



Xuanye Ma

Dual model sensors for viral RNA and protein detection for SARS-
CoV-2 in saliva

School of Water, Environment and Energy Research Degree
MSc by Research

Academic Year: 2021 - 2023

Supervisor: Professor Zhugen YANG

Associate Supervisor: Dr Iva Chiarelli
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ABSTRACT

The coronavirus disease 2019 (Covid-19) pandemic emerged as an infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) over the past 3 years, causing enormous threats to humans and economic loss. It was reported that currently, nearly 68% of the population shows a degree of immunity to the disease. Though vaccine has played a critical role to protect the population, people are at risk of second or third infections with fading antibody titers against the virus. The detection of the viral load was crucial for monitoring the spread of this disease, and the detection of the antibody concentration to the virus was significant as well to understand the neutralization activity and vaccine response. This thesis reports the development of a rapid paper-based platform, which provides nucleic acid detection and immunoassay to test both active infections and body immunity. The project focused on a non-invasive sample, human saliva, as an alternative to nasopharyngeal swabs for diagnosis. The nucleic acid test, employing reverse transcription loop-mediated isothermal mediated amplification (RT-LAMP), was further integrated into a novel paper microfluidic platform, where the result could be reported within 30 mins. Under the optimized conditions, RT-LAMP assay correctly detects above 135 copies μL^{-1} of synthetic SARS-CoV-2 sequence. Moreover, a paper-based immunoassay was devised and constructed, following meticulous refinement of detection parameters and comparative analysis against a conventional 96-well plate assay for the identification of immunoglobulin G (IgG) targeting the SARS-CoV-2 spike protein. For the paper-based immunoassay, the dynamic range of the IgG was 0.5 $\mu\text{g mL}^{-1}$ to 50 $\mu\text{g mL}^{-1}$, which was sensitive enough compared to clinical values. It should be noted that only commercially available artificial saliva was utilized to test the analytical performance of the developed assay. In conclusion, these results demonstrated a rapid and easy-to-use paper microfluidics platform with the potential to be further implemented as a comprehensive detection tool for monitoring both pathogenetic infection and immune levels.

Keywords: COVID-19, nucleic acid, antibody concentration, point-of-care diagnosis

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CRISPR	Clustered, regularly interspaced short palindromic repeats
Ct	Cycle threshold
FDA	Federal drug administration
dPCR	Digital polymerase chain reaction
EUA	Emergency-Use-Administration
ECLIA	Electrochemiluminescence immunoassay
EGF	Epidermal growth factor
ELISA	Enzyme immunosorbent assay
FRET	Fluorescence resonance energy transfer
HR1	Hepated repeat 1
HRP	Horseradish Peroxidase
HUDSON	Heating unextracted diagnostic samples to obliterate nucleases
ISO	International organization for standardization
IVD	In vitro diagnosis
LAMP	Loop-mediated isothermal amplification
LFA	Lateral flow immunoassay
LOD	Limit of detection
LOQ	Limit of quantification
NAAT	Nucleic acid amplification tests
Nab	Neutralizing antibody
NGF	Nerve growth factor
NPMA	National medical products administration
NTC	Non-template control
NTD	N-terminal domain
ORF	Open reading frames
P-ELISA	Paper-based ELISA
PBS-T	Phosphate buffer saline-tween
PMMA	Polymethyl methacrylate
PDMS	Polydimethylsiloxane
POCT	Point-of-care testing
PRPs	Proline-rich proteins
RBD	Receptor binding domain
RCA	Rolling circle amplification
RdRp	RNA-dependent RNA polymerase
RPA	Recombinase polymerase amplification
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	Serve acute respiratory syndrome coronavirus
SD	Standard deviation
SDA	Strand-displacement amplification
SOP	Standard operating procedure

TGF-α	Transforming growth factor alpha
TMB	3, 3', 5, 5'-tetramethylbenzidine
TMPRSS2	Transmembrane protease serine 2
VOC	Variant of concern
VOI	Variant of interest

1 INTRODUCTION

1.1 Background

1.1.1 The COVID-19 pandemic

On 31 December 2019, the World Health Organization (WHO) announced a severe pneumonia outbreak in Wuhan, Hubei Province, China. After additional cases were reported, it was confirmed that the disease was caused by a novel coronavirus, which was subsequently named SARS-CoV-2. The origins of SARS-CoV-2 are hypothesized to have begun at the Huanan market, and further investigations have confirmed that the market was the early epicenter of the pandemic, strongly associated with vendors selling living mammals¹. In March 2022, WHO officially declared COVID-19 a global pandemic, and it was regarded as 'the most crucial global health calamity of the century and the greatest challenge humankind faced since the 2nd World War'. The clinical presentation of COVID-19 is highly heterogeneous, with a significant proportion of infected individuals (approximately 80%) presenting with symptoms. As of 3 February 2023, 676,101,245 confirmed cases have been reported globally², with significant heterogeneity in the distribution of cases across countries, as shown in Figure 1.1 .

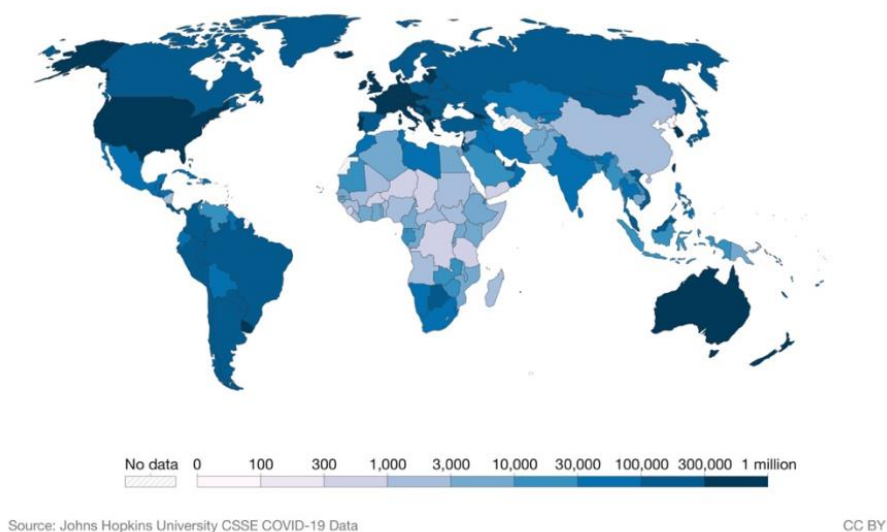


Figure 1.1 Cumulative confirmed COVID-19 cases per million, FEB 3, 2023².

1.1.2 Current detection methods for SARS-CoV-2

The development of SARS-CoV-2 diagnosis has been accelerated due to the demand for clinical testing during the pandemic. Initially, cases were confirmed based on blood counts and serum biochemical tests, such as renal function tests, liver function tests, or creatine kinase tests. However, it would take at least half a day to produce results from these chemical tests³. Therefore, alternative detection methods focusing on detecting viral antigens, viral nucleic acids or antibodies against the virus are being investigated to expedite the turnaround of test results.

Rapid antigen tests have gained popularity due to their relatively low cost, ease of use, and rapid turnaround time. The lateral flow immunoassay (LFIA) is commonly used and involves the application of chromatographic principles⁴. LFIA strips comprise a sample pad, a releasing pad, a nitrocellulose (NC) membrane, an absorbent pad, and a polyvinyl chloride backing. This test operates by detecting specific SARS-CoV-2 antigens like various proteins in a person's nasopharyngeal or oropharyngeal swab sample. If the virus presents in the sample, the antigens will bind to the antibodies, producing a colour change for signal, and any excess liquid from the sample reaches the aspiration pad⁵. However, it should be noted that these tests have demonstrated a lower sensitivity (30%-40%) than the reverse transcription quantitative PCR (RT-PCR) test⁶, particularly when used in asymptomatic individuals or populations with low viral loads. Numerous LFIA tested by Cubas-Atienzar et al.⁷ confirmed that the limit of detection (LOD) from swab buffer was 2-3 orders of magnitude higher than RT-qPCR, which was above 200,000 viral load copies mL⁻¹.

PCR based on DNA polymerase enzyme and a pair of primers, is driven by a thermal cycler and continues provoking double helix strand separation, primers annealing and extension which forms exponentially amplified DNA⁶. With the addition of fluorescence markers, PCR has transitioned from a non-quantitative analytical assay to a real-time tracking method, officially known as quantitative PCR (qPCR), facilitating the assessment of amplification profiles. A reverse

transcription (RT) is required due to the detection of an RNA virus. Within the RT-qPCR, the cycle threshold (Ct) value is a strong indicator for reporting diagnosed results. Typically, the assay should have a Ct value <40 with no amplification in the no template control⁸. Furthermore, the function of known concentrations by combining a calibration curve, quantified standards and Ct values enables the quantification of viral RNA gene copy⁹. RT-qPCR is generally performed in high-complexity large batches with RNA extraction and purification then followed with automation to conduct the assay set up by trained personnel. The strategy of RT-qPCR for diagnosing SARS-CoV-2 is roughly the same but varies in basic reagents and protocols used by different routine laboratories including primer sets, probes or reaction types. Currently, the corresponding LOD for SARS-CoV-2 assay is 10-1000 viral copies mL⁻¹ in most commercial platforms like Roche Cobas or Abbott ID NOW⁸.

Isothermal amplification is an alternative method that could amplify nucleic acid at a constant temperature, which avoids thermal cycling with a shorter time than RT-qPCR¹⁰. In this case, this method also leads to the exclusion of the need for advanced instrumentation like a thermocycler. The loop-mediated isothermal amplification (LAMP) utilized a strategy using 4-6 primers and a DNA polymerase with strand displacement activity without subduing analytical performances. LAMP is believed to have better performance than crude samples; therefore, RT-LAMP is an advantage over RT-qPCR since sample extraction is not part of the workflow. Although RT-qPCR presents higher sensitivity when detecting low genomic copy, a LAMP assay can obtain 100% sensitivity and 98% specificity within 30 mins for results in most cases¹¹. A commercially available isothermal technology from Color Genomics approved by EUA shows a LOD of 750 viral copies mL⁻¹ tested in 543 patients¹². While the majority of house LAMP LOD is around 3000-5000 viral copies mL⁻¹ without the need for RNA extraction^{12,13,14}. LAMP can also be coupled with other techniques, such as clustered, regularly interspaced short palindromic repeats-Cas (CRISPR-associated) systems to improve the overall sensitivity and specificity. Relying on its ability to recognize and cleave specific DNA and RNA sequences¹⁵, this system has adapted and

coupled with well-developed systems to improve their performances. Generally, the detection works on two steps: the amplification step based on LAMP prior to CRISPR-mediated signal step. Cas proteins have different features, and the most renowned ones are Cas9, Cas12, Cas13 and Cas14. Credits to the trans cleavage activity (i.e., non-specific single stranded DNA cleaved when cis cleavage presents) of Cas12a, it is the most popular CRISPR/Cas detection system in SARS-CoV-2 diagnosis¹⁶. The integration of LAMP technique with the Cas system could also enhance the specificity and sensitivity of the assay reducing the false-positive rate solely based on LAMP detection.

Following the implementation of the vaccination program, the demand for antibody testing has escalated, as a considerable portion of the population remains vulnerable to potential reinfection.¹⁷ The antibody protection against SARS-CoV-2 remains temporally for a median interval from five months to one year. Further data analysis also revealed that specific demographic and environmental factors, including older age, pre-existing immunological conditions, and living in congregate care settings, were associated with an increased risk of reinfection¹⁸. In this context, the requirement for antibody tests is becoming demanding, as it would provide an understanding of a comprehensive immune status.

The most common type of immunoassay is called enzyme-linked immunosorbent assay (ELISA) with various formats including direct ELISA, indirect ELISA or sandwich ELISA, and many regulatory agencies have released various formats for detecting SARS-CoV-2 antibodies¹⁹. The result is measured by the intensity formed from enzyme catalysis activity via antigen-antibody reaction¹⁹. However, this test is typically performed in a lab environment with a microplate reader, which takes at least four hours.

LFIA has also been applied to antibody detection to address these issues. This test utilizes capillary action, allowing the sample liquid to flow through the

conjugation pad to the detection zone, where detected analytes usually pre-immobilized SARS-CoV-2 spike protein bind to the anti-SARS-CoV-2 antibody in the sample. This specific binding would produce a colour indicator with gold conjugate in the test zone where a band is shown²⁰. LFIA is a more accessible and faster alternative to equipment-based immunoassay, as it can be performed outside of a laboratory setting and provides results in a shorter time frame. In July 2021, over 3,000 patients participated in a self-administered Fortress LFIA on finger-prick blood for the assessment of anti-SARS-CoV-2 IgG²⁰. The obtained LFIA results were subsequently cross-validated with those derived from the Roche Elecsys® Anti-SARS-CoV-2 electrochemiluminescence immunoassay (ECLIA), which provided specific concentration measurements. Self-administered LFIA is less sensitive than a quantitative antibody test, but the positivity in LFIA correlates better than the quantitative ECLIA with virus neutralization.

In conclusion, those assays mentioned above are widely deployed in laboratories and testing settings, providing reliable results for the surveillance of the pandemic. However, all the assays carried out in the lab and the self-test is mainly based on conventional samples like a serum, which requires a needle to collect blood, or nasopharyngeal swabs, which are painful and invasive. For this reason, using a less invasive sample type, such as saliva, holds significant promise.

1.1.3 Saliva diagnosis

Saliva is a complex mix of fluids secreted from major and minor salivary glands. The major gland secreting saliva is usually found on the mouth floor, while minor glands are in several locations, such as the lower lip, tongue, cheek and pharynx²¹. Most saliva is produced from major parotid, submandibular, and sublingual glands. However, the saliva generated from the minor gland shares the most protective components such as electrolytes (Na⁺, K⁺, Ca⁺, COOH⁻ and

PO43-), immunoglobulin, enzymes, nitrogenous products, and proteins²² as shown in Figure 1-2.

Saliva acts as a mirror of body condition that is beneficial for expanded research in diagnosis. The saliva's primary role is not limited to dentistry but can be analysed for diagnosing periodontal diseases, and it is also an important sample for virology and bacteriology analysis. For example, saliva is a valuable material for detecting the existence of viral DNA or RNA, as was demonstrated in 1997 by Scully and colleagues, who were able to detect HIV in human saliva. In addition, it has been proved that DNA isolated from saliva can tolerate room temperature for up to 5 days²³. A clinical case study confirmed that the sensitivity test for HIV in saliva was up to 77%²⁴. In addition, the current study has revealed a strong correlation between salivary viral burden with symptoms, in conjunction with the salivary antibody response to SARS-CoV-2 nucleocapsid and spike proteins. These findings underscore the potential utility of saliva as an innovative diagnostic medium²⁵.

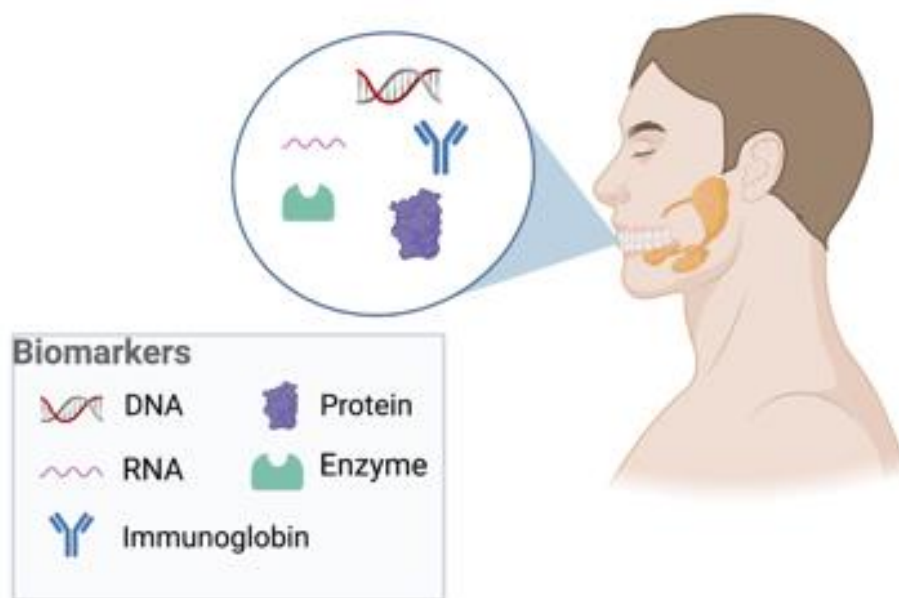


Figure 1-2 Potential salivary biomarkers used for diagnosis and disease monitoring.

1.1.4 Paper-based microfluidic device for saliva diagnosis

Manz and Winder first proposed the lab-on-chip concept in 1990²⁶ it benefited the control of the fluid on a micron scale; hence, the laboratory scale of biochemical analytes could be performed on several square centimetres. The most common microfluidic materials are polymethyl methacrylate (PMMA) and polydimethylsiloxane (PDMS), and PDMS is dominant in the current microfluidic chip manufacture. Martin and Synge²⁷ in 1941 invented the first paper-based model with chromatography paper that brought public attention to paper usage. In 2007, Whitesides' group managed to manipulate liquid flow via hydrophobic patterns on paper which made the paper an alternative for microfluid materials²⁸.

Paper-based microfluidic devices are ideal for point-of-care testing (POCT), as they provide people with diagnostic technology that is simple, inexpensive, portable, and disposable. It has been an advanced tool for many applications, such as clinical diagnosis, food quality control, and environmental monitoring. The first lateral flow assay, the pregnancy test, is based on paper and is the most commercialized diagnosis kit ²⁹. Additionally, the paper-based microfluidic devices fully match the WHO-ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid/Robust, Equipment-free and Deliverable) requirements for POCT^{29,30,31}. In view of clinical diagnosis, current paper-based microfluidic has shown great potential in detecting different body fluids such as blood, urine, and plasma. As previously mentioned, the saliva diagnosis landscape could also be expanded to paper-based microfluidic devices. Some literatures have succeeded in detecting nitrite or glucose^{32,33,34} (Figure 1-3), and acceptable detection limits are achieved after modifications as well as possibility on a large manufacturing scale. For example, four identical (Figure 1-A) channels modified with 5% H₃PO₄ could detect nitrite in saliva with a LOD of 10 $\mu\text{mol L}^{-1}$. Furthermore, some designs shown in Figure 1-B are easy to produce with large amounts simultaneously.

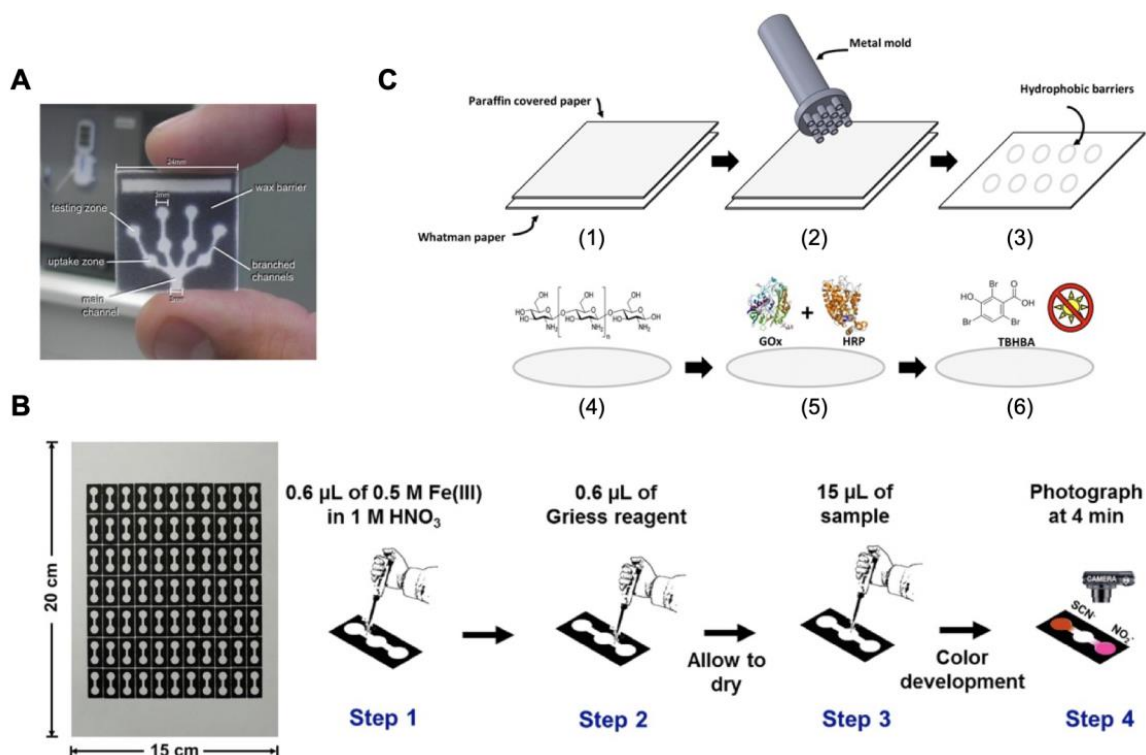


Figure 1-3 Paper-based microfluidic in saliva diagnosis. **A** The design for nitrite detection in saliva containing the hydrophilic main channel, branched channels, uptake zones, and testing zones surrounded by the hydrophobic wax barrier³². **B** A fabric ink-printed paper substrate with 77 dumbbell-shaped devices per production, and operating procedures for determination of thiocyanate and nitrite in synthetic saliva³³. **C** An illustration of the glucose determination strategy, and a smartphone camera used to capture the colour change³⁴ (All images are reprinted with permission from Talent, Analytical chemistry).

Overall, the developments in paper-based microfluidic diagnostics have enhanced POCT capabilities due to the low-cost and versatile features. However, it also has been pointed out that paper devices are still limited by some aspects associated with material properties. Table 1-1 briefly summarizes the pros and cons of this technology, and it could be seen that paper-based microfluidic devices possess great advantages in clinical aspects with high sensitivity in detecting saliva biomarkers. Those established platforms highlight the possibility of using paper-based devices to test more biomarkers such as nucleic acid and immunoglobins in saliva with adjustable device design as it is the goal of this thesis.

Table 1-1 The paper-based microfluidic device for biosensing applications in saliva sample

Biomarkers	Detection methods	LOD	Advantages	Disadvantages	References
Nitrite	Colourimetric	10 $\mu\text{mol L}^{-1}$	Easy to use	Hard to quantify	[32]
Glucose	Colourimetric	0.37 mgdL^{-1}	Easy to use; Quantification; Multiplex analysis	Lightning optimizations	[33]
	Electrochemical	0.15 mmol L^{-1}	Ease of production; High environment tolerance		[35]
Metalloprotein ase-8	Electrophoretic	/	Rapid, reliable	Single test	[36]

1.2 Aims and Objectives

This thesis aims to develop a state-of-art dual paper-based sensor for detecting SARS-CoV-2 in human saliva. At this early developmental stage before further evaluations with human saliva, artificial saliva was substituted to circumvent stringent regulatory approval processes and potential variability in real saliva samples. The artificial saliva components could closely mimic those found in actual human saliva, thereby allowing for the successful demonstration of the proof-of-concept using this substitute sample matrix. There are four main objectives in this project:

1. Transcribe synthetic ORF1ab dsDNA to ssRNA to evaluate the limit of detection (LOD) in RT-qPCR and RT-LAMP assay with designed primers.
2. Integrate the developed assay onto the paper-based platform and test the spiked artificial saliva samples to evaluate the analytical performance for future human saliva testing.
3. Adopt indirect ELISA on a 96-well plate to optimize the detection conditions and determine the optimal conditions for paper-based ELISA assay in artificial saliva samples. Managed to provide a conceptual platform for real saliva sample detection.

4. Evaluate limitations of paper-based platform for nucleic acid assays and immunoassays, with a discussion of further work could be done to get to the point that a real-world analytical test would be available.

1.3 Thesis structure

The thesis is formatted into four chapters illustrated in Figure 1-4. The experiments were all conducted by Xuanye Ma at Cranfield University under the guidance of Drs Zhugen Yang and Iva Chianella.

Chapter 1 mainly discussed the impacts of SARS-CoV-2 and current detection methods, suggesting that there are limitations to the current point-of-care testing. As an alternative, saliva is recommended to be the tested sample.

Chapter 2 used conventional gold standard RT-qPCR to test the artificial saliva sample. Then, the colourimetric RT-LAMP was utilised as the final amplification method and integrated with the paper-based device to test the SARS-CoV-2 ORF1ab RNA sequence in artificial saliva.

Chapter 3 developed a paper-based indirect ELISA that could quantify a range of IgG against SARS CoV-2 spike protein in artificial human saliva. The assay conditions were initially optimised on a 96-well plate and transferred to the paper-based ELISA. Both chapters 2 and 3 explored the sample processing of artificial saliva compatible with the assay.

Chapter 4 Concluded the current results derived from the dual platform and discussed the possibility and challenges of transforming this platform to a real analytical testing scope.

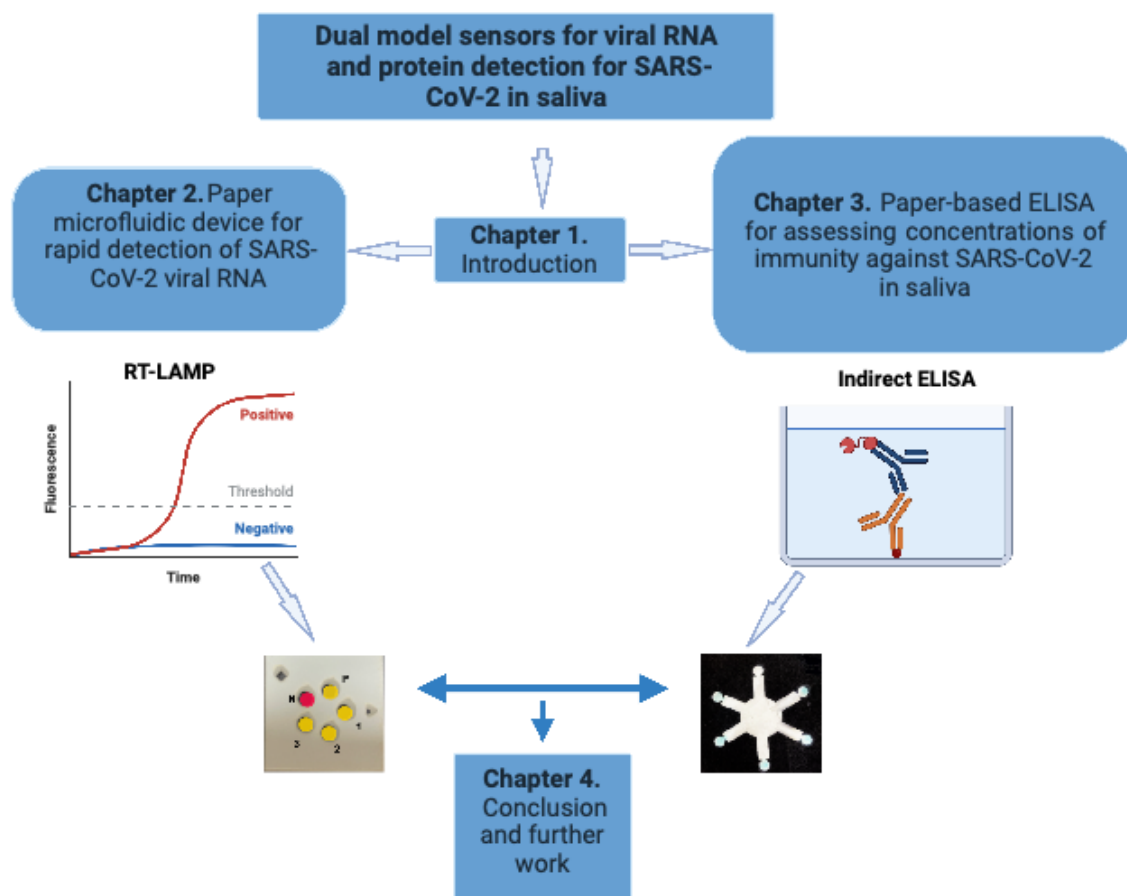


Figure 1-4 The overall thesis plan. The project is divided into two parallel research developments to validate the use of artificial saliva as matrix for both RT-LAMP and paper-based ELISA

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2 Paper-based microfluidic devices for rapid detection of SARS-CoV-2 viral RNA in saliva

2.1 Abstract

RT-qPCR enables the sensitive detection of SARS-CoV-2 viral RNA but relies on thermal cycler and skilled personnel, limiting its broader applications in low-resource settings. In contrast, RT-LAMP assays, conducted at a constant temperature, offer a rapid and competitively sensitive way compared to RT-qPCR. Using paper, interoperating colorimetric RT-LAMP by the naked eye addresses the concern of RT-qPCR. The advantages of paper, including affordability, lightweight nature, and widespread availability, support its potential for mass production and commercialization. This study explores the adaptation of artificial saliva samples with a paper-based device. The suitability of the artificial saliva matrix was confirmed through RT-qPCR and RT-LAMP analysis using a fluorescence machine. Subsequent integration of the colourimetric RT-LAMP assay with the paper device facilitated the detection of SARS-CoV-2 viral RNA sequences, with a LOD exceeding 135 copies μL^{-1} , enabling the identification of the majority of infected individuals. Although not yet tested with human saliva, this work underscores the potential of artificial saliva in RT-LAMP detection when integrated into a paper-based platform, offering a novel point-of-care testing (POCT) device based on the artificial saliva model.

2.2 Introduction

2.2.1 SARS-CoV-2 genome structure

The COVID-19 caused by severe acute respiratory coronavirus 2 is an enveloped, positive-sense, single-stranded RNA that is categorised as the genus Beta coronavirus, and among those coronaviruses, SARS-CoV-2 features a unique RNA-dependent RNA polymerase (RdRp) characteristic. Similarly, this virus is dependent on an enzyme as its obligate receptor, angiotensin-converting enzyme (ACE2), to penetrate the cells¹. The SARS-CoV-2 virion consists of four structural proteins shown in Figure , which are nucleocapsid (N), membrane (M),

envelope (E) and spike (S) proteins. The SARS-CoV-2 genome recently sequenced and submitted to the NCBI genome database (NC_045512.2) is approximately 29.9 kilobases in size. The genome contains 13 to 15 open reading frames (ORFs), of which 12 are functional. It spans roughly 30,000 nucleotides with a precise GC content of 38%.² The ORFs are organised into replicase and protease (1a–1b), and the major S, E, M, and N proteins are shown in a typical 5'-3' order of appearance. The truth is that most viral genome content is held by the two non-structural proteins ORF1a and ORF1b, followed by the structural proteins. ORFs 1a and 1b encode the polyproteins pp1a and pp1ab, respectively, with pp1ab encoded through the ribosomal frameshift mechanism of the gene 1b. These gene products play crucial roles in viral entry, fusion, and survival in host cells and are considered important targets for target choice and vaccine development³.

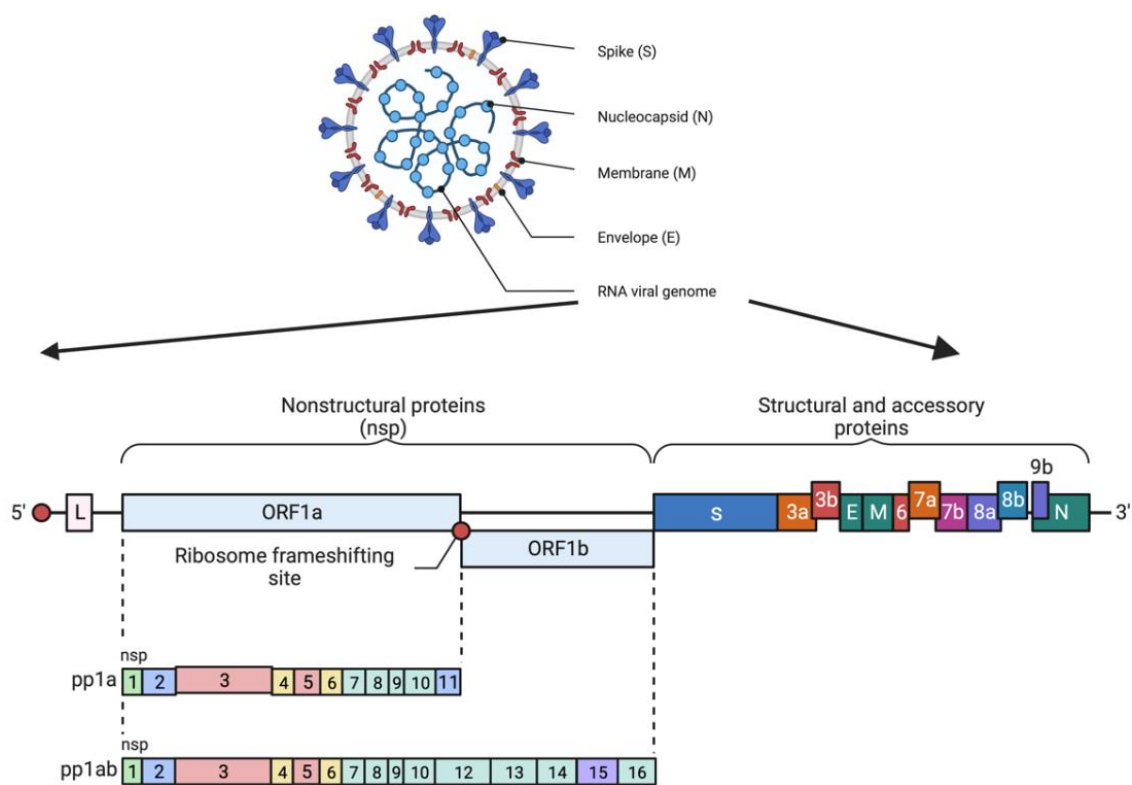


Figure 2-1 The structure of SARS-CoV-2 virion protein and its related viral genome. The viral genome organisation displays the sequential arrangements of the expression

of non-structural and structural, and accessory proteins (The figure is reprinted from the version of Hartenian, E. et al.) ⁴

The proliferation of viral variants poses a significant impediment to comprehending viral characteristics, thereby leading to several challenges in the realm of diagnostic procedures. Variants are caused by nucleotide changes that naturally emerge within viral genome replication, and the mutation is more likely to occur in RNA viruses⁵. Based on the preliminary circulation, there are more than 4,000 mutation strains, but not all are dominant. SARS-CoV-2 variants are classified into two groups by the WHO: variants of concern (VOC) and variants of interest (VOI) ⁶. VOI is referring to specific genetic changes associated with the receptor binding. VOC expresses similarly to VOI but is attributed to a greater disease severity across the globe. Currently, the circulating strain worldwide is Omicron (XBB 1.5) which has increased transmissibility, virulence, and resistance to the vaccine. This variant has a recombination of previous BA.2 lineages and a similar spike genetic profile as XBB.1.9, which distinguishes evolutionary pathways compared to other variants⁷. The reported cases suggest that milder symptoms present with the Omicron. Another gene, like the N gene mutation, is also reported to affect the sensitivity of nucleic acid tests due to variable incongruities between the primers⁷. Conversely, non-structural protein genes are less prone to mutations, and some regions like NSP1 and NSP14 are quite conserved⁸. In addition, over 100 clinical patients indicate that targeting NSP1 (ORF1ab) primers is highly expressed in saliva sample⁹. Thus, opting for the amplicon derived from ORF1ab could potentially ensure a consistent rate of detection.

2.2.2 RT-LAMP assay with saliva samples

The landscape of nucleic acid test for SARS-CoV-2 comprises different sectors including nucleic acid amplification tests (NAATs), and sequencing. Sequencing as a high-throughput measure could scale up performed tests, especially in determining the origin of pathogens and novel variants in the COVID-19 pandemic. Moreover, uncovering the viral lineage helps detect the transmission pattern of outbreaks even before the clinical diagnosis. This method is already

used in wastewater surveillance for monitoring SARS-CoV-2¹⁰. However, sequencing is costly (up to £880 for one whole genome), making it unsuitable for low-cost demands¹¹. As for the individual diagnosis, other nucleic acid approaches are more common. Therefore, this work would focus on NAAT approaches that are more suitable for a POCT. PCR utilizes a pair of primers and DNA polymerase enzymes to yield exponential DNA amplifications. With the addition of a fluorescence marker, this technique enables the semi-quantitative and visual measurements¹². Regarding SARS-CoV-2, a single-strand RNA virus, the inclusion of reverse transcriptase (RT) is required, and its performance depends on many demands, such as a standard curve with Ct values of known target concentrations so that an unknown sample concentration can be determined. Other parameters are considered, including viral load, which strongly relies on the successful sample choice and virus isolation and sampling methods¹³. The mean viral concentrations observed from initial symptoms range from 10^4 to 10^7 copies mL⁻¹, and RT-qPCR, considered as the gold standard, is widely used to detect the virus for its good sensitivity and specificity. Without including a standard curve, a derivative RT-qPCR called digital PCR (dPCR) can detect 625 copies mL⁻¹ of nucleic acid¹⁴.

The alternative amplification is isothermal nucleic acid amplification; compared to RT-qPCR, isothermal amplification benefits from its simple, and rapid detection methods. Generally, this method generates the detected signal by increasing the target nucleic acid concentration at a fixed temperature. The signal is usually presented with fluorescence or colourimetric indicators that colour changes or turbidity suggest the presence of the target¹⁵. The standard approaches are not limited to LAMP; recombinase polymerase amplification (RPA), strand-displacement amplification (SDA), and rolling circle amplification (RCA) are frequent to see as well. LAMP is a powerful and rapid detection method which uses strand displacement DNA polymerase; however, the LAMP primer design is complex and contains inner and outer primers (four to six specific primers) (Figure 2-2). The multiple primes secure the high specificity, especially targeting SARS-CoV-2 up to 96%¹⁶. Common primers are usually located on open-reading

frame 1ab (ORF1ab), N and S gene and some studies indicate that primers targeting the ORF1ab region have higher amplification efficiency, particularly in saliva samples¹⁷. For example, the TaqPath C OVID-19 from Thermo Fisher has also managed to test saliva samples¹⁸ targeting ORF1ab. However, saliva LOD is not confirmed yet, and this assay and other commercial kits are based on RT-qPCR conducted on the equipment for up to at least 1 hour. To help understand the saliva role in a POCT device, this study will focus on the artificial saliva sample amplification targeting the ORF1ab region.

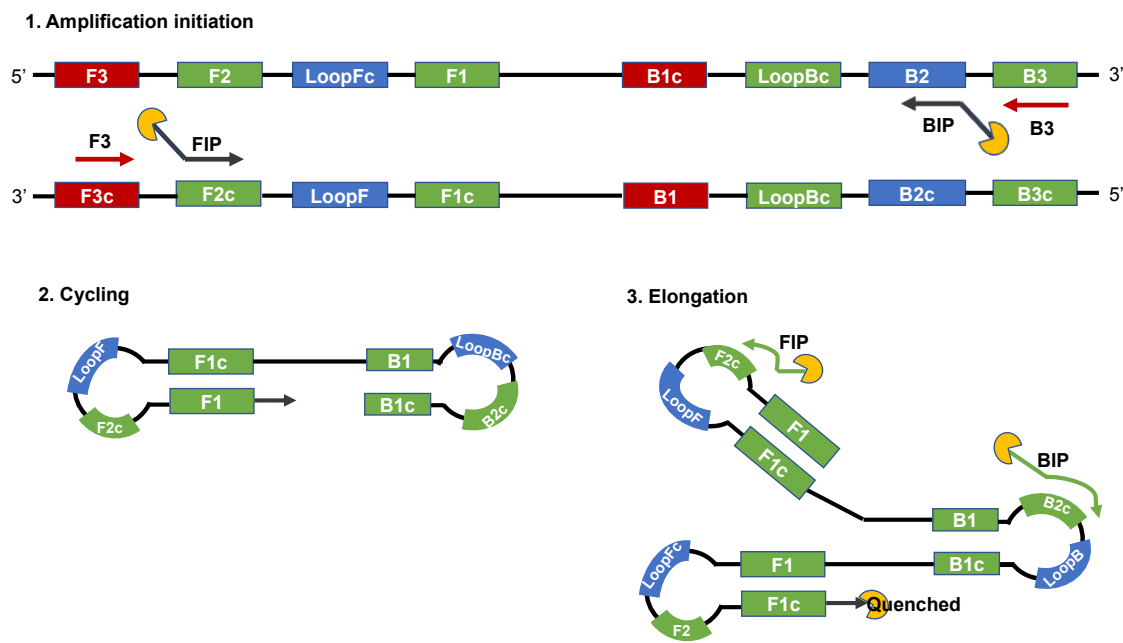


Figure 2-2 The mechanism of LAMP. The LAMP reaction involves amplification initiation, cycling, and elongation. 1. To initiate the reaction, LAMP and displacement primers facilitate the strand invasion of double-stranded DNA. The recognition sites for the displacement primers are highlighted in red, while those for the LAMP and loop primers are shown in green and blue, respectively. 2. This initiation step leads to the formation of dumbbell-like structures, which undergo cycling between two forms. 3. The reaction then progresses to elongation, forming cauliflower-like structures with multiple loops. A fluorophore (yellow) is attached to the FIP primer, and its fluorescence is quenched by proximity to guanine bases through fluorescence resonance energy transfer (FRET). FIP: Forward Inner Primer; The FIP consists of a F2 region at the 3'end and a F1c region at the 5'end. BIP: Backward Inner Primer; The BIP consists of a B2 region at the 3'end and a B1c region at the 5'end.

The interest in detecting COVID-19 in saliva drew attention since saliva is less invasive than nasal swab tests, and saliva can be analyzed more easily by individuals, reducing the risk of transmission to healthcare workers. The first study using saliva was reported in Hong Kong; 11 patients were found to have a detectable amount of viral load in saliva¹⁹, and there were consistent declines in salivary viral RNA levels till recovery. Combined with other studies²⁰, the salivary viral load from collected specimen were measured between 9.9×10^2 to 1.2×10^8 copies mL⁻¹. The sensitivity and specificity were also assessed to confirm further the plausibility of using saliva samples. According to a summary report, the ratio ranged from 66% to 91.7% and from 97% to 100% prospectively, based on comparing RT-qPCR-analysed saliva specimens and the gold standard diagnostic nasopharyngeal swabs²¹. Another critical perspective is that saliva is a less invasive sample in collection than serum. Although the benefits are noticeable, using saliva as the sample faces some challenges. For example, the saliva's viral load is relatively low compared to the nasopharyngeal swab sample, which makes it necessary to optimize the assay protocols to meet the sensitivity. On the other hand, the saliva pre-treatment differs from a general sample; therefore, it is worth trying to develop a standardized way of treating the saliva, making it compatible with nucleic acid detection. In this section, saliva processing was proposed, and with the aid of isothermal amplification, the advantages of using artificial saliva as a sample were elevated. Furthermore, to achieve the goal of POCT, it is also investigated whether the SARS-CoV-2 RNA could be detected based on the paper-based device within an acceptable sensitivity^{21, 22, 23}.

2.3 Materials and Methods

2.3.1 Primer design and transcription of SARS-CoV-2 fragment

LAMP primers were previously described by Jamwal et al.⁹ The LAMP primer set targeting ORF1ab of SARS-CoV-2 was designed with PrimerExplore V5 (<https://primerexplorer.jp/e/>) and synthesised by Merck (Sigma-Aldrich®).

Solutions, US). BLAST results of F3/B3 showed high similarity with SARS-CoV-2 ORF1ab region. The ratio of each primer was prepared by mixing thoroughly with a primer mix (16 μ M FIP/BIP, 2 μ M F3/B3, 4 μ M LF/LB). The specific sequences were listed in

Table 2-1, and qPCR primers were directly used from LAMP F3 and B3 primers.

Table 2-1 LAMP primers set for SARS-CoV-2 targeting ORF1ab sequence

Primer set	Sequence (5'-3')
F3	CTGCACCTCATGGTCATG
B3	GATCAGTGCCAAGCTCG
FIP	GAGGGACAAGGACACCAAGTGTGGTAGCAGAACTCGAAG
BIP	CAGTGGCTTACCGCAAGGTTTTAGATCGGCGCCGTAAC
LF	CACTACGACCGTACTGAAT
LB	CGTAAGAACGGTAATAAAGGAG

The plasmid DNA targeting ORF1ab (spanning from 200 to 800bp, 601bp) of the SARS-CoV-2 whole genome was synthesised by ThermoFisher Scientific (US). The forward primer containing T7 RNA Polymerase promoter (Merck KGaA, Germany) and reverse primer designed (TAATACGACTCACTATAGGG) for this fragment were used to amplify and obtain a segment for further transcription. The agarose gel (85 constant voltage, 45 mins) was run to confirm the size of the amplicon. After confirming the PCR product, a gel was recovered with a kit (ZYMO RESEARCH, US). Then HiScribe T7 High Yield RNA synthesis kit (NEW ENGLAND, BioLabS Inc, US) was used to transcribe the clean product, and 1 μ g PCR fragment was used in a 20 μ L in *vitro* reaction. The reactions were incubated at a constant 37°C, and the product was purified via Monarch total RNA miniprep kit (#T2010S).

2.3.2 RT-qPCR assay

The ssRNA SARS-CoV-2 fragment was acquired via transcription. The concentration of the fragment was initially measured through Invitrogen Qubit 4 fluorometer (ThermoFisher Scientific, US), and the starting concentration was 2.4ng μL^{-1} . According to the formula:

$$\text{RNA copies} = \frac{\text{Amount} \times 6.022 \times 10^{23}}{\text{Length} \times 1 \times 10^9 \times 340} \quad (2-1)$$

The copy number based on the formula was 4×10^9 copies μL^{-1} . Then the sample was serially diluted from 4×10^7 copies μL^{-1} to 4×10^{-2} copies μL^{-1} and subjected to RT-qPCR to generate a standard curve. For the RT-qPCR, the following reaction components were used: 5 μL TaqMan™ Fast Virus 1-step master mix (ThermoFisher Scientific, US), 0.5 μL forward primer (20mM), 0.5 μL reverse primer (20mM), 1 μL Eva Green, 5 μL single-stranded RNA, and 8 μL RNase-free water. The reactions were performed on a QuantStudio 3 Real-Time PCR system (ThermoFisher Scientific, US). Real-Time RT-qPCR system using the following amplification conditions: hold at 50°C for 2 minutes, followed by 95°C for 20 seconds, and 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds, and a final hold step at 60°C for 30 seconds. A negative control was included in which 5 μL RNase-free water was added in place of the template.

2.3.3 RT-LAMP assay

In brief, there are two RT-LAMP assay reaction systems. The standard one is detected by FAM fluorescence signal by QuantStudio3, and the conditions were: 12.5 μL WarmStart® LAMP 2x Master Mix (DNA & RNA) from New England Biolabs (New England Biolabs, UK), 0.5 μL fluorescence dye(50X), 2.5 μL LAMP primer mix, 7.5 μL Rnase-free water and 2 μL RNA template. The conditions were set as 63°C for 40 minutes. Another RT-LAMP reaction was based on a

colourimetric one: 10 μ L WarmStart® LAMP 2x colourimetric Master Mix (DNA & RNA) from New England Biolabs (New England Biolabs, UK), 2 μ L LAMP primer mix, 7 μ L Rnase-free water and 1 μ L RNA template. With the same conditions, the reactions in the PCR tubes were also incubated in a heat block for 40 minutes. The volume of the paper-based RT-LAMP reaction is adjusted to 1/2 for each reagent.

2.3.4 Artificial saliva treatment

Due to health and safety issues, this study does not have access to real human saliva, and artificial saliva was purchased (Sigma-Aldrich® Solutions, US) to mimic the real ones. The specific composition of this product is not disclosed, but the general description mentioned that the artificial saliva is produced from peptide substances and contains growth factors: epidermal growth factor (EGF), nerve growth factor (NGF), and transforming growth factor alpha (TGF- α). Non-enzymatic proteins include mucins, lactoferrins, proline-rich proteins (PRPs), calprotectin, interferon, albumins, and globulins (IgG and IgMg-globulins, and IgA-produced only in the parotid glands) ²⁴.

10 μ L of 4x10⁴ copies μ L⁻¹ SARS-CoV-2 ORF1ab fragment was spiked into 90 μ L saliva to mimic the actual saliva sample as a starting concentration. Before measuring with relevant assays, the mimic samples had to be pretreated based on HUDSON (heating unextracted diagnostic samples to obliterate nucleases) ²⁴. A protocol was adapted from Huang et al.²⁶ and optimized particularly on the dilution buffer. A ratio of 8 μ L Tris-EDTA (pH=8.0), 0.1 μ L Rnase inhibitor and 1.9 μ L protease K (ThermorFisher, Scientific, USA) were mixed homogenously for a 1: 1 diluting saliva sample. The heating conditions were as follows: incubate at room temperature, usually 20-25°C) for 10 mins, then move the sample to 65°C, incubate for 10 mins and finally incubate at 100 °C for 12 mins. A follow-up RNA extraction and purification step were conducted using kits (Qiagen, Germany). RT-qPCR then amplified the purified RNA to confirm the efficiency of saliva treatment.

2.3.5 Design and fabrication of the paper device

The paper-based design was previously described in Yang et al.²⁷ The microfluidic device (Figure 2 -3A) consists of 5 panels sharing different functions, which are printed with hydrophobic wax (Xerox®, colourQube 8570, US). Here are some design details: the black square's length is 3 cm, and the biggest circle's diameter is 1.2cm. The diameter of panel 1 is 4 mm, and the chamber reaction circle (panel 5) diameter is 3mm. The wax-printed paper is heated at 120°C for 1 min on an isothermal hotplate so that the wax can penetrate via both sides, forming a hydrophobic layer; thereby, when loading the reagent, the liquid would be fixed on the hydrophilic area not diffusing to the other places. Figure 2-3A shows panel 6a compatible (size aligning to panel 5 in) manufactured transparent plastic sealed with two acetate films (MicroAmp® Optical Adhesive Film, Thermo Scientific) which can prevent liquid evaporation during the amplification stage.

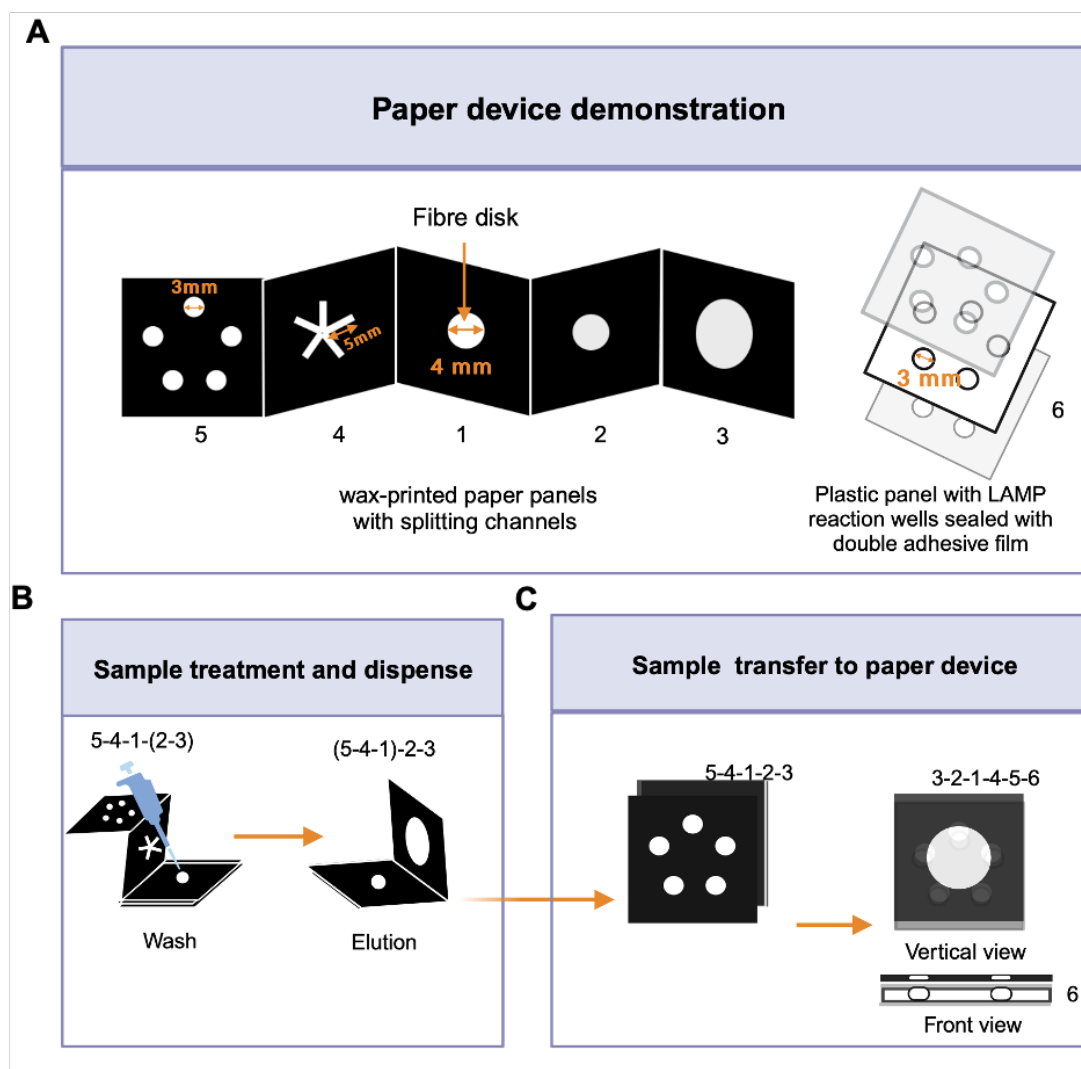


Figure 2-3 Paper-based LAMP detection of SARS-CoV-2 in artificial saliva. **A** Foldable paper device demonstration Dark area is printed with hydrophobic wax; Detailed paraments are noted, and 1-5 panels order is shown at the top. **B** Steps for microfluidic manipulation of assay steps. The broken arrows indicate the folding direction. Other arrows indicate operational directions. The bracket indicates the folded panels. **C** Treated sample transferring from the paper device (left side) to the reaction chamber (right side) which are shown in two different views.

In the context of the paper-based device, as illustrated in Figure 2-3A; the central panel incorporates a fibre disc for sample dispensation, while panel 2 features a perforation facilitating the use of supplementary buffers. The procedural workflow is akin to origami techniques: firstly, panels 2 and 3 are folded and secured to create a dual-layered structure (Figure 2-3B, left). Subsequently, the treated artificial saliva sample is introduced, followed by the application of washing buffer,

and then panels 4 and 5 are folded to affix panel 1 (Figure 2-3B, right). Panel 2 is positioned in the positive slot, facilitating the addition of an elution buffer. Eventually, panel 5, housing the treated sample, is attached to the plastic chamber (Figure 2-3C). Preceding the attachment, paper strips are inserted into the aperture (visible as the white column in the front view), facilitating the transfer of treated samples from the paper-based device to the reaction chamber for subsequent amplification. The entire workflow can be referred to Figure 2-4, and the process can be simply divided into two sections. Figure 2-4A shows a recommended approach to treat the saliva sample, and Figure 2-4B illustrates how the paper-based device works.

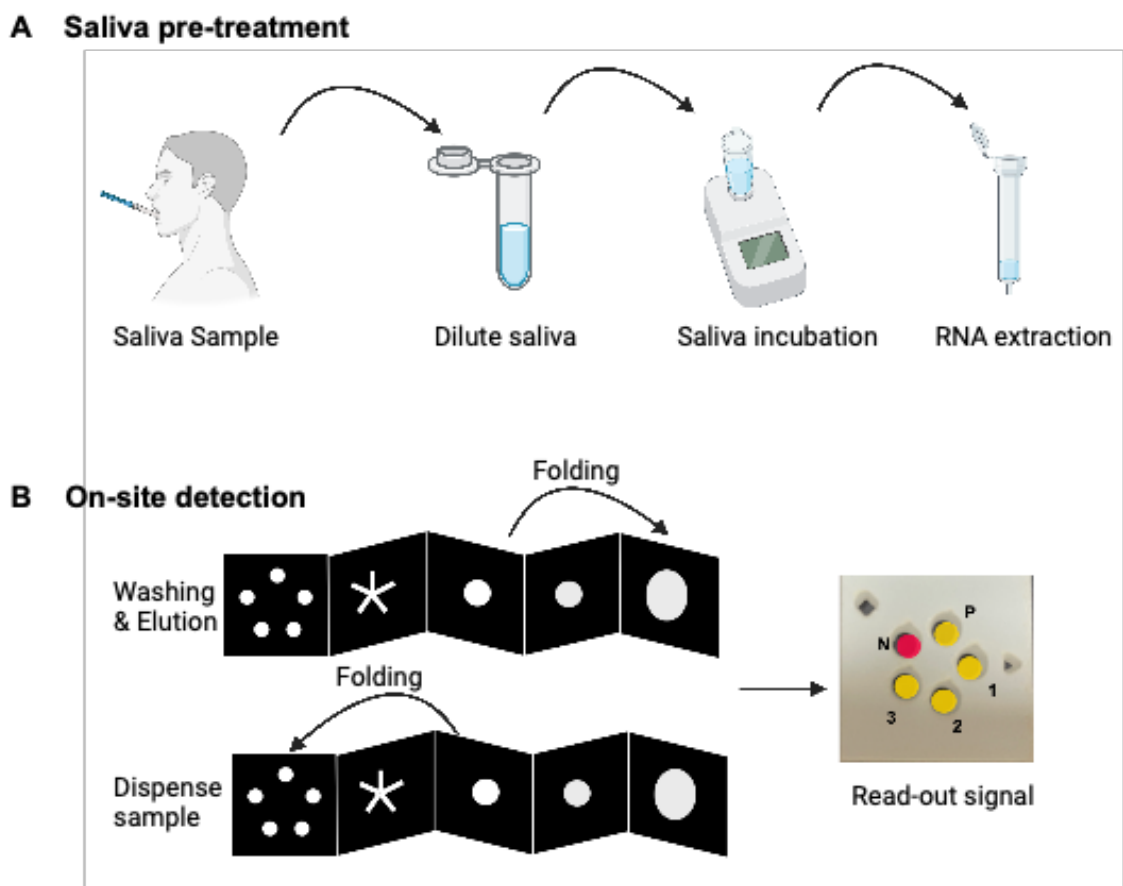


Figure 2-4 The simplified workflow on colourimetric RT-LAMP saliva paper-based detection. A saliva treatment via homemade 1:10 dilution buffer. The incubation conditions are: First at room temperature for 10 mins, and 65°C for 10 mins, then 100°C for 12 mins. RNA extraction can be omitted in LAMP. **B** The treated artificial saliva is

loaded on a paper-based device and incubated to present the results. Proposed test results are shown with different colour indicators. The pink represents negative colour, meaning there is no amplification in the chamber. P is a positive control. 1,2,3 can be used as a triplicate or testing different targets.

2.4 Results and Discussion

2.4.1 Analytical performance of LAMP

RT-qPCR and RT-LAMP result which were measured from the synthetic ssRNA transcribed from plasmid to confirm that the designed primers for the transcription was efficient (Figure 2-5A, C). The gel results confirmed no specific amplification in negative controls (Figure 2-5 B, D).

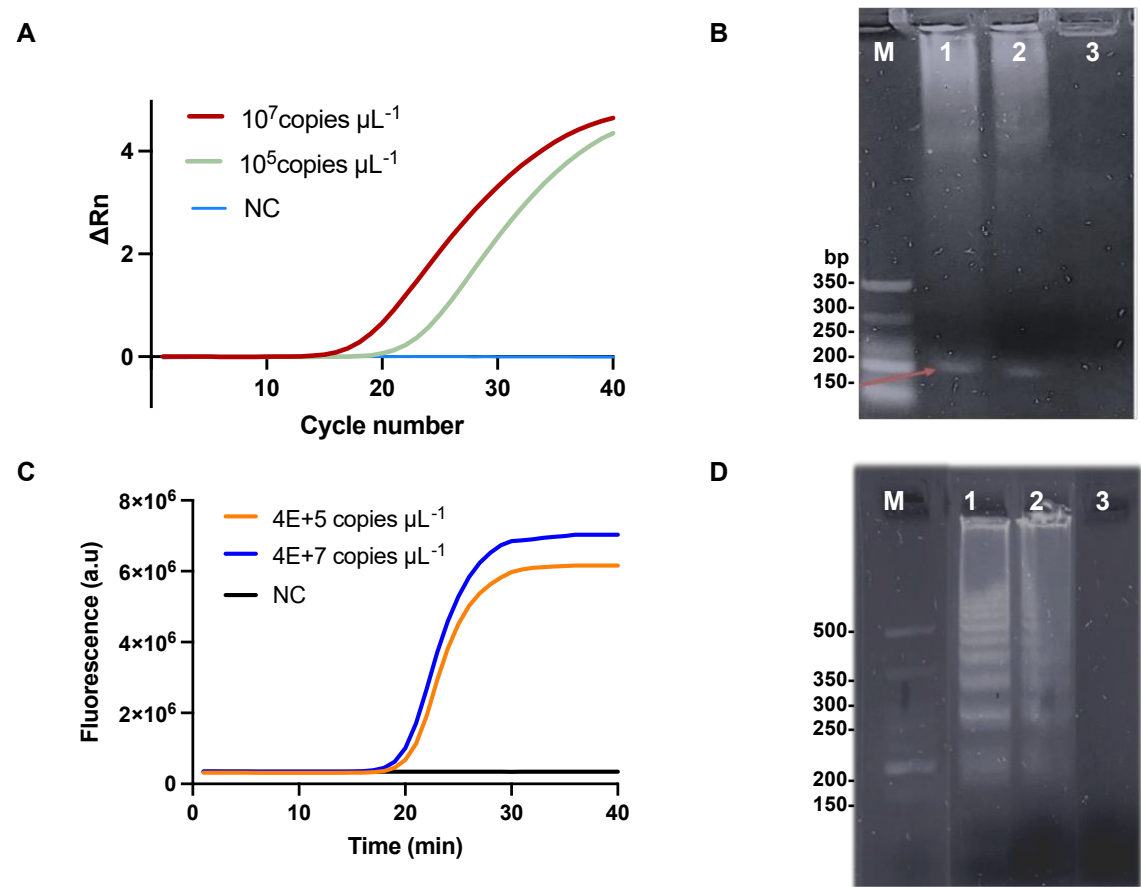


Figure 2-5 The validation of primers performance. A The T7 transcription assay. **B** 2% agarose gel electrophoresis, the product is around 250 bp. The arrow shows amplified products; 1: 10^7 copies μL^{-1} ; 2: 10^5 copies μL^{-1} ; 3: negative control; bp: base pair. **C**

LAMP primers validation assay. **D** 2% agarose gel electrophoresis. Each amplification line represents an independent biological triplicate; 1: 4×10^7 copies μL^{-1} ; 2: 4×10^5 copies μL^{-1} ; 3: Negative control.

To ensure the successful demonstration on the paper device, the plasmid samples were initially analyzed by RT-LAMP to evaluate the LOD. The New England Biolabs WarmStart kit, containing an engineered reverse transcriptase (RTx) and a strand-displacing polymerase (Bst 2.0), allowed the reaction to be performed in one step. The plasmid concentration range between 4×10^6 copies μL^{-1} to 4 copies μL^{-1} were tested with FAM dye, and the LOD was determined to be 4 copies μL^{-1} (Figure 2-6A). After the plasmid samples were validated, artificial saliva samples to mimic real samples. RT-qPCR was loaded with serial dilutions to plot a standard curve to show the sample's concentration, and the recovery rate was calculated (Figure 2-6C). Before pre-treatment, no amplification was observed in the saliva sample, indicating that sample treatment was necessary. The standard calibration curve was used to establish a linear relationship (Figure 2-6D), and the saliva sample recovery rate was within the recommended range of 80-120%²⁸.

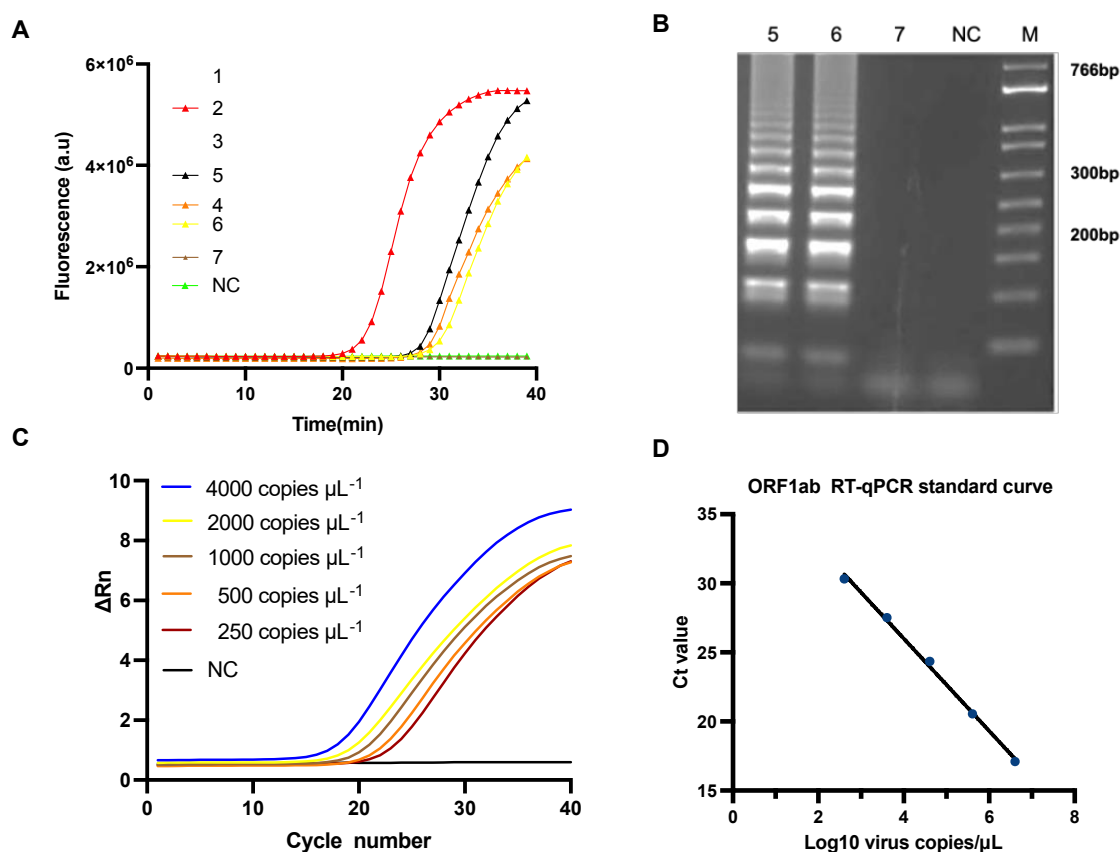


Figure 2-6 The validation of RT-LAMP and spiked-in saliva assay. **A** LAMP assay for ORF1ab. **B** 2% gel electrophoresis for LAMP products. M: Marker; NC: Negative control. **C** Spiked in sample RT-qPCR assay. **D** ORF1ab RT-qPCR standard curve. The formula is $Y = -3.34X + 39.37$. 1-7: 4x10⁶ copies μL⁻¹- 4 copies μL⁻¹.

After the successful amplification of the reverse transcribed RNA, artificial saliva samples were detected using colourimetric LAMP. The extraction step was omitted because the LAMP reaction was tolerant to crude sample environments. Concentrations ranging from 276 copies μL⁻¹ to 12 copies μL⁻¹ were detected, and the LOD was 28 copies μL⁻¹(Figure 2-7A, B). All samples, except the negative control, were observed to turn yellow due to the gradual change in pH to acidic ones with heat. In some cases, water in the reaction mix could lead to false negatives²⁹. Gel results (Figure 2-7C) confirmed no amplification in sample 5, but the yellow indicated otherwise. Based on the results, it could be concluded that the detection time with the colourimetric method was within 35 minutes. In view of quality assessment, the adoption of internal control could guarantee the

reliability and standardization of results. The internal human mRNA (e.g. β actin) was added as a reference ²⁹. No real sample was involved in the detection, thus, this series of experiments included water control rather than human mRNA.

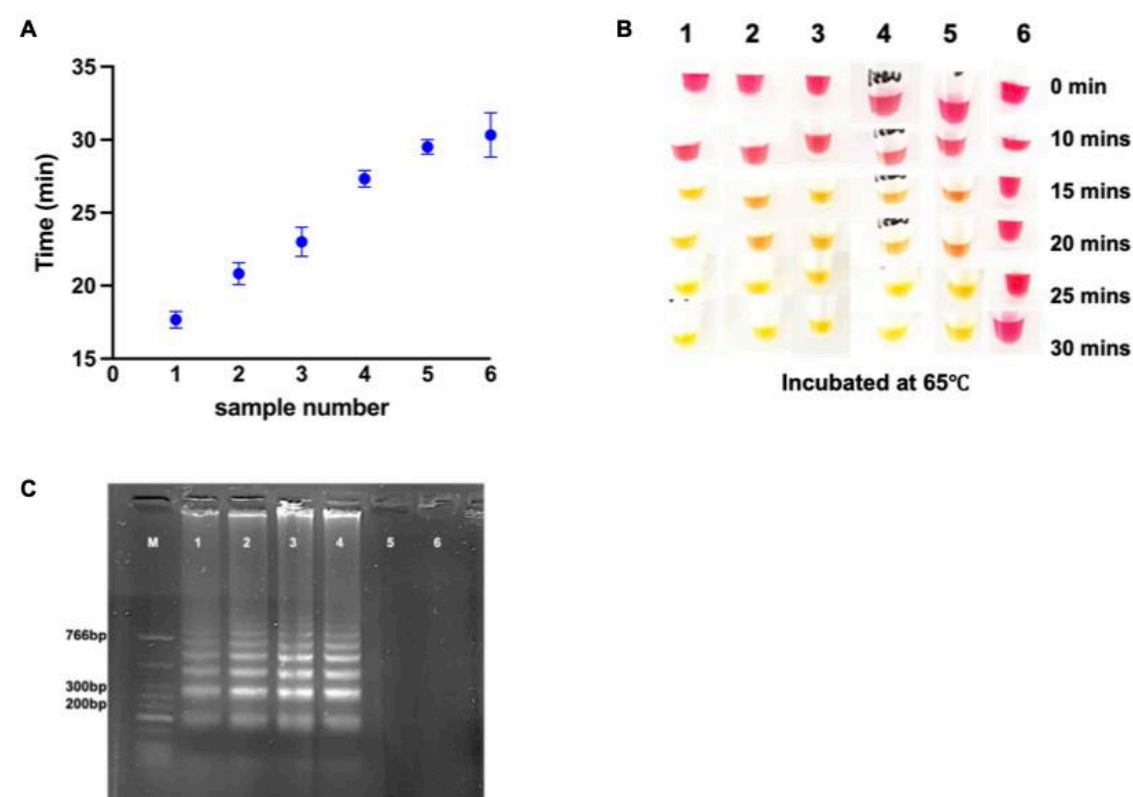


Figure 2-7 LOD of the colourimetric RT-LAMP assay determined by simulated saliva sample. **A** The following numbers 1-6 are indicated as 276 copies μL^{-1} , 135 copies μL^{-1} , 64 copies μL^{-1} , 28 copies μL^{-1} and 12 copies μL^{-1} , negative control. Each concentration was performed with triplicates for standard deviation. **B** The colour illustration in tubes changed with time going as long as the decreased concentrations. The reactions were stopped when the tubes were placed on ice. Photos were taken 50 cm distance from the tube with iPhone 12 MP sensor wide camera with 1.4 μm pixels, ISO32, 26 mm-equivalent f/1.6 lens at dark environment. **C** 10 μL RT-LAMP product was analysed on a 2% agarose gel. The band patterns were visible in the artificial saliva sample with 28 copies μL^{-1} .

2.4.2 Paper-based microfluidic device for saliva detection

To further validate the assay, this naked-eye reaction was transferred to a paper-based device. Carryover contamination is a serious issue in LAMP, as amplified LAMP products can easily form aerosols while handling the reaction mix. Other factors that can cause contamination and result in a false negative result include gloves, pipettes, and work surfaces^{30,31}. To reduce the risk of contamination, the paper device and plastic plate were placed under UV light for 30 minutes while the reaction mix was prepared. The paper strip allowed for a relatively straightforward extraction and elution of the treated sample without a centrifuge, which was then combined with the loaded RT-LAMP reagent for subsequent reaction. The initial detection was displayed in Figure 2-8 A, which showed that the device could detect saliva samples at the concentrations of 716 copies μL^{-1} approving that this device could detect the sequence of synthetic viral RNA in artificial saliva (Figure 2-8 A bottom). The results can also be read with the colorimetric change, as shown in the figure 2-8. Importantly, the operation of the paper device would impact the assay. If the plastic plate was not sealed properly, it would influence the amplification results because the fluid might flow to a random chamber causing non-specific amplifications. An acceptable performance was shown with a broader range of detected concentrations when sealing properly (Figure 2-8 B). The paper device was tested with artificial saliva samples without prior RNA extraction to simplify the on-site detection. The current limit of detection is above 135 copies μL^{-1} and at least lower than 276 copies μL^{-1} , which failed to reach the relatively same testing threshold as quant studio results or in the tube results. The only similar work on saliva colourimetric test showed a relatively comparable LOD (200 copies μL^{-1})². However, their work was carried out in LFT format so that only one target could be tested. Another drawback of that test was that the assay would not generate results up to 60-minute incubation, which was longer than the assay in this study. Carryover contamination and variations in colour perception limited LFT platform and this study, and both these which should be addressed when operating the assay.

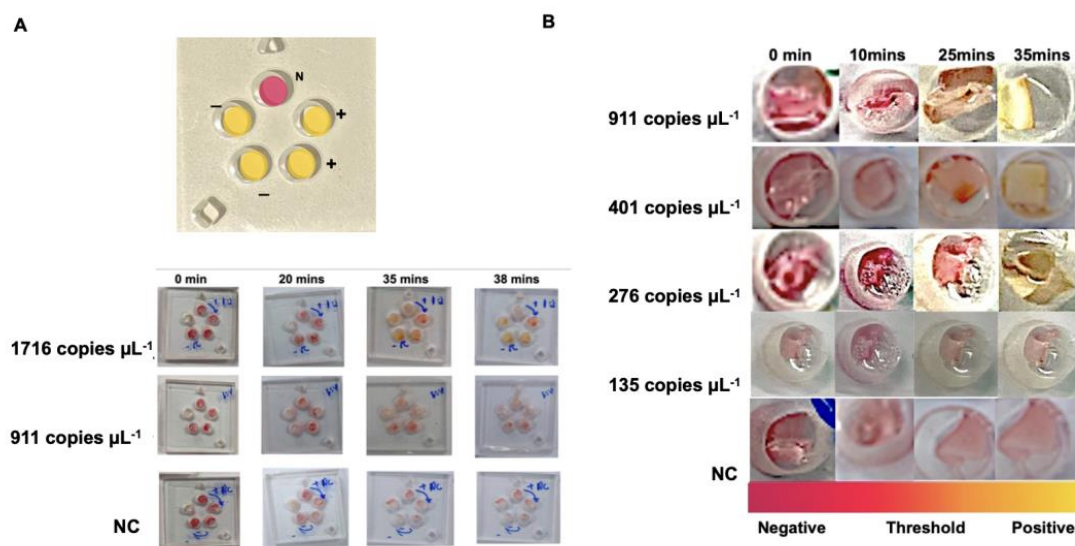


Figure 2-8 The colourimetric RT-LAMP on a paper-based device with an artificial saliva sample. **A** above: The demonstration of a paper-based device for the SARS-CoV-2 detection of spiked saliva samples; bottom: Initial test on a paper device; + indicated the reaction with guanidine hydrochloride; - indicate the reaction without guanidine hydrochloride. **B** LOD of paper-based device. The negative control was tested with the water; The colour bar is created by python with package of matplotlib.pyplot. color bar.

Another requirement for POCT device is time efficiency. Thus, experiments were conducted to speed up the reaction time and achieve rapid detection. Guanidine hydrochloride was added to the reaction mix to accelerate the reaction, based on previous research by Zhang et al ³³. According to their findings, guanidine hydrochloride could significantly impact the reaction, reducing the reaction time to from 40 minutes to 25 minutes at a concentration of 40-50mM. The mechanism for this acceleration is not fully understood, but it is thought to enhance base pairing between primers and target DNA. Based on this finding, the reaction conditions were adjusted to 1 μ L guanidine hydrochloride and 7.5 Ranse-free water added up to 25 μ L total, however, the addition of this reagent seemed not to have an impact on the paper-based device of this study. In Figure 2-8 A (bottom), no time differences were detected with the wells with or without this reagent. Those who have done some work on paper with saliva samples also showed that the speeding up was also associated with pH because the phenol red indicator would change from alkaline to acid. The normal pH of saliva (natural or artificial) was 6 to 7, which is slightly acidic. However, the salivary pH flow with

LAMP master mix was 832. Therefore, a lower initial pH level in buffering activity was proposed as the optimal condition so that it could reach to an acid environment more rapidly. The pH adjustment was not implemented in this project; however, it could be worthy of testifying it upon this condition. In addition, a colour bar (Figure 2-8 C) was formatted to provide a reference for interpreting the RT-LAMP results and assessing the potential for quantification. Some advanced work also enabled the measurement of RT-LAMP results with portable devices. For example, Nguyen et al. developed a smartphone-equipped device that provided the research with qualitative and quantitative results of the RT-LAMP that could advance the POCT diagnosis³⁴. Their work provided an approach to improving this immature device for human saliva SARS-CoV-2 quantification of diagnostic results in the future.

In brief, paper-microfluidic RT-LAMP of this is viable to test artificial saliva in a portable and economic way. The treatment procedure involving the use of proteinase K and RNase inhibitor at pH 8.0 provided a sensitive LAMP-based detection method, eliminating the need for RNA extraction with extra tools. Saliva Direct tested⁴⁰ in multiple vendor kits over 3,000 patients, which showcased a simplified saliva treatment in the individual testing method aligned to the pre-treatment in this study. Furthermore, the required reagent volume for the paper-microfluidic chamber is reduced by half compared to the volume tested on the fluorescence machine, thereby enabling increased detection within the same conditions. While the extent of sensitivity compromised in human saliva remains unclear, the current limit of detection (LOD) (135 copies μL^{-1}) is comparable to some commercially available assays listed in below. It is worth noting that these assays require sophisticated instruments⁴¹ (approximately £44,500 per unit), highlighting the cost benefits of the presented paper-based device (£5) for individual testing.

Table 2-2 The performance of different SARS-CoV-2 nucleic acid detection methods in saliva testing.

Methods	Developer	instrument	Sample processing	Target gene	Time (min)	LOD (copies μL^{-1})	Number of sample (n)	References
RT-qPCR	Phosphorus Diagnostics	Yes-Bio-Rad CFX384	Full extraction	N,RdRp	N/A	5	91	[35]
RT-qPCR	NeuMoDx Molecular Inc	Yes-automated equipment	Automated full extraction	ORF1ab, N	N/A	0.05	72	[36]
RT-LAMP	Pennsylvania State University	Yes-Mobile device (SLIDE) platform	No-specific extraction	N	45	5	N/A	[37]
CRISPR-LAMP based (Cas13)	University of California, Berkeley	Yes-customized microfluidic cartridge	Manually extraction	N	60	40	63	[38]
CRISPR-RPA based LFA	Noodle Analytics Pvt Ltd	No-mobile phone for analysis	Manually extraction	S, ORF1ab	N/A	200	76	[39]
Saliva Direct for RT-qPCR	Kits from multiple vendors NEB Lunar, Thermor Taqpath, BioRad Reliance	Yes-Bio-Rad CFX	Full extraction	N, S, ORF1ab	Over 70	6-12	3,779	[40]
RT-LAMP LFA based	Prude University	No	Extraction-free	ORF1ab	60	200	N/A	[32]

Paper-microfluidic RT-LAMP	This study	No	Extraction- free (Artificial saliva)	ORF1ab	35	Above135	N/A	This study
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There are two main limitations of this paper-based microfluidic device research. In terms of operations, compulsory steps in all platforms including this study are a separated saliva treatment and viral RNA extraction. This demand suggests that no platform could finish the detection in one step. Although RNA extraction is not necessary for LAMP detection, saliva treatment is compulsory which brings difficulties into a POCT device. The literature also indicates that analyzing non-extracted specimens could result in inadequate RNA loads, particularly for individuals who exhibit mild or no symptoms⁴². When this paper-based device is tested with real samples, it may result in false negative results. Another major is the use of artificial saliva since real saliva compositions is affected by complex factors: i) demographic; ii) age; iii) sexes that men with higher levels of sodium and calcium; IV) body nutrition, body posture⁴³. Each factor may impact the measure of detection outcome, and the artificial saliva is quite stable, which would weaken the experimental condition and lead to a lower sensitivity. However, using artificial saliva is crucial for the standard protocol established at the early stage. The history of using artificial saliva can be traced back in 1931²⁴ for toxicity analysis. Their report gave rise to a formula of artificial saliva for use in in vivo and in vitro studies, which means that except for the external variances, the artificial saliva compositions are nearly close to the real oral environments. From this perspective, artificial saliva is a reasonable choice to be involved with the theoretical assay, but more real saliva samples should be introduced to testify to the current method, thus further optimizations could be operated.

The COVID-19 outbreak has emphasized the significance of diagnostic techniques that can be rapidly modified and utilized in diverse situations. The criteria assessment encompasses both detection chemistry and platforms. In addition, it is important to consider other parameters such as the duration of the entire process, detection range, and the ability to perform simultaneous detection of different amplicons. Certification of the diagnostic solution is also a crucial aspect to consider. To ensure the quality and reliability of diagnostic assays before they are made available in the market during the current pandemic, established verification processes exist. These processes vary globally, with European Union (EU) countries relying on CE marking for validating new In vitro diagnoses (IVDs), while the United States relies on Emergency-USE-Administrations (EUAs) ⁴⁴. It should be noted that most POCTs relying on isothermal amplification are not mature technologies in the market, the majority of commercially available SARS-CoV-2 diagnosis test on the regulatory status is PCR or antigen technology, and only a few isothermal kits have been approved without implements on paper-based device ⁴⁵. The current microfluidic paper-based device is beyond gaining regulatory approval due to its uncertain reliability on real saliva samples. A more detailed discussion about the plausibility of introducing a commercialized microfluidic product is addressed in chapter four.

2.5 Conclusion

The microfluidic device has a great protentional accelerating the development of rapid detections in different sectors. The advent of the Covid-19 pandemic has spurred increased research efforts within this burgeoning field. Diverse designs integrated with optimized detection methodologies have demonstrated relatively promising levels of sensitivity and specificity.

In light of this, the study attempts to use paper-based microfluidic to enable the detection of SARS-CoV-2 RNA virus sequence in artificial saliva via RT-LAMP. The treatment procedure provided a sensitive LAMP-based detection method, eliminating the need for RNA purification. The LOD for the colourimetric RT-LAMP is 28 copies μL^{-1} . Furthermore, the sample was successfully loaded onto

the paper-based device, with the LOD of above 135 viral load copies μL^{-1} confirming the effectiveness of detection.

It was observed that when combined with the paper-based device, saliva samples exhibited superiority over conventional nasopharyngeal swabs, with the device's performance being comparable to another paper-based device with a shorter detection time. Nevertheless, improvements in the design are still required, including embedding the RT-LAMP reagent on paper in advance to minimize contamination. Validation with clinical samples is also necessary to confirm the workflow's accuracy.

Despite the sensitivity being lower than analytical equipment, the paper-based platform utilizing RT-LAMP demonstrated promising potential for saliva diagnostics. In general, this approach offers a rapid and less invasive clinical testing option, making it an attractive choice for diagnostic applications once successful validation with clinical samples.

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3 Paper-based ELISA for assessing concentrations of immunity against SARS-CoV-2 in saliva

3.1 Abstract

Characterizing human immunity response to SARS-CoV-2 is significant for diagnosing and estimating herd immunity following vaccination. The standard method to measure antibody concentration against SARS-CoV-2 uses a serum sample that allows a sensitive and specific evaluation; saliva, as the first line of defence against the virus, is also a promising biological sample to assess immunity levels. Here, this section aims to develop a paper-based indirect ELISA to show the possibility of using artificial saliva to quantify the IgG antibody against SARS-CoV-2 spike protein receptor binding domain (RBD). In this assay, following the capture of the anti-RBD antibody, achieved by immobilizing the antigen (RBD) on a microplate and the paper-based device, a colourimetric reaction was utilized, in which a secondary antibody (anti-human IgG) conjugated with horseradish peroxidase (HRP) was used in the presence of 3,3',5,5'-tetra-methylbenzidine (TMB) substrate. The resulting colourimetric readout was recorded, and the specificity and sensitivity of the assay were assessed using a formula. An assay detection threshold of around $0.5 \mu\text{g mL}^{-1}$ was obtained after optimizing reaction conditions, performing artificial saliva treatment, and obtaining linear regression curves. It is believed that this proof-of-concept of the platform has the potential to become a standardized test able to assess the individual immune response to SARS-CoV-2 or to evaluate vaccine efficiency after carrying out validation with real clinical saliva samples.

3.2 Introduction

SARS-CoV-2 has caused a devastating pandemic worldwide. Several approaches to detect this virus have been fully deployed. The two most common tests are RT-qPCR, done in professional labs, and the rapid antigen test, suitable for onsite diagnosis. During the first and second waves of the pandemic, the antigen test played an essential role in monitoring the dynamics of the disease. Until 19 May 2021, it was recorded that up to 4.9 million registered tests were undertaken in England¹. However,

this diagnosis is developed to detect the actively replicating virus, and patients only with acute symptoms or early infectious can be identified. Generally, the accuracy is around 35% ². Therefore, the actual spread situation is unlikely to be defined due to the rapidly evolving nature of the virus, and it cannot be revealed until the middle or post-pandemic time. A series of German studies found that after 5 days of illness onset, patients were hard to test with RT-PCR (less than 50%)². In contrast, the antibodies were detectable on day 6, and their detection could also identify and quantify the body's neutralization ability. Herein, the measurement of antibodies against SARS-CoV-2 is another assessment to estimate the prevalence of SARS-CoV-2 infection. In addition, assessing the antibody levels against the virus also reflects the dynamic vaccine efficiency, which helps adjust the injection timeline.

The previous chapter explained that SARS-CoV-2 produces four structural proteins: Nucleocapsid, Spike, Envelop, and Membrane³. The humoral immune response triggered by the infection primarily targets Nucleocapsid protein and Spike protein⁴. Nucleocapsid antigen is more conserved than Spike protein, which may cause cross-reactivity with SARS-CoV-2 viruses. Conversely, Spike protein is less conserved and located on the surface of the virus. It contains a receptor-binding domain (RBD) that interacts with the ACE2, resulting in virus entry into the host cell ⁵. According to research, RBD is the least conserved region on Spike protein ⁶, making it the most appropriate antigen for the specific detection of antibodies.

Selecting detection antibodies is also crucial for indicating an immune response against SARS-CoV-2. Different antibodies have variable pattern levels, and it was confirmed that IgG is detectable after 1 week and is maintained at a high level for an extended period, while IgA responds approximately 10 days after the infection¹². The antibodies generated by the immune response are diverse. It is stated that when three immunoglobulin classes (IgM, IgG, and IgA) are detected, a maximum neutralization activity is achieved. The neutralizing antibody (NAb) is vital for protecting against SARS-CoV-2, where they reach the goal by preventing the binding of RBD to ACE2, blocking the virus' entry into the host cell, and disrupting the viral genome structure⁷. The relationship between anti-RBD antibodies and neutralizing remained elusive; however, the IgA and IgG are identified as the primary components of Nab in the development against SARS-CoV-2⁸. Some studies reveal that IgA against SARS-CoV-

2 spike protein RBD may correlate with the severity of disease infection at the early beginning, but its levels decay faster than IgG levels in mRNA vaccine groups ^{9,10}. Due to this, findings suggest that immobilizing RBD as the antigen, the capture of anti-SARS-CoV-2 spike protein RBD (anti-S-RBD) IgG may show a trend of neutralizing antibody titers and assess the immune protection against SARS-CoV-2 infection.

Saliva has been demonstrated to be a valuable body fluid for detecting SARS-CoV-2 viral RNA; thus, it is worth studying for measuring antibodies to SARS-CoV-2. One of the most used methods for quantifying antibodies is ELISA ¹¹ used to measure the different immunoglobulin responses in hospitalized patients. They found that the salivary immunoglobulins antibodies positively correlated with the serum samples. In addition, IgA response to spike and RBD proteins were the most observed. At the same time, it was demonstrated that the level of IgG was maintained for a more extended period in COVID-19 patients in salivary samples. Though the serological immunoassay combined with nucleic acid testing can explicitly increase the accuracy of viral tests, especially with the progressive vaccine among the public, a new focus can be placed on the saliva matrix.

As mentioned above, ELISA is a standard and common antibody measurement method. The traditional ELISA tests are generally conducted on a 96-well plate, which requires many steps, including overnight coatings, prolonged incubation of samples, and multiple washing procedures. It will take nearly 1 day to obtain results from ELISA tests. However, adapting the immunoassays to a paper-based ELISA (p-ELISA) would significantly shorten the time for this assay. The simple mechanism of the paper capillary is that the paper cellulose fibre introduces the liquids to penetrate the hydrophilic matrix, and there is no extra need for external support like an active pump ¹³. The capillary action is caused by the surface tension and stands for the ability of a liquid to flow spontaneously in one direction.

Moreover, unlike LFAs that only give a yes or no answer ¹⁴, the p-ELISA can present a more detailed diagnosis result via the blue colour degree generated by the HRP/colourimetric substrate TMB reaction. The colour metric application has been applied to some mature platforms. For example, integrating the colourimetric with a metal nanoprobe is the classical design on a paper platform. Vegas et al.¹⁵ have

developed a paper device that could differentiate the specific bacterial isolate belonging to the *Mycobacterium tuberculosis* complex (MTBC). To shed light on the possibility of rapid antibody testing, the development of an indirect p-ELISA to detect the IgG against SARS-CoV-2 in artificial saliva samples is described. The antigen spike RBD, strongly associated with human ACE-2, was immobilized on the paper to monitor specific neutralizing IgG. This platform can potentially shorten the laborious immunoassay time and give a rapid, accessible, precise, and practical (RACE) diagnosis. This device's exploration also aims to supplement the NAAT to lead to a novel integrated POCT.

3.3 Materials and methods

3.3.1 Reagents and equipment

The immunoassay components were all purchased from Abcam (Cambridge, UK). These included Recombinant human coronavirus SARS-CoV-2 spike glycoprotein (His Tag) (ab275986), Human monoclonal to SARS-CoV-2 spike glycoprotein (IgG) (ab272854), Goat anti-human IgG Fc (HRP modified) (ab97225), HRP Conjugation kit (ab102890). The received proteins were aliquoted and stored at -20°C to avoid a thaw cycle. Bovine Albumin (BSA) was purchased from Sigma-Aldrich® Solutions (US). The coating buffer for immobilizing antigen was 0.1M Phosphate Buffered Saline (PBS) pH 7.4 (Sigma-Aldrich® Solutions, US), and the washing buffer was 0.1 M PBS coupling with 0.1TWEEN20® (Sigma-Aldrich® Solutions, US). PBS was also used as the dilution buffer for the detection antibody. The artificial saliva (Sigma-Aldrich® Solutions, US) for NAAT was used again for the complex biological sample to simulate tests. The blocking buffer for the residual reactive sites on the antibody immobilized was 1:10 Pierce™ Clear Milk Blocking Buffer (10X) (ThermoFisher Scientific, US) in 0.1M PBS. The stop solution consisted of 1M H₂SO₄. Whatman chromatography paper (3mm) was purchased from Sigma-Aldrich® Solutions (US). All images for p-ELISA were captured by iPhone 12 (Apple Inc, USA) MP sensor with a wide camera at ISO 32, 1/14s exposure, 0ev, 1.4µm pixels and 26 mm-equivalent f/1.6 lens in a dark environment and a room temperature.

3.3.2 Indirect ELISA assay on 96-plate well

The detection method followed the indirect ELISA mechanism¹⁶. The detection antibody (IgG) was captured by the immobilized antigen (Ag, spike protein RBD), and the signal was visualized by the specific HRP-secondary antibody reacting with TMB. In this study, the ELISA technique was employed to detect IgG antibodies against the spike protein RBD antigen of the SARS-CoV-2.

For the assay, 50 μL RBD antigen ($1.6 \mu\text{g mL}^{-1}$ in PBS buffer) was immobilized on a microplate surface and incubated at 4°C overnight. Following this, a blocking step was performed with 100 μL of 1:10 clear milk blocking buffer diluted in phosphate-buffered saline with Tween 20 (PBST), and incubated for 1 hour at 37°C . Next, different concentrations of the detection antibody (100 μL ranging from 5 to 80ng mL^{-1}) were loaded and incubated. An HRP-labelled secondary antibody (1:10,000 dilution in 1:40 clear milk blocking buffer diluted in PBST) was then added and incubated. In the end, 75 μL TMB solution was added to each well for signal detection. The reaction was stopped with 50 μL of 1M sulfuric acid, and the signals were measured by recording the absorbance at 450nm using a Varioskan™ LUX multimode microplate reader (Thermo Fisher SCIENTIFIC, US). Three times 200 μL PBST washing was applied between each step. This assay provides a sensitive and specific method for detecting IgG antibodies against the SARS-CoV-2 RBD antigen. Crude absorbance (OD) is prone the substantial variations in reagent batches, technical performance, and other laboratory conditions. Therefore, the crude OD was calculated and formatted into a normalized absorbance. OD(s) is the value of sample absorbance and OD(c) is the value of control absorbance.

$$\text{Normalized Absorbance} = \frac{\text{OD(s)} - \text{OD(c)}}{\text{OD(max)} - \text{OD(c)}} \quad (3-1)$$

3.3.3 p-ELISA device design, fabrication, and operation

The fabrication process of a paper-based chip, as illustrated in Figure 3-1A, involves transferring the ELISA procedure onto 96-well wax-printed paper. Despite its advantages, the use of paper in microfluidics presents some challenges. Limited paper

permeability can lead to fluid inhibition, which may affect the reliability of the assay. This phenomenon can be described by Darcy's Law (3.1), which relates the volumetric flow rate (Q) to the permeability of paper (κ), width of the paper (W), thickness of the paper (H), the viscosity of the fluid (μ), length of paper (L), and pressure difference along the paper fluidic channel (ΔP). Thus, to maintain the signal intensity of the assay within a relative scope, it is necessary to control the permeability by adjusting the length of the paper. To this end, a waste channel is included in the detection zone to remove the waste buffer, as shown in Figure 3-1A.

$$Q = -\frac{\kappa WH}{\mu L} \Delta P \quad (3-2)$$

The present study describes the fabrication procedure of a paper-based chip for ELISA. This procedure is shown in Figure 3-1A. Many previous studies have explored different designs to transfer the ELISA procedure to paper. In most cases, paper-based ELISA employs 96-well wax-printed paper instead of plastic microtiter plates, making the assay cheaper and more accessible. However, the limited permeability of the paper can lead to fluid inhibition in a defined zone, following Darcy's Law. A waste channel is accommodated in the detection zone for waste buffer removal to control the permeability within a relative scope, as depicted in Figure 3-1A.

The pattern design of the paper-based chip consisted of a circle in the centre surrounded by six channels, as illustrated in Figure 3-1A. The central circle had a diameter of 25mm, which was the waste zone, and the smaller circles were connected to the central one through 5 mm long channels. The pattern was created using CorelDraw2021 standard version, and the paper was printed using a wax printer (Xerox®, colorQube 8570, US) and a 210mmx297mm Whatman™ chromatography paper. After printing, the Whatman paper was cured at a 120°C heat block until the ink was fully melted, as depicted in Figure 3-1A. The paper was then modified with 5% BSA to maximize its antigen binding capacity. The paper was incubated for 30 minutes with 5% BSA in a petri dish and then kept at room temperature for further use, as shown in Figure 3-1B.

After the modification, 1.5 μL Ag (1.6 $\mu\text{g mL}^{-1}$) was immobilized on each detection zone for 20 mins, followed by twice 5 μL PBST washing. The artificial saliva spiked-in sample was centrifuged at 15,000 rpm at 4 $^{\circ}\text{C}$ for 20 minutes to remove debris. 5 μL supernatant was immediately transferred to a clean centrifuge tube and diluted at 1:10. The 2.5 μL detection sample and HRP-labeled secondary antibodies with the same conditions as plate ELISA were loaded on each zone. The detection zone was washed every time with 5 μL PBST before new reagents were loaded. A piece of tissue was placed at the bottom to avoid direct contact between p-ELISA and the surface. Finally, 2.5 μL of TMB- H_2O_2 solution was added to each detection zone. The blue colour intensity was recorded using the main wide camera of the iPhone 12, equipped with a 12 MP sensor featuring dual 1.4 μm pixels, phase detection autofocus (PDFA), optical image stabilization (OIS), and a 26 mm-equivalent f/1.6 lens. This measurement was conducted in a low-light environment at room temperature, with the camera positioned at a distance of 30 cm from the object., and the image was analysed by Fuji/Image J (National Institutes of Health, Bethesda, USA). To quantify the colour on the paper, each detection zone was cropped within a 17mmx17mm (Image J pixels scale) circle and divided into Red (R), Green (G), and Blue (B) channels to measure the mean greyscale, as shown in Figure 3-D. The control, which was the absence of the HRP signal, was used as a reference to eliminate the background signal noise. The mean grey value was calculated for each detection zone using Equations 3.2 and 3.3.

$$\Delta\text{RGB} = \sqrt{(R - R_0)^2 + (G - G_0)^2 + (B - B_0)^2} \quad (3-3)$$

$$\text{RGB} = \Delta\text{RGB}_S - \Delta\text{RGB}_C \quad (3-4)$$

In this formula, R , G , and B are the mean grey values of samples captured from the detection zone, while R_0 , G_0 , and B_0 are the same but captured on the control sample. Furthermore, the negative control (without the primary antibody immobilized) was calculated and subtracted from ΔRGB_S to obtain the final RGB intensity. ΔRGB_S Stands for the original sample value is the original control value.

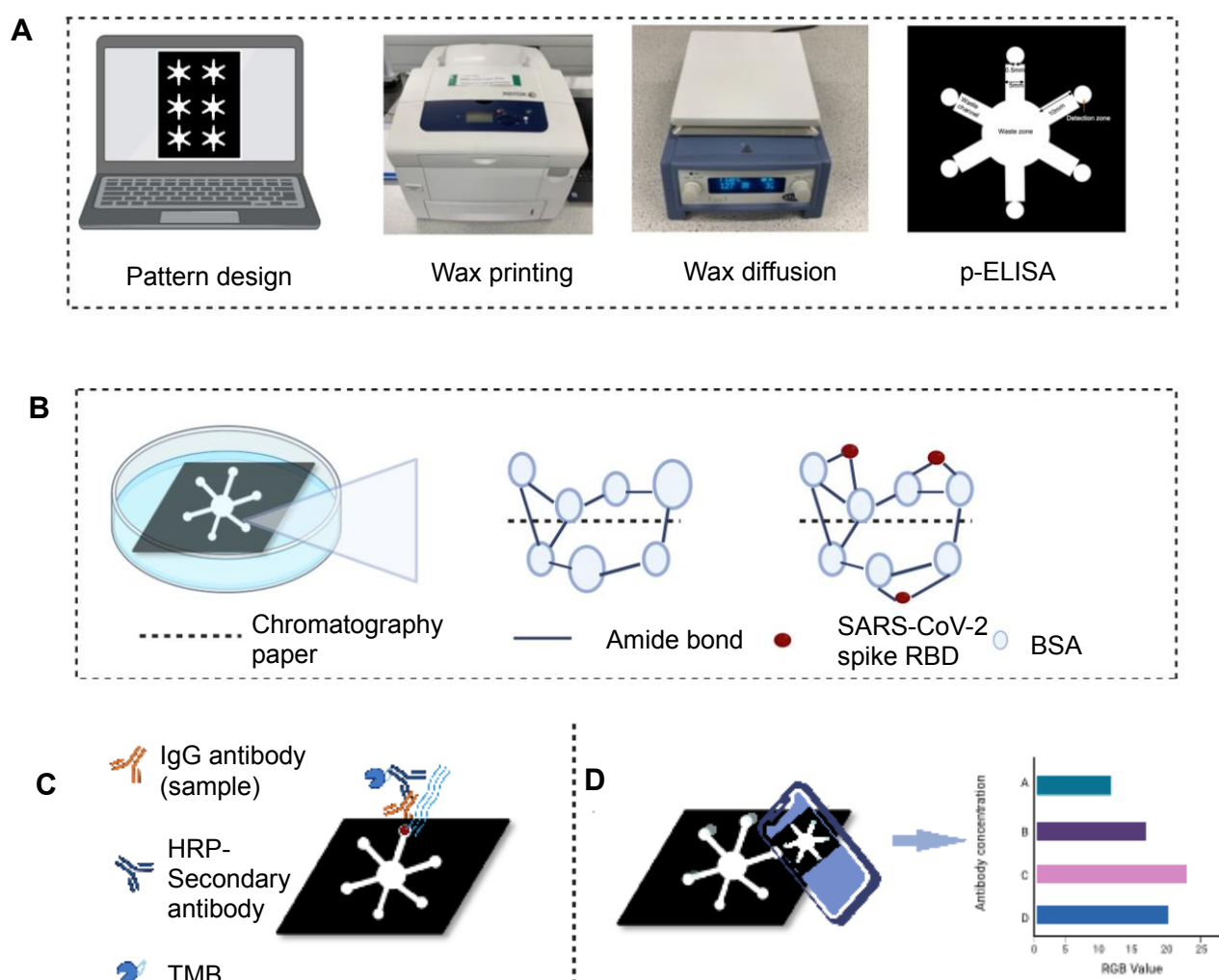


Figure 3-1 Schematic of the detailed design and operation for P-ELISA detecting anti-S RBD IgG in artificial saliva. **A** p-ELISA preparation. The design consists of a detection zone linked to a waste channel with a 0.5mm gap and a waste zone. **B** paper modified with 5% BSA forming amide bonds on both layers. **C** Indirect ELISA reaction on p-ELISA. Each step is followed with three times washing, and the buffer plus unspecific binding flow via the waste channel to the waste zone. The arrow indicates the waste flow direction. **D** p-ELISA image capture and analysis.

3.4 Results and Discussion

Overall, Figure 3- demonstrates p-ELISA detection from step to step. The presence of the IgG antibody for SARS-CoV-2 spike RBD in artificial saliva, when combined with the antigen and secondary antibody containing HRP, results in the oxidation of TMB, producing a blue complex that can be easily seen with the naked eye. The intensity of the blue colour is directly proportional to the amount of the IgG antibody for SARS-CoV-2 spike RBD in artificial human saliva. The p-ELISA may look similar to a later

flow to immunoassay test; however, it varies in the detection area and its sample-multiplex detection. For example, a typical lateral flow test is shown in Figure 3-2, where the analyte is capillary flowing from left to right, and a test line is formed when the analyte is binding specificity-coated reagents. This test has two significant drawbacks: 1) a single readout that only one sample is tested each time; 2) the test line is hard to quantify. The novelty of the p-ELISA in this study is that once the sample is treated, several analytes of interest can be measured simultaneously in saliva, and they cannot only be detected by also quantified.

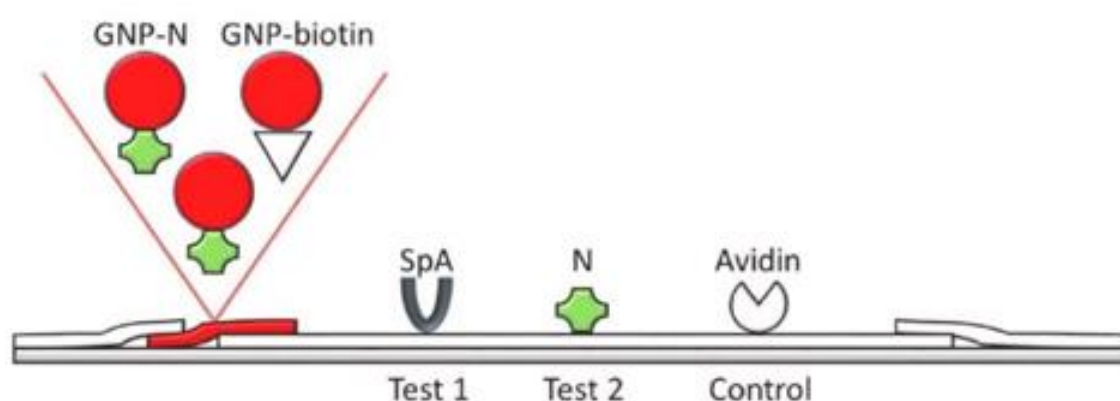


Figure 3-2 Scheme of the LFIA device for the rapid serological diagnosis of SARS CoV-2.

The strip is composed of the analytical membrane onto which the protein A(SpA), the SARS CoV-2 nucleocapsid protein (N), and avidin are coated to form the two test (T1 and T2) and the control (C) lines, respectively ¹⁷.

As previously discussed, the flow dynamics, determined by several factors such as the characteristics of the paper channel, its thickness, and pore size, were found to be critical in the successful implementation of p-ELISA¹⁸. Here, to define a relatively efficient and economical design of the paper, three sets of mock trails are tested with calcein reagent (2.5mM) to visualize the capillary flow (Figure 3-3). The dimensions of the designated detection zone in trail 1 exceeded the required parameters, extending beyond the optimal scale for efficient sample immobilization. The passage channel in trail 2 failed to transport the undesired analyte or reagent to the waste zone efficiently. It was concluded that trial 3 was the best to obtain a high flow rate while using the least paper materials (Table 3-1).

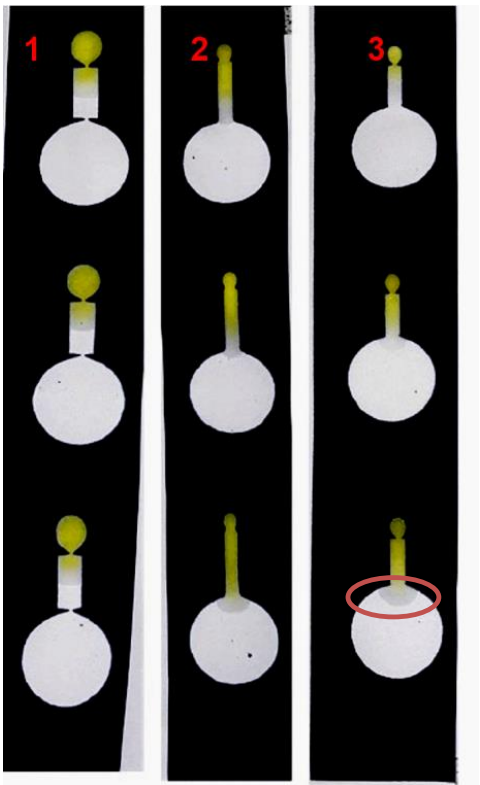


Figure 3-3 An illustration of three designs of p-ELISA (only one channel listed). The detailed information of trail 1, 2, 3 is recorded in Table 3-1. The same volume of liquid (10 μ L) was loaded on the detection zone. Three trails vary in detection zone diameter, waste channel length and width, respectively. In trail 1, most liquid accumulated in the detection zone or the upper position of the waste channel. In trial 2, a portion of the liquid stagnated in the middle of the waste channel. In trail 3, most residual fluid (circled in red) was passively transported to the waste detection zone. Each trail was demonstrated in triplicate.

Table 3-1 The p-ELISA design with different parameters

Microfluid performance	Detection zone diameter (mm)	Waste channel length (mm)	Waste channel width (mm)
Low	10	18	8
Medium	5	18	5
High	5	10	5

In developing any diagnostic assay, optimizing varying parameters is essential to ensure a test's sensitivity, specificity, and accuracy. This study optimized several factors in the in-house indirect ELISA for detecting anti-S-RBD IgG. One of the critical factors was the amount of immobilized antigen. Initially, a preliminary antigen

concentration range ranging from 0.2 to 2 $\mu\text{g mL}^{-1}$ was employed, revealing that a concentration of 0.5 $\mu\text{g mL}^{-1}$ yielded the most discernible colourimetric response (refer to Figure 3-4A). Subsequently, a refined antigen concentration range (0.5 to 2 $\mu\text{g mL}^{-1}$) was reassessed to ascertain the optimal immobilized antigen concentration. After blocking with 1%BSA, it was observed that 1.6 $\mu\text{g mL}^{-1}$ (Figure 3-4B) was the most suitable concentration, as this produced the steepest and most accurate calibration curve. Other protocols¹⁹ have reported a performance similar to those shown here, using 2 $\mu\text{g mL}^{-1}$. However, as the optimal antigen concentration in the assay described here seemed to be lower than 2 $\mu\text{g mL}^{-1}$, this was selected as the best since the purified spike RBD protein is quite expensive. The next step was the choice of blocking buffer as it is known to contain inert proteins or a mixture of other inert compounds capable of blocking the surface of the plate not occupied by the immobilized antigen, minimizing the nonspecific adsorption of components, including other proteins present in the biological fluids. The most used commercial blocking buffer is BSA, which was used in one protocol developed for SARS-CoV-2²⁰, in this work, a blocking buffer containing only BSA did not seem sufficient to remove the nonspecific binding. According to Figure 3-4C, a commercial blocking buffer known as clear milk blocking buffer had better blocking effects than 1%BSA (5% was tried as well, but no colour differences were detected by the naked eye or plate reader), and the significance was $p=0.0002$. The cross-specificity was also investigated; an IgG anti-human epidermal growth factor receptor 2 (HER2) (ab187288, Abcam, Cambridge, UK) applied with Goat pAb to IgG (ab205719) was used to determine the specific interaction between SARS-CoV-2spike RBD and its IgG. The comparison between the two groups (Figure 3-4D) revealed that this proposed protocol substantially impacted removing any other nonspecific bindings, so the in-house indirect ELISA for detecting SARS-CoV-2 was considered fully optimized.

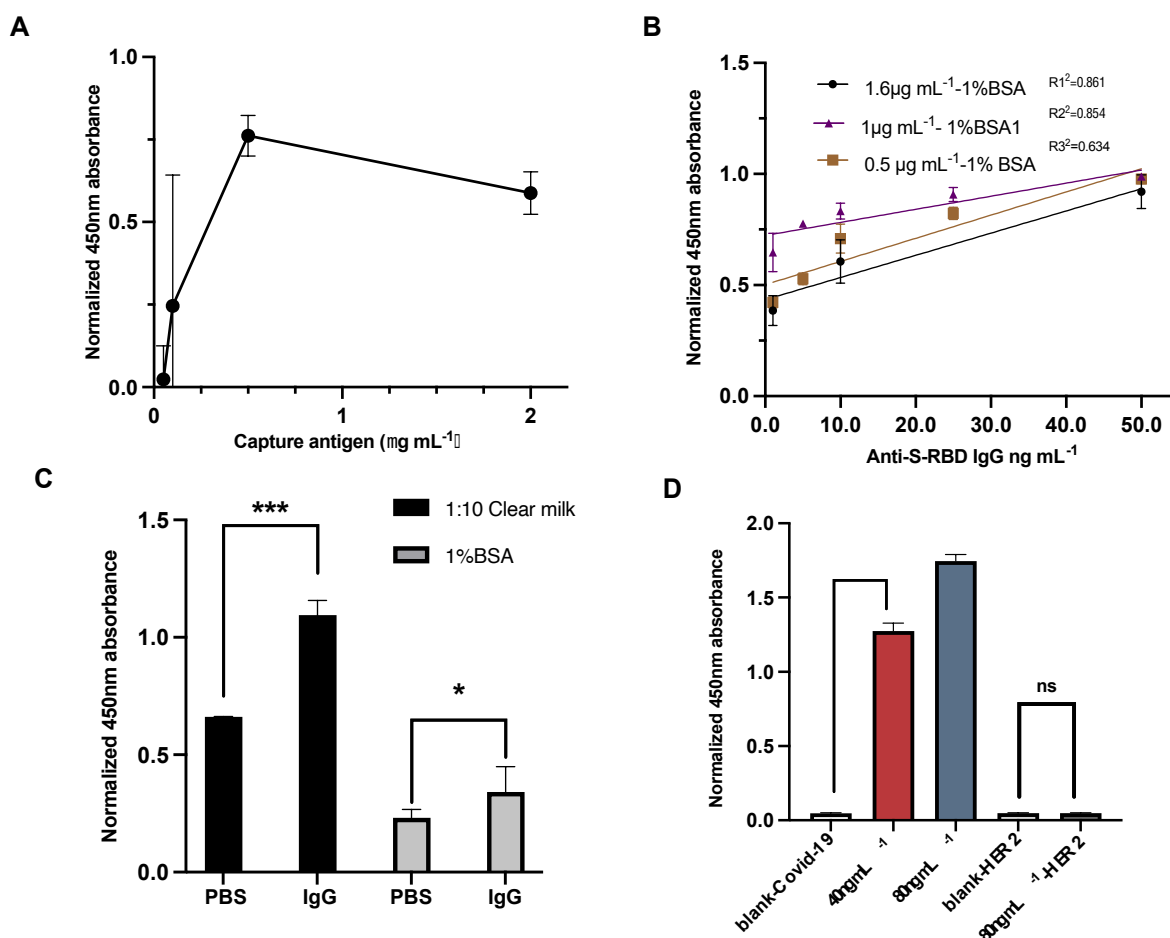


Figure 3-4 The optimization conditions for generating good performance indirect ELISA. **A** The choice of antigen concentration range. **B** The specific antigen concentration refinement. **C** The blocking buffer choice Paired T-test was used to compare the function of two buffers in blocking effects. *** $p=0.0002$ * $p=0.019$. **D** The specificity test with established indirect ELISA protocol. Ordinary one-way ANOVA and Paired t-tests were used to compare the antibody detection specificity. **** $p<0.0005$, ns is no significance by t-test (two-tailed). Each error bar represents an independent triplicate experiment shown with mean $\pm$ SD

The standard curve displayed the results from the optimized assay (Figure 3-5A). The $R^2=0.99$ indicated the strong correlation between the signal and the anti-S-RBD IgG detection. The high coefficient of determination ($R^2 = 0.99$) signified a robust association between the signal and the detection of anti-S-RBD IgG. Notably, the calculated limit of detection (LOD) was determined to be 10 ng mL^{-1} , underscoring the considerable sensitivity of the test. This LOD value markedly surpassed those reported in existing clinical literature, where the seropositive LOD was noted to be 3.6 μg mL^{-1} IgG, corresponding to 56.5% neutralization antibody²¹. Those findings mainly focused on real clinical samples, whilst it was derived from serum, it revealed a relatively real-

world antibody concentration range. Under this circumstance, the stated protocol using artificial saliva was re-assessed on the paper-based device, and the antibody concentration range was adjusted. The saliva spike-in recovery rate was calculated based on the standard curve to confirm whether the saliva could be an acceptable matrix. After the centrifugation without further treatments, the pure saliva significantly impacted the specific binding (Figure 3-5B); the spike-and-recovery rate was an outlier in most concentration ranges (80%-120%)²². At least two concentrations were in the upper limit of quantification (LOQ) (approximated by multiplying the LOD signal ratio by 3.3), suggesting the need for a suitable treatment to increase the validity of the saliva matrix. Accordingly, after centrifugation, the artificial saliva spike-in sample used to prepare the standards was at a dilution factor of 1:10 with PBS buffer, and the same assay was carried out. This treatment considerably improved the saliva tolerance of detected immunoglobins. Figure 3-5C showed that the Sigmoidal four-parameter logistic (4PL) fitted the two lines indicating the saliva matrix was suitable for analysis with this treatment. Overall, these results demonstrated the optimization of the indirect ELISA assay for detecting SARS-CoV-2 IgG and highlighted the suitability of saliva as a potential matrix for analysis, which provided that the appropriate treatment was applied.

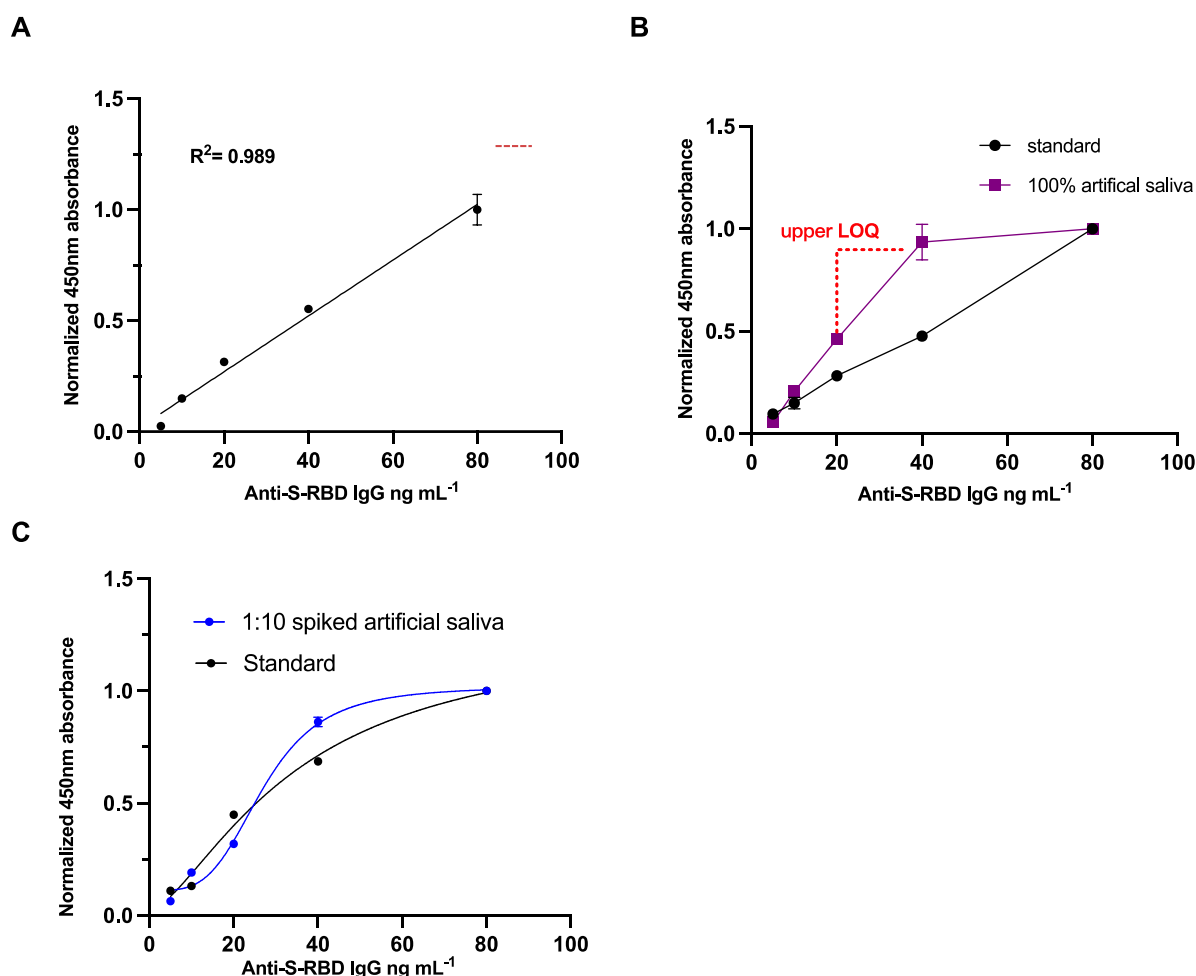


Figure 3-5 The assessment of spike-in saliva validity. **A** The standard curve of indirect ELISA. **B** The comparison between pure saliva sample and standard. R^2 was below 0.8 with no apparent linear regression. **C** Sigmoidal four-parameter logistic (4PL) curve fitting to recombinant antibody compared with the standard plot. R^2 was 0.99. Each error bar represents an independent triplicate experiment, and data were shown as mean $\pm$ SD.

To demonstrate the paper-based assay, chromatography paper adjustments were performed to make the assay compatible with the paper. BSA can form amide bonds so that proteins and other polymerases can be linked *via* these functionalities while other substances are removed²³. Therefore, a paper treatment with BSA was introduced prior to the immobilization of the antigen. The initial trial with a 2% BSA concentration failed to modify the paper, and no colour was displayed on the assay. Therefore, the concentration of 5% BSA was embedded on the paper, and more apparent colours were shown at the end (Figure 3-6). Another modification was adjusting the sample's concentration range to test on paper. Though the traditional indirect ELISA optimized in the first phase of the work was rather sensitive, the clinical

antibody titer reported in biological fluids is much higher than the previously tested range. Therefore, the p-ELISA assay was evaluated in a different IgG concentration range. According to the research on the serum levels of IgG and neutralizing antibody observed in vaccinated, uninfected, infected individuals, and infected people before the vaccine, an IgG range between 0.6-3.3 $\mu\text{g mL}^{-1}$ was detected. This range raised to around 88 $\mu\text{g mL}^{-1}$ after vaccination²⁴. In contrast to infected cases, the seronegative people (uninfected) had only 0.2 $\mu\text{g mL}^{-1}$ IgG before the vaccine²⁴. In one study, serum and saliva response to SARS-CoV-2 spike protein RBD antigen in COVID-19 patients were assessed, and it was concluded that the salivary IgG responses are correlated with serum IgG responses²⁵. Based on this evidence, the IgG against SARS-CoV-2 spike RBD detection range on the p-ELISA test was adjusted between 0.5-50 $\mu\text{g mL}^{-1}$ to make the assay results clinically meaningful.

Next, to measure the specificity, the positive control prepared by diluting different IgG concentrations was analysed with the formularized protocol. In addition, p-ELISA was also performed with different types of antibodies. The samples contained an anti-HER-2 antibody were used as this would be nonspecific to the SARS-CoV-2 spike RBD antigen immobilized on the paper. It was noticed that without an immobilized antigen, the HRP-labelled secondary antibody was generating a faint colour on the paper by the HRP-based colourimetric reaction (Figure 3-6A), which would diminish the actual detection signal. The absence of Goat anti-human IgG Fc (HRP modified, Figure 3-6A, 1) was shown to reduce the background signal. The sample concentration could be quantified as a linear regression relationship by subtracting this control signal plus the negative control signal (formulas 3.2 and 3.3). Figure 3-6B depicted the colorimetric assays with different anti-S RBD IgG concentrations. An apparent colour change can be observed by the naked eye as the concentration of the antibody increases. However, the determination by the naked eye was not potent enough to support the hypothesis that the colour changes were strongly associated with the detected increasing antibody concentration. Therefore, a more analytical analysis was required.

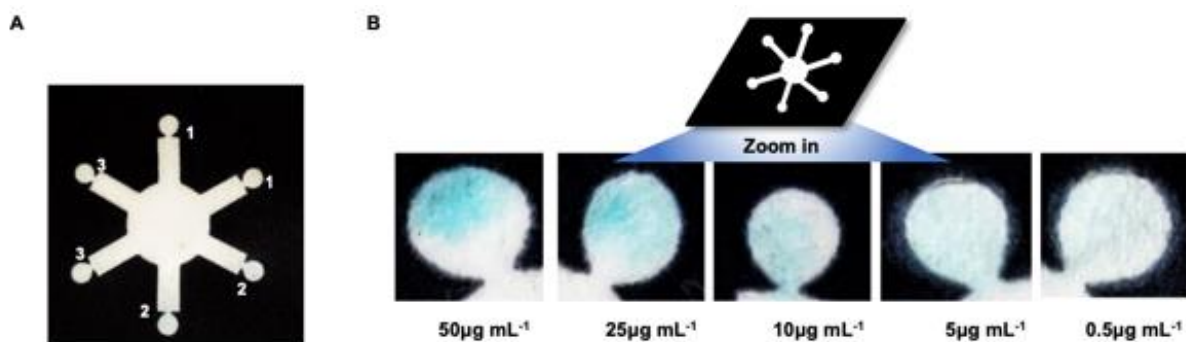


Figure 3-6 The illustration of the p-ELISA concept with specificity. **A** Groups of images of the TMB analysis. 1: the absence of Goat anti-human IgG Fc (HRP modified); 2: without antibody (negative control); 3: immobilized with anti-HER2 primary antibody. **B** Captured colourimetric images of anti-S- RBD IgG detection from the known concentration.

Hence, image software quantified the colours generated by the TMB/HRP reactions. As described in the method and (3.2) formula, the *RGB* value was calculated and used as the final indicator for assessing the colour range. Figure 3-7A plotted the calculated *RGB* values versus the increased antibody concentration obtained in a calibration curve. $R^2=0.93$ and a linear equation expressed by $Y = 0.48X + 9.939$ were obtained in the standard plot (Figure 3-7B). Therefore, it was confirmed that the colour degrees from the TMB/HRP responses were associated with the amount of IgG in the samples, which meant the indirect p-ELISA was able to indicate the antibody titers either stimulated by natural infection or vaccine injection, proving it has the potential to become an established platform for further analysis.

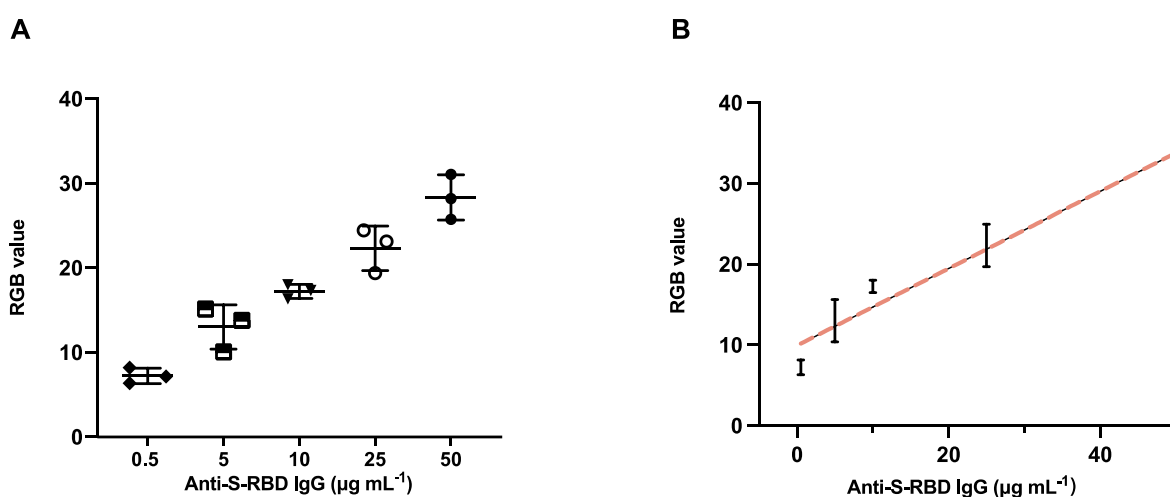


Figure 3-7 The colorimetric quantification assay. A The ΔRGB value with 0.5 $\mu\text{g mL}^{-1}$ to 50 $\mu\text{g mL}^{-1}$ range of anti-S-RBD IgG concentrations with increasing RGB values. **B** A linear algorithm calibration curve in this range. Each error bar represents independent triplicate experiments, and data were shown as mean $\pm$ SD

Finally, to further quantify p-ELISA for detecting IgG, saliva dilution was optimized. In the first reaction, the saliva/buffer 1:1 dilution ratio was hypothesized to be sufficient to reduce the matrix effect. However, the assay signal obtained by p-ELISA in the 1:1 dilution showed that such dilution partially hindered the specific binding between the antigen-antibody complex as the colour was not as intense (Figure 3-8A). To resolve this issue, the saliva dilution ratio was adjusted to minimize the effect on the assay signal. For this, dilution ratios between 1:1 and 1:10 were explored, and it was found that although 1:4 was more sensitive to the lower concentrations (Figure 3-8B), the 1:10 dilution ratio could lead to a detectable proportional trend, particularly in the full range between 0.5-50 $\mu\text{g mL}^{-1}$ making the assay particularly useful for onsite detection. The further RGB (Figure 3-8C) values on this saliva: buffer dilution ratios, were also analyzed and calculated to confirm the naked eye inference. According to the bar comparison, the colourimetric results were generally consistent with the quantitate analysis. The current LOD was calculated to be below 1 $\mu\text{g mL}^{-1}$, allowing for discerning the immune or unimmune groups. This LOD was also more sensitive than other commercial ELISA kits, usually reported as 10 $\mu\text{g mL}^{-1}$ ²⁶. The time of the assay to perform the test was below 2 hours shorter than most-based assays (<5 hrs). However, the assay time was not comparable to rapid LFA, but the platform could

allow multiplex detection and quantification. These promising achievements indicate that the p-ELISA can potentially become an onsite detection device.

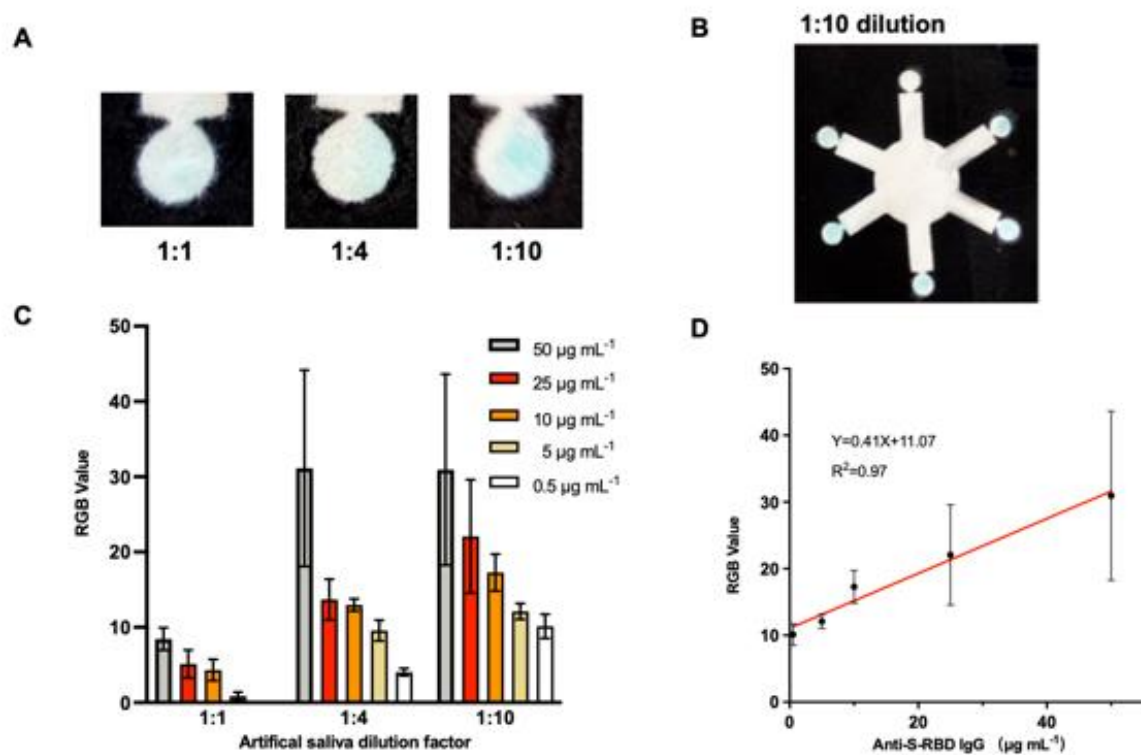


Figure 3-8 The saliva dilution treatment analysis. **A** The comparison between three different saliva dilution ratios. Therefore, the saliva was mixed with PBS in 1:1, 1:4, and 1:10 to make a saliva matrix. **B** The display of saliva antibody detection in a dilution ratio of 1:10. The clockwise orientations were NC, 50 $\mu\text{g mL}^{-1}$, 25 $\mu\text{g mL}^{-1}$, 10 $\mu\text{g mL}^{-1}$, 5 $\mu\text{g mL}^{-1}$, 0.5 $\mu\text{g mL}^{-1}$, respectively. **C** RGB analysis of saliva dilution ratio. **D** The linear relationship of 1:10 artificial saliva dilution ratio. Each bar or line represented an independent triplicate experiment and was shown with Mean+SD.

This p-ELISA still suffered from a few shortcomings. In contrast to the plastic plate, the wax-printed paper led to variable features. For example, due to its low resolved quality, it was difficult to control the end-point fluid and achieve an even distribution on the paper, especially after the BSA modification. These variations caused issues when analyzing the RGB values, which can be seen in Figure 3-8D, where there are high standard deviation (SD) values, especially for the highest concentration tested. These variations probably would also result in a false negative in the clinical testing. Limited by a simplified platform, the paper could not allow pre-treatment, and saliva still requires a 20-minute centrifugation step, increasing the difficulties of conducting it on-site. It might be possible to use specific filters like polypropylene (PP) filters as these

have been successfully utilized as a pre-processing material to treat saliva pepsin²⁷. It is also suggested that using a scanned colour scale rather than taking and analysing a picture could contribute to better results interpretation. By using this, people could quickly identify the antibody levels utilizing the colour threshold without the need to calculate RGB values and, therefore, without requiring software for the analysis. Finally, using paper-based sensors facilitates the freedom in design that permits many benefits in detection, such as detection sites.

Overall, by optimizing in-house ELISA and p-ELISA conditions, the LOD could be sensitive to $0.5 \mu\text{g mL}^{-1}$ and 10 ng mL^{-1} for detecting anti-S-RBD IgG in artificial saliva. Table 3-2 summarizes the recent research on antibody detection in saliva, it is likely to conclude that the assay in-house ELISA has achieved similar LOD in saliva testing as other assays. Although p-ELISA is not comparable to most analytical methods, this simple operation with paper is advantageous. More importantly, this p-ELISA is the most time-efficient platform, which is especially friendly to on-site testing. Others demand more time and complex machines. According to the commercially available detection kits, the dominant assay type is a serological test approved by EUA³⁴; however, an antibody test in saliva could only be found in a research study or clinical studies that is not intended for diagnostic use. Furthermore, as discussed in the previous chapter, LOD is only valid for artificial saliva while the assay has not been tested on any real clinical sample. With real saliva, complex components may inhibit the immunoassay and generate strong background noises as the first-time spike-in saliva was tested in this study. Finally, when testing with real saliva in immunoassay, a saliva stability test is considered. Present studies for saliva stabilization at ambient temperature are only valid for DNA& RNA³⁵, and it seems that other components stabilization in saliva is limited. An alternative suggested way of testing real saliva includes the inclusion of 1% Triton-100 at room temperature for 1hr or heat treatment at 56°C for 30 mins¹¹. Another crucial point is that in real testing, a collection of saliva sample may be shipped at low temperatures (-4°C - 8°C), and incomplete thawed saliva after centrifugation would affect the assay efficiency as well. Centrifuging pellet mucins would interfere with specific binding and effect results³⁷. These all should be addressed in the future. However, from the view of self-testing, p-ELISA is easy to

handle. Thereby, after further testing on clinical saliva to ensure the adapted LOD, self-testing at home is potentially possible.

Table 3-2 The comparison between different analytical platform performance of SARS-CoV-2 antibody detection in saliva testing

Methods	Developer	Sophisticated instrument	Target antibody	Time (hour)	LOD	Number of samples (n)	References
Saliva-ELISA	University Hospital Tübingen	Yes-CLARIO star microplate reader	Anti-S-RBD IgG	36	6 ng mL ⁻¹	91	[28]
ELISA	University of California, Los Angeles	Yes-Amperial™ reader	Anti-S1 IgG	2	2 ng mL ⁻¹	34(Self-collected)	[29]
Automated solid phase DELFIA	University of Helsinki and Helsinki University Hospital	Yes-GSP™ instrument	Anti-S1 IgA, IgG	N/A	N/A for saliva	1231(self-collected)	[30]
Indirect Serology	MSD Isotyping Panel 1 Human/NHP Kit (K15203D)	Yes-high-throughput automation from MSD	Total anti-S salivary antibodies	2	0.96 AU *mL ⁻¹ *	N/A	[31]
Zinc bead-based	Simoa® technology	Yes---HD-X™	Anti-S-RBD total antibody	4	96.17 ng mL ⁻¹	N/A	[32]
Paper-based ELISA (artificial saliva)	This study	No	Anti-S-RBD IgG	1	1000 ng mL ⁻¹ 10 ng/mL ⁻¹ ¹ (in-house)	N/A	This study

*AU: arbitrary unit

3.5 Conclusion

The development of a paper-based platform (p-ELISA) enables colourimetric assay of anti-SARS-CoV-2 spike RBD IgG in artificial saliva samples ranging from $1 \mu\text{g mL}^{-1}$ to $50 \mu\text{g mL}^{-1}$, where the responses are visible to the naked eye. The p-ELISA is one of the first examples of a POCT detecting the human antibody IgG in artificial saliva samples. The detection range of this p-ELISA is reconciled with clinical measurements; thus, the detection of IgG enables tracking an individual's immune response level potentially at home. The immobilized antigen can also be changed to an E or N protein to evaluate the total antibodies. Briefly, the merits of this p-ELISA are as follows:

- Less invasive and non-painful sample collection--- saliva
- The device fabrication can be implemented on a large scale (compatible with mass production)
- Detection is equipment-free, and the results can be read by naked eyes
- Rapid (less than 2 hours) and almost meets the POCT standard

The limitations of this platform are briefly addressed next to make improvements in the future. Saliva treatment by removing mucin and other components could enhance the assay validation because mucin other components, and real clinical samples have not yet been tested. So, whether the platform is genuinely helpful for practical applications cannot be fully concluded. Overall, this platform demonstrates a proof of concept of conducting saliva antibody tests on paper microfluidic devices. It paves the way for vastly deployed to quantify SARS-CoV-2 IgG antibodies and other antibodies against emerging pathogens showing great potential for community surveillance testing to augment clinical workflows.

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4 Overall conclusions and outlook

4.1 Conclusions

The thesis focuses on two parts of SARS-CoV-2 detection, which is aimed at detecting RNA virus sequences via paper-based RT-LAMP and IgG concentrations against SARS-CoV-2 spike protein RBD with indirect ELISA assay. Both assays are based on a collection of artificial saliva samples, and the results are shown on a paper-based device.

The identification of spiked RNA virus sequences entails the utilization of a colourimetric isothermal loop-mediated amplification (LAMP) technique, employing an RNA extraction-free methodology. Following a straightforward elution process, the sample undergoes incubation at a stable temperature, enabling amplification, coinciding with a visual colour change from red to orange or yellow. The limit of detection (LOD) for the identification of SARS-CoV-2 ORF1ab is determined to be above 135 copies μL^{-1} under the optimized conditions, placing it in alignment with conventional laboratory results. Conversely, the paper-based device, while exhibiting reduced sensitivity, demonstrates heightened rapidity and on-site applicability. The incorporation of loop-mediated isothermal amplification (LAMP) contributes to a notable reduction in the overall detection time to 35 minutes. Moreover, the employment of paper substrates for both sample pre-treatment and result manifestation eliminate the need for expensive instrumentation. Additionally, the user-friendly properties of paper support convenient handling, further enhanced by its customizable design. The inclusion of multiple reaction chambers in the design facilitates multiplexing, allowing for the simultaneous detection of various genes or pathogens or triplet tests. Consequently, this feature endows the paper-based microfluidic device with enhanced efficiency when contrasted with similar LFA methods, where LFA is only capable of testing one sample at a time, and the result is not available to tell the antibody concentration of the SARS-CoV-2.

Another pivotal aspect of the study is to use of immobilized spike protein RBD on the paper-based device (p-ELISA) to detect the IgG concentrations to reflect an immune response activity, indicating the individual immune status, especially the ability to

combat reinfection. The development of p-ELISA was able to detect around $1 \mu\text{g mL}^{-1}$ IgG with clear colour differences analyzed by RGB value through image analysis software. The platform outweighs the traditional immunoassay on detection time, as the whole assay requires less than 2 hours from collecting the sample to the result. The premise for the final device is that the paper will be distributed already treated with 5% BSA and incubated for half an hour with the antigen for its immobilization. The identification of an appropriate company for routine production prior to the widespread distribution of the paper-based device for the assay is currently under consideration. However, a comprehensive search has yielded no large-scale custom manufacturing firm on paper modification with BSA. Consequently, manual alterations represent the sole available alternative at present.

Remarkably, the advantageous attributes of paper have been prominently illustrated, leading to reductions in both detection time and associated costs. Leveraging the initial platform designed for artificial saliva, successful detection within human saliva will be effectively achieved through the current protocol. With regards to disease management, the dual platform enables testing both for the virus and the immunity levels allowing a more accurate estimation of patients' disease progression. For example, following vaccination, the antibody concentrations will vary differently among individuals. It is suspected that antibody titer will wane drastically after three months of infection, but it does not universally apply across all cases. The dual platform assures the individual is aware of their immune conditions and whether they are actively infected. With the aid of saliva, this platform can also help ensure compliance with routine testing practices that are carried out at regular intervals. One study has reported that a combined detection assay, including RT-PCR and lab-based antibody testing, could increase the sensitivity for diagnosis from 67.1% to 99.4%¹ in hospitalized patients. Herein, this dual platform could be highly beneficial for individuals or for a community scale analysis, which would be significantly helpful for endemic management.

4.2 Future Work

As discussed in previous sections, the dual platform approach for SARS-CoV-2 antibody detection has limitations. The primary challenge is to validate the efficacy of

the saliva treatment method on real clinical samples, as the artificial saliva used in this study cannot fully represent the variability of saliva deriving from real patients. The natural composition of saliva may present a more complex and variable matrix, requiring further exploration and optimization.

In terms of the paper-based device, modifications are necessary to enable lower viral load sample detection. The high sensitivity of the colourimetric RT-LAMP in centrifuge tubes provides a promising avenue for achieving comparable detection limits as those achieved with the paper device. Adjusting the pH and optimizing the sealing process for the plastic film cover are recommended for improving sensitivity and reducing contamination, respectively. Additionally, a method for validating the amplification results should be established to supplement the colour signal interoperability of the current paper device.

For the p-ELISA, improvements are necessary to address the loss of detected antibodies during the washing step. Including a control design and formatted colour threshold bar will improve the accuracy and precision of antibody detection without the need for software and data manipulation. The time-consuming RGB analysis process needs to be streamlined for practical use.

Overall, interpreting the results from the integrated dual platform requires a more comprehensive report that accounts for the limitations of each method. A thorough understanding of the strengths and weaknesses of each detection approach will provide better guidance for immune surveillance in actual patients.

Microfluidics is an enabling platform with promising market share due to the feature of replacing manual processing and bulky bench equipment. As indicated by Yole Development, the valuation of the microfluidic product market stood at approximately GBP 9.19 billion in 2019. It is projected to ascend to GBP 19.3 billion by 2025, exhibiting a compound annual growth rate (CAGR) of 14% especially in the POCT segment². The emergence of the SARS-CoV-2 outbreak has also significantly expedited the development pace of POCT. As reported by Our World in Data (OWID), the United States alone conducted approximately 2×10^8 COVID-19 tests in the initial six months of 2021, with the global figures far exceeding this count³.

However, it is noteworthy that despite the prevalence of conventional lateral flow tests and the development of automated high-throughput devices by global enterprises, successfully commercialized microfluidic products remain relatively limited. Only a minority of biotechnology innovators have endeavoured to manufacture devices suitable for potential markets in developing countries⁴. Notably, Daktari Diagnostics Inc. implemented a microfluidic differential counter capable of quantifying CD4+ or CD8+ immune cells in blood specimens in Africa⁵. However, the company ceased its operations in 2019. Similarly, Diagnostic for All has been focusing on establishing DNA/RNA testing using paper-based microfluidic devices in developing nations⁶, albeit operating as a non-profit organization. The underlying reasons for limited commercialized real-world testing are complicated, and include the following factors: platform components, technology development, manufacturing, authorized audit, and secured target. To gain deeper insights into the distribution process of microfluidic devices to end-users, a concise depiction of the commercialized chain for such devices is illustrated in Figure 4-1. The procedure suggests that achieving a market-ready microfluidic device demands substantial financial resources and rigorous endeavours. While the utilization of paper as an alternative material may reduce certain costs associated with platform components, this investment represents only a fraction of the entire business operation. Numerous challenges exist within the domain, and the subsequent challenges are emphasized concerning real-world testing from the standpoint of commercialized products.

- 1) Lack of market sense and huge investment in the research and development (R&D) stage. In the early stage, regarding the need of customer requirements, it is crucial to fully understand the current applications and potential specific areas that will benefit the adoption of microfluidic. Whilst most academic researchers are unfocused and random, they usually aim to develop an invention with novel materials and techniques without sufficient clinical and market investigations. Another barrier in the early and middle stages is the investment in constructing a compatible prototyping involving with selection of materials, components, and reagents. To maintain the validity and stability of assays, there would be increasing consumption of these items to support the continuous R&D⁷. In this study, most of the time was invested into the search of

proving the plausibility of saliva diagnosis, finding the most optimized saliva treatment and paper modification. Upon the eventual production of the prototype, the prevalence of Covid-19 had substantially diminished.

- 2) Lack of practical integration for standardization. Most research focusing on inventing individual microfluidic components^{8,9} neglects the critical aspect of integrating these components seamlessly. Additionally, reproducibility is less provided in the development process for detection or prototyping. The lack of this data poses challenges in effectively assembling these pioneering components into fully functional, integrated devices. To unlock the complete potential of microfluidic devices, researchers need to prioritize the final commercial application right from the initial design phases, ensuring the mutual compatibility of each component. The presented platform faced the same issue that the dual platform failed to treat the sample within it, and both platforms still work in their function without being linked to show the result.
- 3) Complex scale-up manufacturing. The manufacturing cost plays a key role in the successful transition from a laboratory prototype to a marketable product, which is typically contingent upon the profit margin per unit and the volume of sales achievable by the company. Approximately 57% of the capital allocated for new product development is estimated to be directed toward manufacturing¹⁰. Many variables including mould fabrication, reagent storage, and surface treatment influence the cost of manufacturing, therefore, careful consideration and optimization of various aspects are essential to streamline the manufacturing process thereby controlling the budget.

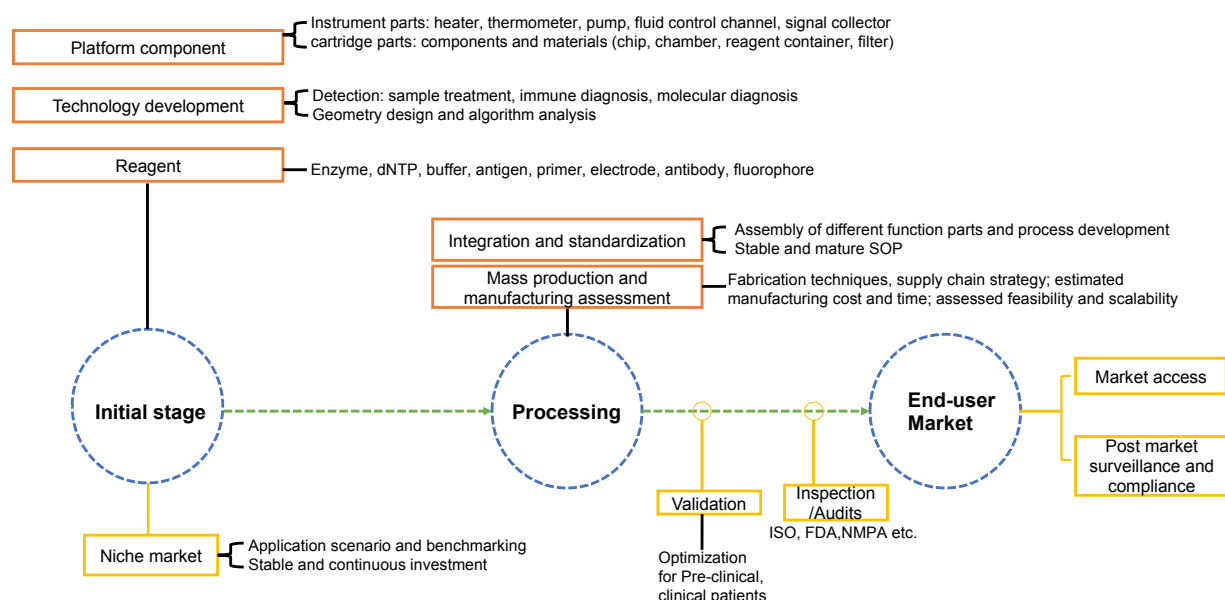


Figure 1-1 Commercialized chain for microfluidic device development. The development is segmented into three parts: initial stage, processing, and end-user market. Each segment has multiple factors impacting its event. SOP: Standard Operating Procedure; ISO: International Organization for Standardization; FDA: Federal Drug Administration; NMPA: National Medical Products Administration

The mentioned points are the main challenges for the development of microfluidics being introduced to the market, surely, there are other obstacles during the process. For example, the approval from relevant authorities and regulators⁴. The conclusion is that the success of a new microfluidic product requires timely collaborations of many parts. It is imperative to synchronize industrial advancements with academic initiatives. Active involvement of industrial collaborators possessing marketing acumen is instrumental in evaluating the potential success of a product within a targeted market and offering valuable insights into end-user needs and expectations. Simultaneously, academic researchers can direct their efforts toward delivering a seamlessly integrated product tailored to a specific application.

4.3 References

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