing real samples containing multiple variants will provide an opportunity to assess the assay's robustness in identifying specific strains amidst the presence of other variants. Such scenarios reflect the complexity of real-world wastewater samples, where co-circulation of multiple variants is common. A thorough evaluation of the assay's performance in such conditions will validate its ability to accurately detect and distinguish between strains, even in mixed-variant samples. Finally, deploying the device and assay in real field settings, such as wastewater treatment facilities or community surveillance projects, will allow for the evaluation of operational feasibility, user-friendliness, and consistency in performance. This step will also uncover potential challenges

related to sample collection, processing, and data interpretation, guiding further refinements to improve their practical applicability.

#### **E) Expanding the application of the analytical platform**

The adaptability of the diagnostic platform developed in this study can be leveraged to detect a wide array of pathogens across various environmental and clinical samples, significantly broadening its scope and utility. By tailoring the platform's design, incorporating gRNA sequences and amplification strategies, it could be adapted to monitor a range of viral, bacterial, or fungal pathogens, thereby addressing various public health challenges.

A particularly promising area of application is the detection and surveillance of antimicrobial resistance (AMR) genes. The platform's adaptability renders it a valuable asset in the global fight against antibiotic-resistant bacteria, as it can be employed to identify and track AMR genes in wastewater, hospital sewages, and environmental samples. This capability would provide critical data to inform strategies for mitigating the spread of antimicrobial resistance and assessing the effectiveness of intervention programs. Beyond public health, the diagnostic platform has the potential to play a vital role in agriculture and food safety monitoring. For instance, it could be employed to detect pathogens or contaminants in irrigation water, livestock, or food products, ensuring compliance with safety standards and protecting public health. Potential applications include monitoring for foodborne pathogens such as *Salmonella*, *Listeria monocytogenes*, or *Escherichia coli* in supply chains, thus minimizing the risk of outbreaks associated with contaminated food.

## 6.5 Conclusion

This thesis contributes to the advancement of WBE by addressing key challenges in viral concentration from wastewater, molecular detection, variants identification, and on-site diagnostics. The technology presented here for concentrating viruses from wastewater lays the foundation for a scalable and efficient wastewater monitoring tool. It demonstrates the importance of WBE in COVID-19 outbreaks with practical long-term monitoring studies. Meanwhile, using novel CRISPR/Cas12 technology for the analysis of SARS-CoV-2 and its variants enables more efficient diagnostics. Furthermore, combining CRISPR/Cas technology with paper microfluidics bridges the gap between laboratory methods and field-based solutions. These advancements hold promise for improving public health surveillance, particularly in the context of unknown infectious diseases in the future.

The potential of this research is not limited to SARS-CoV-2 detection or wastewater surveillance. By combining state-of-the-art molecular diagnostics, the CRISPR/Cas technology, with a cost-effective portable platform, the paper microfluidics, this work lays the groundwork for future innovations in clinical diagnostics, environmental monitoring, disease control and public health management. These contributions are essential for strengthening health diagnostics, especially in developing regions, and global preparedness for future pandemics. Through continued research, the vision of universal access to rapid, accurate and affordable testing methods can become a reality, enhancing the ability to address current and future global health threats.

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# Appendix

## Appendix A Ethical approval letter



16 January 2025

Dear Miss Hui ,

Reference: CURES/24145/2025

Project ID: 14881

Title: CRISPR/Cas-enabled paper microfluidic device to detect SARS-CoV-2 and its variants for wastewater-based epidemiology

Thank you for your application to the Cranfield University Research Ethics System (CURES).

**We are pleased to inform you your project title change request, reference CURES/24145/2025 has been approved.**

If you have any queries, please contact CURES Support.

Regards,

CURES Team

## **Appendix B: Optimization of concentration methods for SARS-CoV-2 detection in wastewater and insights from wastewater surveillance in Cranfield University**

### **B.1 Information of raw wastewater samples collected at Cranfield University**

| <b>Collected Date</b> | <b>Sample label</b> | <b>Collected Date</b> | <b>Sample label</b> |
|-----------------------|---------------------|-----------------------|---------------------|
| 1/13/2022             | 1-1                 | 4/1/2023              | 13-1                |
| 1/18/2022             | 1-2                 | 12/1/2023             | 13-2                |
| 1/20/2022             | 1-3                 | 19/1/2023             | 13-3                |
| 1/28/2022             | 1-4                 | 31/1/2023             | 13-4                |
| 2/3/2022              | 2-1                 | 7/2/2023              | 14-1                |
| 2/11/2022             | 2-2                 | 14/2/2023             | 14-2                |
| 2/22/2022             | 2-3                 | 16/2/2023             | 14-3                |
| 2/24/2022             | 2-4                 | 23/2/2023             | 14-4                |
| 3/8/2022              | 3-1                 | 7/3/2023              | 15-1                |
| 3/15/2022             | 3-2                 | 14/3/2023             | 15-2                |
| 3/22/2022             | 3-3                 | 22/3/2023             | 15-3                |
| 3/29/2022             | 3-4                 | 30/3/2023             | 15-4                |
| 4/7/2022              | 4-1                 | 6/4/2023              | 16-1                |
| 4/19/2022             | 4-2                 | 13/4/2023             | 16-2                |
| 4/21/2022             | 4-3                 | 20/4/2023             | 16-3                |
| 4/28/2022             | 4-4                 | 27/4/2022             | 16-4                |
| 5/5/2022              | 5-1                 | 3/5/2023              | 17-1                |
| 5/12/2022             | 5-2                 | 10/5/2023             | 17-2                |
| 5/19/2022             | 5-3                 | 18/5/2023             | 17-3                |
| 5/26/2022             | 5-4                 | 25/5/2023             | 17-4                |

|            |      |            |      |
|------------|------|------------|------|
| 6/7/2022   | 6-1  | 1/6/2023   | 18-1 |
| 6/14/2022  | 6-2  | 8/6/2023   | 18-2 |
| 21/6/2022  | 6-3  | 15/6/2023  | 18-3 |
| 23/6/2022  | 6-4  | 22/6/2023  | 18-4 |
| 5/7/2022   | 7-1  | 5/7/2023   | 19-1 |
| 12/7/2022  | 7-2  | 11/7/2023  | 19-2 |
| 19/7/2022  | 7-3  | 18/7/2023  | 19-3 |
| 21/7/2022  | 7-4  | 27/7/2023  | 19-4 |
| 9/8/2022   | 8-1  | 8/8/2023   | 20-1 |
| 18/8/2022  | 8-2  | 15/8/2023  | 20-2 |
| 25/8/2022  | 8-3  | 24/8/2023  | 20-3 |
| 31/8/2022  | 8-4  | 31/8/2023  | 20-4 |
| 6/9/2022   | 9-1  | 7/9/2023   | 21-1 |
| 13/9/2022  | 9-2  | 14/9/2023  | 21-2 |
| 21/9/2022  | 9-3  | 21/9/2023  | 21-3 |
| 27/9/2022  | 9-4  | 28/9/2023  | 21-4 |
| 4/10/2022  | 10-1 | 5/10/2023  | 22-1 |
| 11/10/2022 | 10-2 | 11/10/2023 | 22-2 |
| 18/10/2022 | 10-3 | 18/10/2023 | 22-3 |
| 28/10/2022 | 10-4 | 26/10/2023 | 22-4 |
| 8/11/2022  | 11-1 | 7/11/2023  | 23-1 |
| 15/11/2022 | 11-2 | 14/11/2023 | 23-2 |
| 22/11/2022 | 11-3 | 16/11/2023 | 23-3 |
| 29/11/2022 | 11-4 | 23/11/2023 | 23-4 |
| 1/12/2022  | 12-1 | 29/11/2023 | 24-1 |
| 8/12/2022  | 12-2 | 14/12/2023 | 24-2 |
| 13/12/2022 | 12-3 | 3/1/2024   | 24-3 |
| 15/12/2022 | 12-4 | 5/1/2024   | 24-4 |

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## B.2 The weekly average infected data in England

| 2022        |                        | 2023        |                        |
|-------------|------------------------|-------------|------------------------|
| Week Number | Average Reported Cases | Week Number | Average Reported Cases |
| 1           | 86410                  | 1           | 895                    |
| 2           | 49519                  | 2           | 806                    |
| 3           | 38251                  | 3           | 689                    |
| 4           | 33386                  | 4           | 778                    |
| 5           | 29212                  | 5           | 909                    |
| 6           | 24036                  | 6           | 1013                   |
| 7           | 18018                  | 7           | 1190                   |
| 8           | 16197                  | 8           | 1139                   |
| 9           | 19252                  | 9           | 1109                   |
| 10          | 26659                  | 10          | 1122                   |
| 11          | 34501                  | 11          | 1095                   |
| 12          | 38016                  | 12          | 1042                   |
| 13          | 36779                  | 13          | 880                    |
| 14          | 31662                  | 14          | 681                    |
| 15          | 22924                  | 15          | 613                    |
| 16          | 17914                  | 16          | 529                    |
| 17          | 12376                  | 17          | 484                    |
| 18          | 10590                  | 18          | 442                    |
| 19          | 9005                   | 19          | 378                    |
| 20          | 7893                   | 20          | 314                    |
| 21          | 7050                   | 21          | 290                    |
| 22          | 7896                   | 22          | 227                    |
| 23          | 10818                  | 23          | 207                    |
| 24          | 11754                  | 24          | 147                    |

|    |       |    |     |
|----|-------|----|-----|
| 25 | 16215 | 25 | 106 |
| 26 | 20826 | 26 | 84  |
| 27 | 24329 | 27 | 99  |
| 28 | 22321 | 28 | 124 |
| 29 | 17958 | 29 | 147 |
| 30 | 13941 | 30 | 211 |
| 31 | 10852 | 31 | 265 |
| 32 | 9095  | 32 | 317 |
| 33 | 6907  | 33 | 327 |
| 34 | 6035  | 34 | 340 |
| 35 | 5390  | 35 | 406 |
| 36 | 5069  | 36 | 507 |
| 37 | 5567  | 37 | 423 |
| 38 | 8268  | 38 | 484 |
| 39 | 10466 | 39 | 634 |
| 40 | 11950 | 40 | 647 |
| 41 | 11137 | 41 | 554 |
| 42 | 9715  | 42 | 481 |
| 43 | 7173  | 43 | 432 |
| 44 | 5469  | 44 | 349 |
| 45 | 4816  | 45 | 279 |
| 46 | 4551  | 46 | 290 |
| 47 | 4827  | 47 | 276 |
| 48 | 5599  | 48 | 324 |
| 49 | 6818  | 49 | 432 |
| 50 | 7612  | 50 | 549 |

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### B.3 The daily reported infected cases in Milton Keynes and Bedford

| Date       | Milton<br>Keynes | Bedford | Date       | Milton<br>Keynes | Bedford |
|------------|------------------|---------|------------|------------------|---------|
| 2023-05-01 | 6                | 3       | 2023-08-31 | 5                | 2       |
| 2023-05-02 | 11               | 1       | 2023-09-01 | 4                | 4       |
| 2023-05-03 | 9                | 1       | 2023-09-02 | 4                | 1       |
| 2023-05-04 | 2                | 3       | 2023-09-03 | 6                | 2       |
| 2023-05-05 | 4                | 4       | 2023-09-04 | 6                | 1       |
| 2023-05-06 | 1                | 2       | 2023-09-05 | 10               | 4       |
| 2023-05-07 | 2                | 1       | 2023-09-06 | 10               | 3       |
| 2023-05-08 | 5                | 8       | 2023-09-07 | 7                | 4       |
| 2023-05-09 | 8                | 3       | 2023-09-08 | 13               | 5       |
| 2023-05-10 | 3                | 2       | 2023-09-09 | 8                | 2       |
| 2023-05-11 | 3                | 0       | 2023-09-10 | 6                | 3       |
| 2023-05-12 | 4                | 1       | 2023-09-11 | 15               | 2       |
| 2023-05-13 | 2                | 2       | 2023-09-12 | 3                | 4       |
| 2023-05-14 | 4                | 4       | 2023-09-13 | 5                | 4       |
| 2023-05-15 | 4                | 2       | 2023-09-14 | 4                | 2       |
| 2023-05-16 | 5                | 4       | 2023-09-15 | 7                | 1       |
| 2023-05-17 | 7                | 3       | 2023-09-16 | 7                | 2       |
| 2023-05-18 | 2                | 1       | 2023-09-17 | 6                | 2       |
| 2023-05-19 | 1                | 2       | 2023-09-18 | 5                | 8       |
| 2023-05-20 | 4                | 2       | 2023-09-19 | 3                | 4       |
| 2023-05-21 | 3                | 2       | 2023-09-20 | 8                | 5       |
| 2023-05-22 | 9                | 3       | 2023-09-21 | 5                | 5       |
| 2023-05-23 | 6                | 3       | 2023-09-22 | 5                | 7       |

|            |   |   |            |    |   |
|------------|---|---|------------|----|---|
| 2023-05-24 | 5 | 1 | 2023-09-23 | 9  | 9 |
| 2023-05-25 | 5 | 0 | 2023-09-24 | 6  | 5 |
| 2023-05-26 | 3 | 5 | 2023-09-25 | 10 | 5 |
| 2023-05-27 | 3 | 1 | 2023-09-26 | 13 | 6 |
| 2023-05-28 | 5 | 0 | 2023-09-27 | 10 | 7 |
| 2023-05-29 | 3 | 1 | 2023-09-28 | 8  | 2 |
| 2023-05-30 | 3 | 4 | 2023-09-29 | 6  | 4 |
| 2023-05-31 | 5 | 1 | 2023-09-30 | 10 | 3 |
| 2023-06-01 | 2 | 1 | 2023-10-01 | 11 | 4 |
| 2023-06-02 | 1 | 1 | 2023-10-02 | 20 | 6 |
| 2023-06-03 | 4 | 3 | 2023-10-03 | 13 | 7 |
| 2023-06-04 | 3 | 2 | 2023-10-04 | 8  | 8 |
| 2023-06-05 | 4 | 2 | 2023-10-05 | 15 | 2 |
| 2023-06-06 | 3 | 3 | 2023-10-06 | 4  | 9 |
| 2023-06-07 | 4 | 1 | 2023-10-07 | 5  | 3 |
| 2023-06-08 | 1 | 1 | 2023-10-08 | 5  | 4 |
| 2023-06-09 | 3 | 1 | 2023-10-09 | 8  | 9 |
| 2023-06-10 | 0 | 2 | 2023-10-10 | 8  | 4 |
| 2023-06-11 | 5 | 1 | 2023-10-11 | 5  | 9 |
| 2023-06-12 | 1 | 2 | 2023-10-12 | 2  | 2 |
| 2023-06-13 | 6 | 3 | 2023-10-13 | 3  | 7 |
| 2023-06-14 | 2 | 4 | 2023-10-14 | 1  | 3 |
| 2023-06-15 | 1 | 2 | 2023-10-15 | 6  | 7 |
| 2023-06-16 | 0 | 1 | 2023-10-16 | 7  | 7 |
| 2023-06-17 | 0 | 2 | 2023-10-17 | 7  | 3 |
| 2023-06-18 | 2 | 0 | 2023-10-18 | 7  | 7 |

|            |   |   |            |   |   |
|------------|---|---|------------|---|---|
| 2023-06-19 | 1 | 1 | 2023-10-19 | 5 | 3 |
| 2023-06-20 | 0 | 1 | 2023-10-20 | 4 | 3 |
| 2023-06-21 | 1 | 0 | 2023-10-21 | 6 | 3 |
| 2023-06-22 | 0 | 0 | 2023-10-22 | 6 | 6 |
| 2023-06-23 | 1 | 0 | 2023-10-23 | 7 | 2 |
| 2023-06-24 | 3 | 0 | 2023-10-24 | 5 | 4 |
| 2023-06-25 | 3 | 0 | 2023-10-25 | 2 | 3 |
| 2023-06-26 | 3 | 0 | 2023-10-26 | 2 | 1 |
| 2023-06-27 | 4 | 1 | 2023-10-27 | 2 | 2 |
| 2023-06-28 | 2 | 0 | 2023-10-28 | 2 | 3 |
| 2023-06-29 | 0 | 0 | 2023-10-29 | 6 | 4 |
| 2023-06-30 | 1 | 0 | 2023-10-30 | 3 | 5 |
| 2023-07-01 | 3 | 0 | 2023-10-31 | 5 | 2 |
| 2023-07-02 | 1 | 2 | 2023-11-01 | 7 | 1 |
| 2023-07-03 | 1 | 1 | 2023-11-02 | 5 | 2 |
| 2023-07-04 | 4 | 1 | 2023-11-03 | 5 | 0 |
| 2023-07-05 | 5 | 2 | 2023-11-04 | 7 | 1 |
| 2023-07-06 | 3 | 2 | 2023-11-05 | 3 | 3 |
| 2023-07-07 | 8 | 0 | 2023-11-06 | 1 | 1 |
| 2023-07-08 | 9 | 1 | 2023-11-07 | 4 | 1 |
| 2023-07-09 | 4 | 0 | 2023-11-08 | 2 | 1 |
| 2023-07-10 | 6 | 2 | 2023-11-09 | 4 | 3 |
| 2023-07-11 | 4 | 1 | 2023-11-10 | 2 | 0 |
| 2023-07-12 | 4 | 0 | 2023-11-11 | 4 | 1 |
| 2023-07-13 | 1 | 0 | 2023-11-12 | 2 | 3 |
| 2023-07-14 | 3 | 1 | 2023-11-13 | 6 | 0 |

|            |   |   |            |    |   |
|------------|---|---|------------|----|---|
| 2023-07-15 | 2 | 0 | 2023-11-14 | 4  | 2 |
| 2023-07-16 | 3 | 2 | 2023-11-15 | 4  | 1 |
| 2023-07-17 | 2 | 1 | 2023-11-16 | 1  | 3 |
| 2023-07-18 | 2 | 2 | 2023-11-17 | 5  | 0 |
| 2023-07-19 | 3 | 1 | 2023-11-18 | 3  | 1 |
| 2023-07-20 | 3 | 1 | 2023-11-19 | 3  | 2 |
| 2023-07-21 | 7 | 1 | 2023-11-20 | 4  | 3 |
| 2023-07-22 | 2 | 1 | 2023-11-21 | 8  | 1 |
| 2023-07-23 | 2 | 0 | 2023-11-22 | 4  | 2 |
| 2023-07-24 | 5 | 0 | 2023-11-23 | 2  | 2 |
| 2023-07-25 | 4 | 0 | 2023-11-24 | 1  | 2 |
| 2023-07-26 | 2 | 1 | 2023-11-25 | 0  | 0 |
| 2023-07-27 | 2 | 1 | 2023-11-26 | 0  | 3 |
| 2023-07-28 | 5 | 3 | 2023-11-27 | 3  | 3 |
| 2023-07-29 | 5 | 1 | 2023-11-28 | 1  | 1 |
| 2023-07-30 | 1 | 1 | 2023-11-29 | 0  | 2 |
| 2023-07-31 | 3 | 2 | 2023-11-30 | 6  | 2 |
| 2023-08-01 | 4 | 1 | 2023-12-01 | 2  | 2 |
| 2023-08-02 | 7 | 1 | 2023-12-02 | 0  | 2 |
| 2023-08-03 | 6 | 1 | 2023-12-03 | 6  | 1 |
| 2023-08-04 | 2 | 3 | 2023-12-04 | 6  | 2 |
| 2023-08-05 | 3 | 4 | 2023-12-05 | 2  | 4 |
| 2023-08-06 | 5 | 3 | 2023-12-06 | 14 | 4 |
| 2023-08-07 | 7 | 0 | 2023-12-07 | 4  | 1 |
| 2023-08-08 | 6 | 2 | 2023-12-08 | 1  | 2 |
| 2023-08-09 | 5 | 3 | 2023-12-09 | 7  | 6 |

|            |   |   |            |    |   |
|------------|---|---|------------|----|---|
| 2023-08-10 | 1 | 2 | 2023-12-10 | 2  | 1 |
| 2023-08-11 | 3 | 1 | 2023-12-11 | 7  | 4 |
| 2023-08-12 | 1 | 0 | 2023-12-12 | 7  | 1 |
| 2023-08-13 | 5 | 0 | 2023-12-13 | 6  | 4 |
| 2023-08-14 | 3 | 6 | 2023-12-14 | 6  | 3 |
| 2023-08-15 | 4 | 0 | 2023-12-15 | 3  | 2 |
| 2023-08-16 | 7 | 2 | 2023-12-16 | 1  | 3 |
| 2023-08-17 | 1 | 2 | 2023-12-17 | 0  | 2 |
| 2023-08-18 | 3 | 3 | 2023-12-18 | 12 | 3 |
| 2023-08-19 | 2 | 1 | 2023-12-19 | 5  | 9 |
| 2023-08-20 | 3 | 3 | 2023-12-20 | 3  | 1 |
| 2023-08-21 | 2 | 1 | 2023-12-21 | 4  | 3 |
| 2023-08-22 | 2 | 1 | 2023-12-22 | 6  | 3 |
| 2023-08-23 | 3 | 3 | 2023-12-23 | 2  | 3 |
| 2023-08-24 | 5 | 6 | 2023-12-24 | 2  | 2 |
| 2023-08-25 | 3 | 2 | 2023-12-25 | 1  | 8 |
| 2023-08-26 | 2 | 1 | 2023-12-26 | 5  | 3 |
| 2023-08-27 | 4 | 0 | 2023-12-27 | 5  | 2 |
| 2023-08-28 | 3 | 1 | 2023-12-28 | 9  | 5 |
| 2023-08-29 | 4 | 6 | 2023-12-29 | 3  | 2 |
| 2023-08-30 | 7 | 3 | 2023-12-30 | 2  | 2 |

---

#### B.4 The weekly average infected data in Milton Keynes and Bedford

| Week<br>Number | Milton<br>Keynes | Bedford | Week<br>Number | Milton<br>Keynes | Bedford |
|----------------|------------------|---------|----------------|------------------|---------|
| 1              | 35               | 15      | 19             | 60               | 22      |
| 2              | 29               | 20      | 20             | 47               | 17      |
| 3              | 26               | 16      | 21             | 41               | 43      |
| 4              | 36               | 13      | 22             | 68               | 31      |
| 5              | 21               | 13      | 23             | 70               | 39      |
| 6              | 20               | 11      | 24             | 33               | 41      |
| 7              | 12               | 14      | 25             | 42               | 32      |
| 8              | 9                | 2       | 26             | 26               | 19      |
| 9              | 14               | 3       | 27             | 35               | 14      |
| 10             | 34               | 7       | 28             | 19               | 10      |
| 11             | 23               | 6       | 29             | 26               | 9       |
| 12             | 21               | 7       | 30             | 19               | 13      |
| 13             | 24               | 7       | 31             | 18               | 13      |
| 14             | 30               | 15      | 32             | 36               | 20      |
| 15             | 28               | 8       | 33             | 30               | 19      |
| 16             | 23               | 17      | 34             | 34               | 24      |
| 17             | 21               | 14      | 35             | 33               | 26      |
| 18             | 33               | 19      |                |                  |         |

## **Appendix C: Development of a CRISPR/Cas12a-based fluorescent detection method of SARS-CoV-2 and its Delta and Omicron variants for wastewater analysis**

### **C.1 The sequence information of the N gene of influenza B**

**Influenza B virus (B/Lee/1940) segment 5, complete sequence**

**NCBI Reference Sequence: NC\_002208.1**

**>NC\_002208.1:58-1740 Influenza B virus (B/Lee/1940) segment 5, complete sequence**

```
ATGTCCAACATGGATATTGACAGTATAAATACCGGAACAATCGATAAAACACCAGAAGAACT
GACTCCCGGAACCAAGTGGGGCAACCAGACCAATCATCAAGCCAGCAACCCTTGCTCCGCC
AAGCAACAAACGAACCCGAAATCCATCTCCAGAAAGGACAACCACAAGCAGTGAAACCGAT
ATCGGAAGGAAAAATCCAAAAGAAACAAACCCCAACAGAGATAAAGAAGAGCGTCTACAAAA
TGGTGGTAAAACTGGGTGAATTCTACAACCAGATGATGGTCAAAGCTGGACTTAATGATGA
CATGGAAAGGAATCTAATTCAAAATGCACAAGCTGTGGAGAGAATCCTATTGGCTGCAACT
GATGACAAGAAAACTGAATACCAAAAGAAAAGGAATGCCAGAGATGTCAAAGAAGGGAAG
GAAGAAATAGACCACAACAAGACAGGAGGCACCTTTTATAAGATGGTAAGAGATGATAAAA
CCATCTACTTCAGCCCTATAAAAATTACCTTTTTTAAAAGAAGAGGTGAAAACAATGTACAAG
ACCACCATGGGGAGTGATGGTTTCAGTGGACTAAATCACATTATGATTGGACATTCACAGA
TGAACGATGTCTGTTTCCAAAGATCAAAGGGACTGAAAAGGGTTGGACTTGACCCTTCATT
AATCAGTACTTTTGCCGAAGCACACTACCCAGAAGATCAGGTACAACCTGGTGTGCAATC
AAAGGAGGTGGAACCTTTAGTGGATGAAGCCATCCGATTTATAGGAAGAGCAATGGCAGAC
AGAGGGCTACTGAGAGACATCAAGGCCAAGACGGCCTATGAAAAGATTCTTCTGAATCTGA
AAAACAAGTGCTCTGCGCCGCAACAAAAGGCTCTAGTTGATCAAGTGATCGGAAGTAGGAA
CCCAGGGATTGCAGACATAGAAGACCTAACTCTGCTTGCCAGAAGCATGGTAGTTGTCAGA
CCCTCTGTAGCGAGCAAAGTGGTGCTTCCCATAGCATTATGCTAAAATACCTCAACTAG
GATTCAATACCGAAGAATACTCTATGGTTGGGTATGAAGCCATGGCTCTTTATAATATGGCA
ACACCTGTTTCCATATTAAGAATGGGAGATGACGCAAAAGATAAATCTCAACTATTCTTCAT
GTCGTGCTTCGGAGCTGCCTATGAAGATCTAAGAGTGTTATCTGCACTAACGGGCACCGAA
TTTAAGCCTAGATCAGCACTAAAATGCAAGGGTTTCCATGTCCCGGCTAAGGAGCAAGTAG
AAGGAATGGGGGACAGCTCTGATGTCCATCAAGCTTCAGTTCTGGGCCCCAATGACCAGAT
CTGGAGGGAATGAAGTAAGTGGAGAAGGAGGGTCTGGTCAAATAAGTTGCAGCCCTGTGT
TTGCAGTAGAAAGACCTATTGCTCTAAGCAAGCAAGCTGTAAGAAGAATGCTGTCAATGAA
CGTTGAAGGACGTGATGCAGATGTCAAAGGAAATCTACTCAAATGATGAATGATTCAATG
GCAAAGAAAACCAAGTGGAATGCTTTCATTGGGAAGAAAATGTTTCAAATATCAGACAAAAA
CAAAGTCAATCCCATGAGATTCCAATTAAGCAGACCATCCCCAATTTCTTCTTTGGGAGGG
ACACAGCAGAGGATTATGATGACCTCGATTATTAA
```

## **C.2 The sequence information of the N gene of influenza A virus subtype H7N9**

**Influenza A virus (A/Shanghai/02/2013(H7N9)) segment 5 nucleocapsid protein (NP) gene**

**NCBI Reference Sequence: NC\_026426.1**

**>NC\_026426.1:1-1497 Influenza A virus (A/Shanghai/02/2013(H7N9))  
segment 5 nucleocapsid protein (NP) gene, complete cds**

```
ATGGCGTCTCAAGGCACCAAACGATCCTATGAACAGATGGAACTGGTGGGGAACGCCAG
AATGCTACTGAGATCAGGGCATCTGTTGGAAGAATGGTTAGCGGCATTGGGAGATTCTACA
TACAGATGTGTACAGAACTCAAACCTCAGTGACAATGAAGGGAGGCTGATTCAGAACAGTAT
AACAAATAGAGAGAATGGTACTCTCTGCATTTGATGAAAGAAGGAACAGATACCTGGAAGAG
CACCCCAGTGCAGGAAAGGACCCTAAGAAAACCTGGAGGTCCAATTTACAGGAGAAGAGAC
GGAAAATGGGTGAGAGAGCTGATCCTGTATGACAAAGAGGAAATCAGGAGAATTTGGCGA
CAAGCGAACAATGGAGAGGATGCAACTGCTGGTCTTACCCATCTGATGATATGGCATTCCA
ACCTGAATGATGCTACCTATCAGAGAACGAGAGCTCTCGTGCGTACTGGAATGGATCCCC
GGATGTGCTCTCTGATGCAAGGATCAACTCTCCCGAGGAGATCTGGAGCTGCAGGTGCAG
CAGTGAAGGGGATAGGGACAATGGTGTATGGAACCTGATTTCGGATGATAAACGAGGGATCA
ACGACCGGAATTTCTGGAGAGGGCGAAAATGGAAGAAGGACAAGAATTGCATATGAGAGAA
TGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACAAAGGGCAATGATGGATCAAGT
GCGAGAGAGCAGAAATCCTGGGAATGCTGAAATAGAAGATCTCATTTTTCTGGCAAGGTCT
GCACTCATCCTGAGAGGATCAGTGGCTCATAAATCCTGCTTGCCTGCTTGTGTGTACGGAC
TTGCAGTGGCTAGTGGATATGACTTTGAGAGAGAAGGGTACTCCTTGGTTGGAATAGATCC
TTTCCGTCTGCTTCAAAACAGCCAGGTCTTTAGTCTCATTAGACCAAATGAGAACCCAGCA
CATAAGAGCCAACTAGTGTGGATGGCATGCCACTCTGCAGCGTTTGAGGACCTTAGGGTC
TCAAGTTTTCATTAGAGGGACAAGAATGGTCCCAAGAGGACAGCTATCCACTAGAGGGGTTT
AAATTGCTTCAAATGAGAACATGGAAGCAATGGACTCCAATACTCTTGAACCTGAGAAGTAG
ATATTGGGCTATAAGAACCAGAAGCGGAGGGAACACCAACCAACAGAGGGCATCTGCAGG
ACAGGTCAGCGTTCAACCCACTTTCTCAGTACAGAGAAACCTTCCTTTCGAAAGAGCAACC
ATTATGGCAGCATTTACAGGAAATACTGAGGGTAGAACGTCTGACATGAGGACTGAAATCA
TAAGAATGATGGAAAGTGCCAGACCAGAAGATGTGTTCATTCCAGGGGCGGGGAGTCTTCG
AGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAATAATGAAGG
ATCTTATTTCTTCGGAGACAATGCAGAGGAGTATGACAATTGA
```

### **C.3 The sequence information of the N gene of swine-origin influenza A virus**

**Influenza A virus (A/Puerto Rico/8/1934(H1N1)) segment 6, complete sequence**

**NCBI Reference Sequence: NC\_002018.1**

**>NC\_002018.1:21-1385 Influenza A virus (A/Puerto Rico/8/1934(H1N1)) segment 6, complete sequence**

```
ATGAATCCAAATCAGAAAATAATAACCATTGGATCAATCTGTCTGGTAGTCGGACTAATTAG
CCTAATATTGCAAATAGGGAATATAATCTCAATATGGATTAGCCATTCAATTCAAACTGGAA
GTCAAAACCATACTGGAATATGCAACCAAAACATCATTACCTATAAAAATAGCACCTGGGTA
AAGGACACAACCTTCAGTGATATTAACCGGCAATTCATCTCTTTGTCCCATCCGTGGGTGGG
CTATATACAGCAAAGACAATAGCATAAGAATTGGTTCCAAAGGAGACGTTTTTGTGATAAGA
GAGCCCTTTATTTTCATGTTCTCACTTGAATGCAGGACCTTTTTTCTGACCCAAGGTGCCTT
ACTGAATGACAGGCATTCAAATGGGACTGTTAAGGACAGAAGCCCTTATAGGGCCTTAATG
AGCTGCCCTGTCCGTGAAGCTCCGTCCCGTACAATTCAAGATTTGAATCGGTTGCTTGGT
CAGCAAGTGCATGTCATGATGGCATGGGCTGGCTAACAATCGGAATTTTCAGGTCCAGATAA
TGGAGCAGTGGCTGTATTAAATACAACGGCATAATAACTGAAACCATAAAAAGTTGGAGG
AAGAAAATATTGAGGACACAAGAGTCTGAATGTGCCTGTGTAAATGGTTCATGTTTTACTAT
AATGACTGATGGCCCGAGTGATGGGCTGGCCTCGTACAAAATTTTCAAGATCGAAAAGGG
GAAGGTTACTAAATCAATAGAGTTGAATGCACCTAATTCTCACTATGAGGAATGTTCTGT
ACCCTGATACCGGCAAAGTGATGTGTGTGTGCAGAGACAATTGGCATGGTTCGAACCGGC
CATGGGTGTCTTTTCGATCAAAACCTGGATTATCAAATAGGATACATCTGCAGTGGGGTTTTT
GGTGACAACCCGCGTCCCAAAGATGGAACAGGCAGCTGTGGTCCAGTGTATGTTGATGGA
GCAAACGGAGTAAAGGGATTTTCATATAGGTATGGTAATGGTGTGTTGGATAGGAAGGACCA
AAAGTCACAGTTCCAGACATGGGTTTGAGATGATTTGGGATCCTAATGGATGGACAGAGAC
TGATAGTAAGTTCTCTGTGAGGCAAGATGTTGTGGCAATGACTGATTGGTCAGGGTATAGC
GGGAGTTTCGTTCAACATCCTGAGCTAACAGGGCTAGACTGTATAAGGCCGTGCTTCTGG
GTTGAATTAATCAGGGGACGACCTAAAGAAAAACAATCTGGACTAGTGCGAGCAGCATTT
CTTTTTGTGGCGTGAATAGTGATACTGTAGATTGGTCTTGCCAGACGGTGCTGAGTTGCC
ATTCACCATTGACAAGTAG
```

#### **C.4 The sequence information of the N gene of original SARS-CoV-2 virus**

**Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu<sup>1</sup>, complete genome**

**NCBI Reference Sequence: NC\_045512.2**

**>NC\_045512.2:28274-29533 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome**

```
ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCT
CAGATTCAACTGGCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTC
GGCCCCAAGGTTTACCCAATAATACTGCGTCTTGTTTCACCGCTCTCACTCAACATGGCAA
GGAAGACCTTAAATTCCTCGAGGACAAGGCGTTCCAATTAACACCAATAGCAGTCCAGAT
GACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAAATG
AAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTC
CCTATGGTGCTAACAAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACC
AAAAGATCACATTGGCACCCGCAATCCTGCTAACAAATGCTGCAATCGTGCTACAACCTCCT
CAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCC
TCTTCTCGTTCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTA
GGGGAACCTTCTCCTGCTAGAAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGC
TGCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAAACAAGG
CCAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACT
GCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAACCCAAG
GAAATTTTGGGGACCAGGAATAATCAGACAAGGAAGTATTACAAACATTGGCCGCAAT
TGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGT
CACACCTTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCA
AATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCCACC
AACAGAGCCTAAAAAGGACAAAAGAAGAAGGCTGATGAAACTCAAGCCTTACCGCAGAG
ACAGAAGAAACAGCAAACTGTGACTCTTCTCCTGCTGCAGATTTGGATGATTTCTCCAAAC
AATTGCAACAATCCATGAGCAGTGCTGACTCAACTCAGGCCTAA
```

## C.5 The sequence information of the N gene of SARS-CoV-2 Delta variant

### >Delta\_B.1.617.2\_N\_Gene

```
ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCT
CAGATTCAACTGGCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTC
GGCCCCAAGGTTTACCCAATAATACTGCGTCTTGTTTCACCGCTCTCACTCAACATGGCAA
GGAAGGCCTTAAATTCCCTCGAGGACAAGGCGTTCCAATTAACACCAATAGCAGTCCAGAT
GACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAAATG
AAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTC
CCTATGGTGCTAACAAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACC
AAAAGATCACATTGGCACCCGCAATCCTGCTAACAAATGCTGCAATCGTGCTACAACCTCCT
CAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCC
TCTTCTCGTTTCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTAT
GGGAACTTCTCCTGCTAGAAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCT
GCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAAACAAGGC
CAAAGTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTG
CCACTAAAGCATAACAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAACCCAAGG
AAATTTTGGGGACCAGGAAGTAATCAGACAAGGAAGTATTACAAACATTGGCCGCAATT
GCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTC
ACACCTTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAA
ATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCCACCA
ACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTTATGAAACTCAAGCCTTACCGCAGAAAC
AGAAGAAACAGCAAAGTGTGACTCTTCTCCTGCTGCAGATTTGGATGATTTCTCCAAACAA
TTGCAACAATCCATGAGCAGTGCTGACTCAACTCAGGCCTAA
```

## C.6 The sequence information of the N gene of SARS-CoV-2 Omicron variant

### >Omicron\_B.1.1.529\_N\_Gene

ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACTCCGCATTACGTTTGGTGGACCCT  
CAGATTCAACTGGCAGTAACCAGAATGGTGGGGCGCGATCAAACAACGTCGGCCCCAAG  
GTTTACCCAATAATACTGCGTCTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCT  
TAAATTCCCTCGAGGACAAGGCGTTCCAATTAACACCAATAGCAGTCCAGATGACCAAATT  
GGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAAATGAAAGATCTC  
AGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGTG  
CTAACAAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAAGATCA  
CATTGGCACCCGCAATCCTGCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACA  
ACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGT  
TCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTAAACGAACTT  
CTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACA  
GATTGAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACACAACAAGGCCAAACTGT  
CACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAA  
GCATACAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAACCCAAGGAAATTTTG  
GGGACCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATT  
TGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTC  
GGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAA  
GATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCCACCAACAGAGCC  
TAA

## C.7 Information of LAMP primers for variants amplification

### C7.1 Primers for Delta amplification

| Name   | Sequence                                      |
|--------|-----------------------------------------------|
| D1-F3  | GTTCTTCGGAATGTCGCG                            |
| D1-B3  | CACAGTTTGCTGTTTCTTCT                          |
| D1-FIP | GTCATCCAATTTGATGGCACCTGCATGGAAGTCACACCTT      |
| D1-BIP | TATTGACGCATACAAAACATTCCCCTTGAGTTTCATAAGCCTTCT |
| D1-LF  | GTAGGTCAACCACGTTCCCG                          |
| D1-LB  | CCAACAGAGCCTAAAAAGGACAAAA                     |
| D2-F3  | GACAAAGATCCAAATTTCAAAGA                       |
| D2-B3  | GAGTCAGCACTGCTCATG                            |
| D2-FIP | TAGGCTCTGTTGGTGGGAATAAGTCATTTTGCTGAATAAGCA    |
| D2-BIP | CTTACCGCAGAAACAGAAGAAACATGTTGCAATTGTTTGGAGAA  |
| D2-LB  | CTTCCTGCTGCAGATTTGGATGA                       |

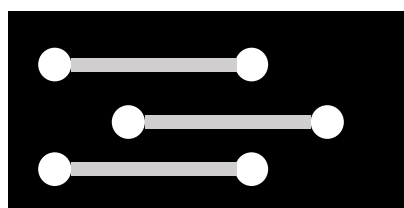


## C7.2 Primers for Omicron amplification

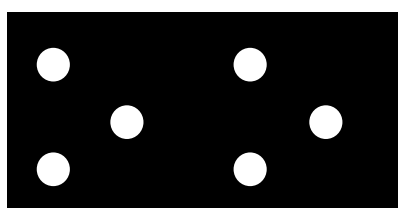
| Name   | Sequence                                   |
|--------|--------------------------------------------|
| O1-F3  | AGATCACATTGGCACCCG                         |
| O1-B3  | CCATTGCCAGCCATTCTAGC                       |
| O1-FIP | TCTGCGTAGAAGCCTTTTGGCACAATGCTGCAATCGTGCTAC |
| O1-BIP | GGCGGCAGTCAAGCCTCTTCTTACTGCTGCCTGGAGTTG    |
| O1-LB  | TCGTTCTCATCACGTAGTCGC                      |
| O2-F3  | GGCTTCTACGCAGAAGGGA                        |
| O2-B3  | TTGCTCTCAAGCTGGTTCAA                       |
| O2-FIP | TTCGTTTACTGCTGCCTGGAGTGCAGTCAAGCCTCTTCTCG  |
| O2-BIP | TTCTCCTGCTAGAATGGCTGGCTCTGTCAAGCAGCAGCAAAG |
| O2-LF  | GTTGCGACTACGTGATGAGGAA                     |
| O2-LB  | GGCGGTGATGCTGCTCTT                         |
| O3-F3  | TGGCTACTACCGAAGAGCT                        |
| O3-B3  | TGCAGCATTGTTAGCAGGAT                       |
| O3-FIP | TCTGGCCCAGTTCCTAGGTAGTGACGAATTCGTGGTGGTGA  |
| O3-BIP | AGACGGCATCATATGGGTTGCAGCGGGTGCCAATGTGATC   |
| O3-LB  | GAGGGAGCCTTGAATACACCAA                     |

## Appendix D: Development of a paper microfluidic device for SARS-CoV-2 on-site detection from wastewater

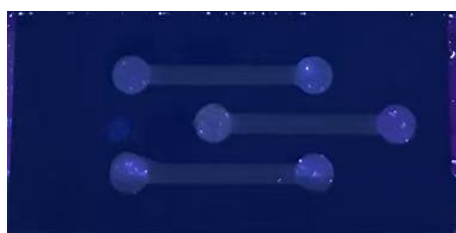
### D.1 The scheme of two-group reaction chamber



Top view



Bottom view



Real sample