

Development of Portable and Easy-to-Use Chemo/Biosensors

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Abstract

Development of Portable and Easy-to-Use Chemo/Biosensors

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Low-cost, portable, and easy-to-use analytical devices are essential for applications ranging from healthcare and environmental monitoring to biotechnology and forensics. Microfluidic paper-based analytical devices (μ PADs) and tablet-based assays represent promising platforms for developing such detection tools, particularly for resource-limited settings. However, challenges and gaps remain. Improvements in μ PAD fabrication are needed to enhance accessibility, while enhanced precision and sensitivity are required for colorimetric and electrochemical sensing. For tablet-based assays, further progress is required to expand applicability to viscous media and to establish integration of bioreagents into auto-mixing tablets.

This thesis originated during pandemic-related laboratory shutdowns with a comprehensive review of COVID-19 diagnostic methods, published in *Clinical Microbiology Reviews*. The review surveys molecular, antigen, and serological assays, providing insights into pandemic preparedness and identifying gaps in diagnostic technologies. Building on these findings, the main body of the thesis comprises four chapters, each corresponding to a manuscript focused on μ PADs and tablet-based assays.

First, a critical review examines the history, fabrication, and detection strategies of μ PADs and tablet-based assays, presenting the first dedicated literature review of tablet-based assays. Next, a simplified μ PAD fabrication method combining Parafilm[®] heating and CO₂ laser cutting enabled high-resolution devices. Novel multi-inlet and segmented detection zones reduced the color gradient by more than half, improving precision in colorimetric glucose detection in urine. Subsequently, a do-it-yourself screen-printing approach for electrochemical electrode fabrication was developed using portable equipment and laser-cut vinyl masks. A simple water treatment enhanced signal intensity by over 75-fold, further amplified by sandwiching electrodes between two paper layers, and the devices were validated for glucose detection in cell culture media. Finally, rapid auto-mixing tablets were developed, with effective operation in viscous media up to

1700 mPa·s. A proof-of-concept application was demonstrated for nitrite detection in saliva. Additionally, a simple mist-sprayer method for reagent encapsulation in trehalose enabled incorporation of bioreagents into tablets without specialized equipment. Using this approach, the first tablet-based biosensor with rapid auto-mixing was validated for glucose detection in human urine.

Overall, this work simplifies fabrication, enhances analytical performance, and broadens applicability, further advancing the translation of μ PADs and tablet-based assays toward diverse real-world uses.

Author's Declaration

I hereby declare that this thesis is the result of my own work and has not been submitted, in whole or in part, for any other degree or qualification at this or any other institution. All sources used have been duly acknowledged.

I also acknowledge that during the preparation of this thesis, an AI-based tool (ChatGPT, versions 3, 4, and 5) was used for assistance in writing and editing to improve grammar, style, and clarity. After using the tool, the content was thoroughly reviewed and revised as necessary, and I take full responsibility for the final work.

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“All is one, one is all”

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*To all my teachers,
who shaped my thoughts and character
and made this possible.*

Contribution of Authors

The majority of the work presented in this thesis was carried out by the author, Seyed Hamid Safiabadi Tali. The contributions included Conceptualization, Methodology, Investigation, Validation, Formal Analysis, Visualization, Project Administration, Writing-Original Draft, and Writing-Review&Editing. Dr. Sana Jahanshahi-Anbuhi supervised all projects, contributing to Conceptualization, Methodology, Funding Acquisition, Resources, Formal Analysis, Validation, Visualization, Writing-Review&Editing, and Supervision. The contributions of authors to each section are outlined below.

Chapter 2 was prepared specifically for this thesis by Seyed Hamid Safiabadi Tali, under the supervision of Dr. Jahanshahi-Anbuhi.

Chapter 3 is adapted from an article titled “[*Engineered detection zone to enhance color uniformity on paper microfluidics fabricated via Parafilm[®]-heating-laser-cutting*](#)” published in *Sensors and Actuators B: Chemical* (2023). Hasti Hajimiri contributed to Investigation and Validation. Zubi Sadiq contributed to Methodology and Writing-Review&Editing.

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List of Abbreviations

μPAD	Microfluidic paper-based analytical devices
2019-nCoV	2019 novel coronavirus
2D	Two dimensional
3D	Three dimensional
ACE2	Angiotensin-converting enzyme 2
AChE	Acetylcholinesterase
Ag-RDT	Antigen rapid diagnostic test
AI	Artificial intelligence
AIOD-CRISPR	All-In-One Dual CRISPR
AKD	Alkyl ketene dimer
ALT	Alanine aminotransferase
ANS	Anterior nasal
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AuNP	Gold nanoparticle
BAL	Bronchoalveolar lavage
BIP	Backward inner primer
BOP	Backward outer primer
BSL	Biosafety Level
bw	Basis weight
c.w.	Continuous wave
CA	Chronoamperometry
Cas	CRISPR-associated
CASdetect	CRISPR-assisted detection
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CDtection	Cas12b-mediated detection

CH	Chromatography paper
CHR	Chromatography
CK	Creatine kinase
CLIA	Clinical Laboratory Improvement Amendments
CLIA	Chemiluminescence immunoassays
CMYK	Cyan-magenta-yellow-key
COVID-19	Coronavirus disease 2019
CP	Cleanroom paper
CREST	Cas13-based, Rugged, Equitable, Scalable testing
CRISPR	Clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
CT	Computed tomography
Ct	Threshold cycle
CTAB	Hexadecyltrimethylammonium bromide
CV	Cyclic voltammetry
CVD	Chemical vapor deposition
CXR	Chest X-ray
d.p.o	Days post onset of symptoms
ddNTPs	Dideoxynucleotides
ddPCR	Droplet digital PCR
DETECTR	DNA Endonuclease Targeted CRISPR Trans Reporter
DI	Deionized
DIY	Do-it-yourself
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotides
dsDNA	Double-stranded DNA
DTT	Dithiothreitol

D-zone	Detection zone
E protein	Small envelope protein
EIA	Enzyme immunoassay
EIS	Impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
ePAD	Electrochemical paper-based analytical device
EUA	Emergency Use Authorization
F	Fluorophore
FAM	Fluorescein amidite
FDA	Food and Drug Administration
FDM	Fused deposition modeling
FET	Field-effect transistor
FFPE	Formalin-fixed paraffin-embedded
FIP	Forward inner primer
FITC	Fluorescein isothiocyanate
FL	Filter paper
FMI	Fluorescent microparticle immunoassays
FOP	Forward outer primer
FRET	Fluorescence resonance energy transfer
FRNT	Focus reduction neutralization test
FT-IR	Fourier-transform infrared
GGO	Ground-glass or reticular opacities
GI	Gastrointestinal
GISAID	Global Initiative on Sharing All Influenza Data
GOx	Glucose oxidase
gRNA	Guide RNA
HBV	Hepatitis B virus
hCoV-19	Human coronavirus 2019
HE protein	Hemagglutinin-esterase

HRP	Horseradish peroxidase
IAT	Isothermal amplification technologies
ICU	Intensive care unit
IDA	Indoxyl acetate
IDF	International Diabetes Federation
IDNCOV	ID Now COVID-19
IFA	Immunofluorescence assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemical
IL-6	Interleukin 6
ILA	Immunoluminescent assay
ILI	Influenza-like illness
IQR	Interquartile range
ISH	In situ hybridization
LDH	Lactate dehydrogenase
LDT	Laboratory-developed tests
LFA	Lateral flow assay
LFIA	Lateral flow immunoassay
LIMA	Laboratory information management system
LOD	Limit of detection
M protein	Membrane/matrix protein
mAbs	Monoclonal antibodies
MDF	Melamine-faced chipboard
MEM	Minimal essential media
MERS-CoV	Middle eastern respiratory syndrome coronavirus
MIS-C	Multi-system inflammatory syndrome
MN	Microneutralization

MRI	Magnetic resonance imaging
MTS	Mid-turbinate nasal swab
MTS	Mid-turbinate nasal swab
N protein	Nucleocapsid protein
Na	Anterior nares
NA	Nasal aspirate
NAAT	Nucleic acid amplification test
nAbs	Neutralizing antibodies
NALC	N-acetyl-L-cysteine
NC	Nitrocellulose membrane
NCBI	National Center for Biotechnology Information
NEAR	Nicking enzyme amplification reaction
NED	N-(1-naphthyl)ethylenediamine dihydrochloride
NFC	Near-field communication
NGS	Next-generation sequencing
NP	Nasopharyngeal
NPA	Nasopharyngeal aspirate
NPA	Negative percent agreement
NPS	Nasopharyngeal swab
NPW	Nasopharyngeal wash
NS	Nasal swab
nsp	Non-structural proteins
NW	Nasal wash
OD	Optical density
OP	Oropharyngeal
OPS	Oropharyngeal (throat) swab
Orf	Open reading frame
OTS	Octadecyltrichlorosilane
PB	Phosphate buffer

PBASE	1-Pyrenebutyric acid N-hydroxysuccinimide ester
PBNA	Pseudovirus-based neutralization assays
PBS	Phosphate-buffered saline
PC	Physical containment
PCL	Polycaprolactone
PCT	Procalcitonin
PEG	Polyethylene glycol
PET	Polyethylene terephthalate
PET/CT	Positron emission tomography-CT
PFU	Plaque-forming units
PHLC	Parafilm [®] -heating-laser-cutting
PK	Proteinase K
PLA	Polylactic acid
POC	Point of care
POU	Point of use
PP	Parchment paper
pp	Polyproteins
PPA	Positive percent agreement
PPE	Personal protective equipment
PPI	Pulses per inch
PRNT	Plaque reduction neutralization assays
PUA	Polyurethane acrylate
PVP	Polyvinylpyrrolidone K30
Q	Quencher
RBD	Receptor-binding domain
RBD	Receptor-binding domain
RdRp	RNA-dependent RNA polymerase
RDT	Rapid diagnostic tests

REST-ASSURED	Real-time connectivity, Ease of specimen collection, Scalable, Transferable, Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and environmentally friendly, and Deliverable to end-users
RGB	Red-green-blue
RH	Relative humidity
RIA	Radioimmunoassay
RLU	Relative light units
RPA	Recombinase polymerase amplification
RSD	Relative standard deviation
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RT-ERA	Reverse transcription-enzymatic recombinase amplification
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Real-time reverse-transcription polymerase chain reaction
S glycoprotein	Spike glycoprotein
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCMC	Sodium carboxymethylcellulose
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SHERLOCK	Specific High sensitivity Enzymatic Reporter unLOCKing
snELISA	Surrogate neutralization ELISA
SPCE	Screen-printed carbon electrodes
SPE	Screen-printed electrode
SSB	Single-strand binding proteins
ssRNA	Single-stranded RNA
STOPCovid	SHERLOCK Testing in One Pot Covid
TCID₅₀/mL	Median tissue culture infectious dose/ml
TCS	Trichlorosilane

THF	Tetrahydrofuran
TMA	Transcription-mediated amplification
TMB	3,3',5,5'-tetramethylbenzidine
UTM	Universal transport media
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
VOC	Variants of concern
VSV	Vesicular stomatitis virus
VTM	Viral transport media
WBC	White blood cell
WEPEAR	Whole-course encapsulated procedure for exponential amplification from RNA
WHO	World Health Organization

Chapter 1

Introduction and Objectives

The development of low-cost and portable analytical devices is crucial for a wide range of applications, including medical diagnostics, environmental monitoring, forensics, and the biotechnology and biopharmaceutical industries, all with significant socio-economic impact. Globally, millions of lives are lost each year due to the consumption of unsafe food and water, and the delayed detection of life-threatening illnesses. For example, according to 2024 data from the International Diabetes Federation (IDF), 589 million people are living with diabetes, with 252 million cases remain undiagnosed, while diabetes now ranks among the top ten causes of death worldwide [1,2]. The COVID-19 pandemic is another instance that emerged in 2019 and recorded about 2.3 million deaths in just 14 months [3]. These statistics underscore the urgent need for accessible, efficient detection tools to help prevent disease, mitigate environmental risks, and manage public health crises. Reflecting this need, the global market for low-cost, portable diagnostic devices is projected to grow from ~USD 15 billion in 2024 to ~USD 23 billion by 2029 [4].

Ideally, low-cost and portable analytical devices, often referred to as point-of-care (POC) in medical settings and point-of-use (POU) in broader applications, should adhere to the REST-ASSURED criteria. This framework, adapted from the World Health Organization's original 2004 recommendations for POC diagnostics, stands for: Real-time connectivity, Ease of specimen collection, Scalable, Transferable, Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and environmentally friendly, and Deliverable to end-users [5–7]. Within this context, microfluidic paper-based analytical devices (μ PADs) and tablet-based assay platforms are particularly promising, especially for use in remote or resource-limited settings.

μ PADs are essentially based on creating microchannels within paper substrates. Owing to the inherent advantages of paper, such as low cost, environmental friendliness, wide availability, and pump-free liquid transport via capillary action, μ PADs offer significant potential to develop lab-on-paper devices that are affordable, multiplexed, and require little to no instrumentation [8,9]. Over the past decade, μ PADs have attracted significant interest and have been applied across various fields, including food and water quality monitoring, disease diagnostics, and forensic

analysis [10–12]. The very first and crucial step in developing a functional μ PAD is the fabrication of microfluidic channels. Ideally, the method should be rapid, high-resolution, straightforward, and cost-effective. Wax printing has been the most commonly used technique, but it suffers from relatively low resolution and risk of nozzle clogging [13,14]. Furthermore, the discontinuation of wax printers by Xerox Inc. has driven the search for alternative fabrication methods.

Regarding detection, colorimetric sensing is the most widely used approach [10]. It enables equipment-free detection by producing or changing color in the presence of analytes, allowing visual readout. However, non-uniform color distribution across the detection zone remains a major challenge, affecting accuracy and precision [13,15]. Another widely used detection technique is the electrochemical method. Electrochemical paper-based analytical devices (ePADs) enable seamless quantification using miniaturized electronics, offering rapid detection times and straightforward integration with digital technologies [16–18]. Among various electrode fabrication techniques, screen printing is one of the most common ones, as it provides high-resolution patterns and allows for rapid production. However, this method typically requires specialized equipment, and the fabrication of the required masks is often outsourced to third parties, making the overall process time-consuming and costly [16,17,19,20]. Another key but under-addressed issue with ink deposition methods such as screen printing is the composition of many commonly used conductive inks (e.g., carbon or graphite) which often contain hydrophobic components. These materials interfere with capillary-driven flow in the paper matrix, reducing liquid-electrode interaction and ultimately compromising sensor performance [18,21,22]. Addressing these challenges is critical for improving both the accessibility and analytical performance of ePADs.

In addition to paper-based devices, tablet-based assays offer a convenient platform for POC/POU testing by incorporating pre-measured quantities of reagents in tablet form. This format enables low-cost, stable assays with extended shelf life. Traditionally, such tablets were fabricated through powder compression; however, since 2014, following the pioneering works of Jahanshahi-Anbuhi et al., polysaccharide-encapsulated tablets have gained popularity for analyte detection [23,24]. To date, tablet-based assays have been reported in a range of applications, including the detection of hepatitis B, glucose, and uric acid [25–27]. Despite this progress, major gaps remain. Powder-compressed tablets have been developed both with and without auto-mixing capability, but the auto-mixing format has been limited to chemosensors, with little investigation into their compatibility with bioreagents such as enzymes. Incorporation of bioreagents in powder-

compressed tablets has also relied on bulky and costly equipment such as freeze dryers, limiting scalability. Polysaccharide-encapsulated tablets, although promising for biosensing, often require vigorous shaking or vortex mixing to achieve complete dissolution, which complicates assay workflows. Moreover, both platforms have been used only with low-viscosity fluids such as urine and water, restricting their applicability in more complex media and reducing their broader utility in POC/POU settings.

Given the aforementioned challenges, the main research goal of this thesis is to enhance both μ PAD and tablet-based assay platforms through the following specific objectives:

- **Obj. 1:** Develop a straightforward and high-resolution fabrication method for μ PADs.
- **Obj. 2:** Address non-uniform color formation in μ PADs to improve detection accuracy and precision.
- **Obj. 3:** Establish a simple, do-it-yourself screen-printing approach for ePADs to increase accessibility in laboratories and resource-limited settings.
- **Obj. 4:** Enhance the sensitivity of ePADs by mitigating ink hydrophobicity and employing novel device designs.
- **Obj. 5:** Develop rapid, auto-mixing tablet-based assays to simplify detection and extend applicability to challenging media such as viscous fluids.
- **Obj. 6:** Create a low-cost method for incorporating bioreagents into tablet formulations and assess their compatibility with auto-mixing agents.

Before outlining the structure of this thesis, here, a brief overview of its chronological development is provided to help readers better understand its contents and organization. Shortly after beginning my PhD, the COVID-19 pandemic caused extended lab shutdowns and lockdowns in 2020. During this time, I identified significant gaps in early literature reviews on COVID-19 detection methods and authored one of the most comprehensive reviews on the topic at that time, which has since received over 500 citations. This work deepened my understanding of the limitations of conventional detection tools and highlighted the urgent need for effective POC/POU devices. Recognizing the potential of μ PADs and tablet-based assay platforms, I then focused my research on enhancing these technologies to broaden their applicability and advance them toward real-world and commercial use. With this context, for the sake of coherence, the published work related to COVID-19 detection is included in Appendix, while the main body of the thesis focuses on the

development of μ PADs and tablet-based assays. The chapter-wise organization of this thesis is demonstrated in Figure 1.1 and described as follows:

Chapter 1 introduces the importance of low-cost, portable analytical devices for POC/POU applications and outlines the ideal criteria they should meet. It then discusses the advantages of μ PADs and tablet-based assays as potential platforms to address these criteria, along with the challenges associated with each approach. Finally, the chapter presents the research objectives and provides an overview of the thesis organization.

Chapter 2 presents a comprehensive literature review on μ PADs and tablet-based assays. It examines the history, fabrication techniques, and detection strategies of μ PADs, as well as the development of tablet-based assays from powder-compressed to polysaccharide-encapsulated formats. Both platforms are critically evaluated in terms of their advantages, limitations, and future prospects.

Chapter 3 addresses Obj. 1 & 2. It reports a simplified fabrication method for μ PADs using a CO₂ laser cutter, a laboratory oven and a sheet of Parafilm[®], a material widely used in laboratories. The technique enables creation of half-laminated μ PADs with a high resolution allowing for complex geometries. A new design with multiple inlets into the detection zone is then introduced, which significantly improves color uniformity without requiring chemical modification. The practical applicability of these enhanced devices is demonstrated through glucose detection in urine for diabetes monitoring.

Chapter 4 addresses Obj. 3 & 4. It describes a do-it-yourself method for fabricating ePADs using a low-cost, portable screen-printing setup with laser-cut vinyl masks, enabling on-demand device production with high resolution and speed. Then, a simple post-printing water treatment is introduced to overcome ink hydrophobicity, significantly improving signal intensity. Also, a novel sandwiched electrode design is presented, in which electrodes are positioned between two paper layers, nearly doubling the electroactive surface area and sensitivity. The practical utility of the platform is demonstrated through glucose detection in cell culture media, relevant for monitoring cell growth and biopharmaceutical production.

Chapter 5 addresses Obj. 5 & 6. It reports the development of rapid, auto-mixing tablets for analyte detection in a simple drop-and-detect format. Their mixing capability is evaluated across solutions of varying viscosity, along with their effect on solution pH. Then, a proof-of-concept is

demonstrated through nitrite detection in saliva, a challenging medium due to its variable viscosity. A straightforward strategy is also presented for encapsulating enzymes and other components in trehalose using only a handheld mist sprayer, a non-stick tray, and a spatula, producing bulkier and more stable powders. From these powders, the first tablet-based biosensor with auto-mixing capability is created and successfully applied to glucose detection in real human urine, highlighting its potential for real-world applications.

Chapter 6 provides an overview of the major findings and contributions of this study. It also outlines potential avenues for future research within the fields discussed. Finally, the chapter presents a list of publications, conferences, media interviews, and workshops used to disseminate the knowledge generated from the works related to this thesis, as well as from other academic endeavors undertaken during this PhD program.

Appendix provides a thorough literature review on detection methods for the diagnosis of COVID-19, written during the pandemic and the shutdown of research laboratories. It presents an overview of COVID-19 detection technologies, including molecular methods for viral RNA detection, antigen detection techniques, and serological immunoassays. Each method is briefly explained, with its advantages and disadvantages discussed, and the chapter concludes with suggestions for future research directions.

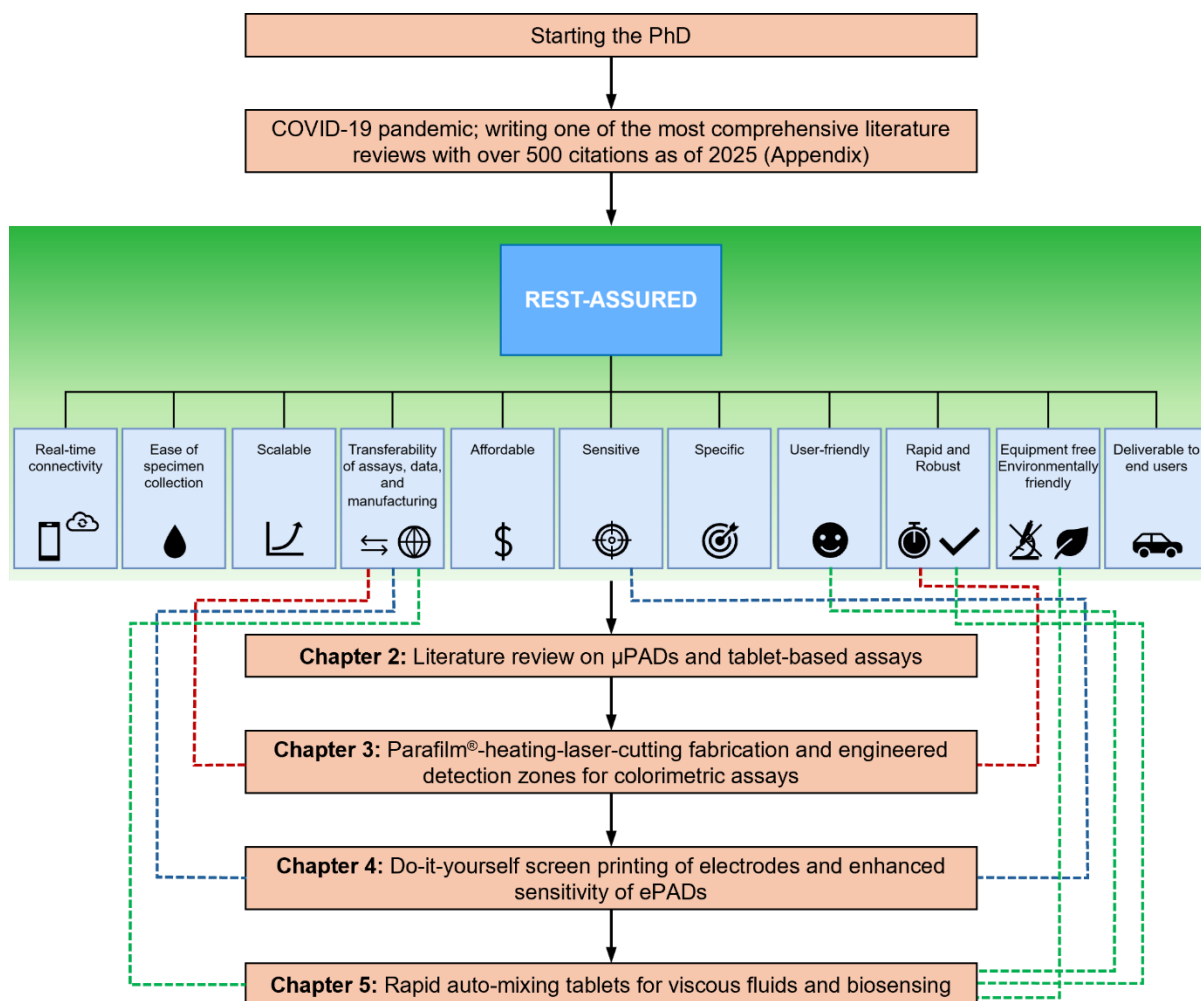
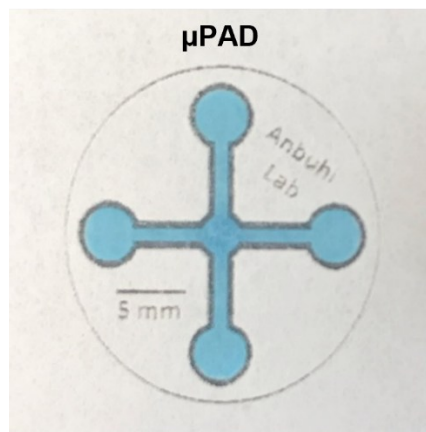


Figure 1.1. Schematic representation of the thesis organization and significance of each chapter with respect to the REST-ASSURED criteria.

Chapter 2

Literature Review of Microfluidic Paper-Based Analytical Devices (μ PADs) and Tablet-Based Assays

This chapter forms the basis of two manuscripts. One has been published in *Clinical Microbiology Reviews* and is presented in full in the Appendix. The other focuses on tablet-based assays and is currently being prepared for submission to a peer-reviewed journal.



2.1. Abstract

The demand for low-cost, portable, and easy-to-use analytical devices is increasing, with applications ranging from diagnostics to food safety and environmental monitoring. This chapter reviews two platforms that exemplify these principles: microfluidic paper-based analytical devices (μ PADs) and tablet-based assays. Harnessing the natural capillarity of paper, μ PADs manipulate fluids without pumps, enabling inexpensive devices that require only small sample volumes and are straightforward to fabricate. Tablet-based assays, on the other hand, simplify reagent handling with pre-measured quantities and improve biomolecule stability, allowing room-temperature storage and more reliable performance. The review discusses the history, fabrication techniques, and detection strategies of μ PADs, as well as the development of tablet-based assays from powder-compressed to polysaccharide-encapsulated formats. Notably, this work presents the first comprehensive literature review dedicated to tablet-based assays. Both platforms are critically assessed with respect to their advantages, limitations, and future prospects.

2.2. Introduction

Millions of people worldwide still lack reliable access to laboratory facilities, creating an urgent demand for analytical tools that are low-cost, portable, and simple to use for point-of-care (POC) and point-of-use (POU) applications. The COVID-19 pandemic further underscored this need, revealing how the unprecedented demand for rapid, large-scale testing strained health systems even in well-resourced regions. In response to this crisis, the author conducted a comprehensive review of COVID-19 diagnostic methods, encompassing molecular, antigen, and serological approaches, while also identifying critical limitations and gaps in conventional testing (Appendix [3]). These limitations included their reliance on centralized infrastructure, expensive equipment, skilled personnel, and time-consuming processes. Insights from this review also highlight general design principles and technological strategies that are also applicable to broader POC diagnostic systems. Together, these challenges have intensified the search for innovative platforms that combine analytical performance with affordability, scalability, and ease of deployment across diverse settings.

Within this landscape, microfluidic paper-based analytical devices (μ PADs) and tablet-based assays are particularly promising. Among them, μ PADs leverage the natural capillarity of paper to manipulate fluids without external pumps or power, operate with minimal sample volumes, and offer a versatile platform that is inexpensive, lightweight, and easily fabricated into complex geometries for analytical applications. Tablet-based assays, on the other hand, simplify reagent handling through pre-measured reagent delivery while also stabilizing sensitive biomolecules in solid form, thereby enabling room-temperature storage and ensuring more consistent assay performance. Together, these two approaches directly address many of the shortcomings highlighted during the pandemic and exemplify the principles of accessibility, simplicity, and robustness in point-of-care (POC) and point-of-use (POU) device design.

This chapter provides a comprehensive review of the state-of-the-art in μ PADs and tablet-based assays. It first introduces the history, fundamental principles, fabrication strategies, and detection methods of μ PADs. It then turns to tablet-based assays, outlining their historical development and recent advancements as platforms for POC/POU applications. Finally, the chapter evaluates the advantages and limitations of each approach and offers remarks on how to best leverage their unique features to maximize their potential.

2.3. Microfluidic Paper-Based Analytical Devices (μ PADs)

In this section, a brief history of μ PADs is first presented, followed by a discussion of the fundamental aspects of μ PADs, including paper characteristics and fluid flow within channels. Various fabrication techniques for creating paper-based channels are then reviewed. Finally, advancements in detection mechanisms are highlighted, with particular emphasis on colorimetric and electrochemical approaches.

2.3.1. A Brief History of μ PADs

The use of paper as a functional material in science has a surprisingly rich and enduring history. Since the invention of modern paper in 105 A.D. in China, it has evolved beyond its original purpose as a writing medium, eventually serving in diverse applications due to its affordability, disposability, and unique capillary wicking ability [28,29]. These features have continued to attract scientific interest, particularly in the development of low-cost analytical devices.

One of the earliest known examples of paper used for chemical analysis is litmus paper, which emerged in 17th and 18th century [30–32]. This simple yet powerful tool, a strip of cellulose paper impregnated with pH-sensitive dyes, enabled the measurement of acidity and alkalinity in samples and places that was not possible before and marked a significant step toward decentralized chemical analysis. In 1861, Schönbein demonstrated that during the capillary rise of an aqueous solution in filter paper, water advances ahead of the dissolved substances, and the relative ascent height varies between compounds. This discovery opened new possibilities for identifying individual components of a salt mixture based on their capillary behavior [33]. By 1930s, various colorimetric spot tests had been developed for the detection of anions and metal ions [33,34].

However, at the time, a major limitation of paper-based analysis was the lack of fluidic control, liquids spread randomly through the fiber network by capillary action, requiring the entire paper area to be chemically treated. This changed in 1937, when Yagoda introduced hydrophobic barriers using paraffin wax to confine spot tests for the detection of nickel and copper ions (Figure 2.1A) [35]. Building on this concept, Müller and Clegg developed the first microfluidic channel in paper in 1949, directing liquid flow along a defined path. They used this device for the chromatographic separation and detection of dye mixtures (Figure 2.1B) [36]. This pioneering work laid the

groundwork for modern paper-based microfluidic devices, as it enabled selective and directional movement of fluids on paper.

Around the same period, in 1941, Martin and Synge introduced partition chromatography [37], which led to the introduction of paper-based partition chromatography in 1944 by Consden et al. for protein analysis [38,39]. These advancements in chromatography awarded Martin and Synge the 1952 Noble prize in chemistry and further solidified the role of paper in analytical chemistry.

By the 1950s, the concept of using paper for diagnostic testing took another leap forward. Comer [40] and Free et al. [41] developed the first dipstick tests to detect urinary glucose in a dip-and-read manner. These devices were also the first paper-based bioassays, incorporating enzymes to detect glucose with high specificity, which were commercialized by Ames Company in 1956 [42]. In parallel, the concept of lateral flow assays (LFAs), also known as lateral flow immunoassays (LFIAs), began to take shape. Between 1956 and 1960, Berson and Yalow introduced radioimmunoassay (RIA) coupled with paper chromato-electrophoresis to detect insulin in plasma [43–45]. This innovation, along with developments in latex agglutination, enzyme immunoassays, and metal particle labelling, culminated in the launch of the first one-step point-of-care LFA by Unipath in 1988 for pregnancy testing [29,46,47]. Since then, LFA technology has been adapted for a wide range of applications, from infectious disease detection (e.g., COVID-19) to cancer biomarkers and foodborne pathogens, making it the most widely used paper-based analytical device [46,48].

Meanwhile, in the 1990s, a new technology emerged that captured the attention of researchers as the next frontier in POU testing: microfluidics and lab-on-a-chip systems. Initially hailed as a potential game-changer, microfluidic systems were compact but typically relied on bulky external equipment such as pumps and valves, limiting their portability and use outside the laboratory. Despite their success in controlled lab environments, e.g., enabling tasks like DNA amplification, cell sorting, and complex chemical analysis, true field-deployable systems remained elusive. This challenge reignited interest in passive, capillary-driven devices [10,49].

A breakthrough came in 2007, when the Whitesides group at Harvard University published a landmark paper demonstrating the fabrication of microfluidic paper-based analytical devices (μ PADs) using patterned photoresist to define hydrophilic channels and colorimetric detection zones (Figure 2.1C) [9]. The outstanding features of this device, requiring no power, minimal

materials, and using paper available in every part of the world, sparked an explosion of research. It allowed chemical and biochemical reactions to be performed affordably and reliably in resource-limited settings, marking a new era for POC/POU technologies. Since then, the field has rapidly expanded to include more complex three-dimensional (3D) architectures, diverse fabrication techniques, and a wide range of detection methods and applications [10,50–53].

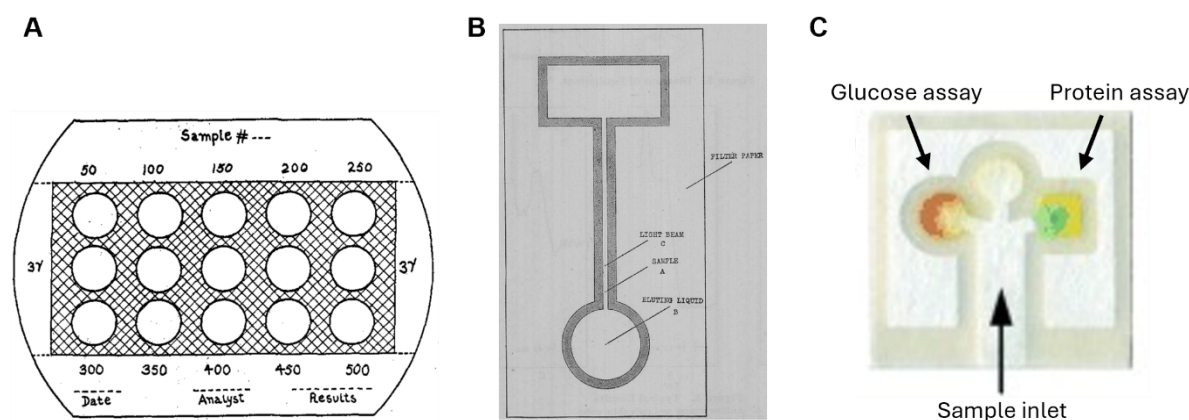


Figure 2.1. Key historical milestones in the development of μ PADs. (A) Schematic of the first report on creating confined spots on paper using paraffin wax to form hydrophobic barriers, published by Yagoda in 1937. Adapted with permission from Ref. [35]; copyright 1937 American Chemical Society. (B) The first paper-based microfluidic channel, reported by Müller and Clegg in 1949. Adapted with permission from Ref. [36]; copyright 1949 American Chemical Society. (C) A multiplex colorimetric test on paper from the landmark 2007 publication by Martinez et al., which catalyzed the modern field of μ PADs. Adapted with permission from Ref. [9]; copyright 2007 Wiley.

2.3.2. Paper and Its Characteristics

Traditionally, paper is defined as a flexible sheet composed of an interlaced network of compressed cellulose fibers. In the context of paper-based microfluidics, however, the definition extends to any porous substrate capable of wicking fluids via capillary action [54]. Selecting a suitable paper for analytical applications requires careful evaluation of its physicochemical properties, including surface chemistry, physical structure, capillary flow rate, substrate uniformity, and optical and electrical characteristics. These parameters have been extensively discussed in the literature [8,29,46,55,56] and are briefly explained below.

Surface chemistry plays a crucial role in determining biomolecule immobilization, non-specific binding, and signal generation in enzyme-catalyzed colorimetric assays [8]. Among the most

widely used porous membranes in paper-based devices are cellulose paper and nitrocellulose membranes. Cellulose-based papers are naturally hydrophilic, composed primarily of cellulose fibers with a high density of hydroxyl ($-OH$) groups and small amounts of carboxylic acid ($-COOH$) groups. Common forms include filter paper and chromatography paper, both frequently used in the fabrication of μ PADs. Nitrocellulose, in contrast, is synthesized by nitrating cellulose, replacing hydroxyl groups with nitrate groups, and casting the resulting polymer into membranes with controlled porosity. These membranes possess high protein-binding affinity and are commonly employed in applications such as LFAs and Western blotting [54]. Figure 2.2 demonstrates the chemical and physical structure of cellulose paper and nitrocellulose membrane.

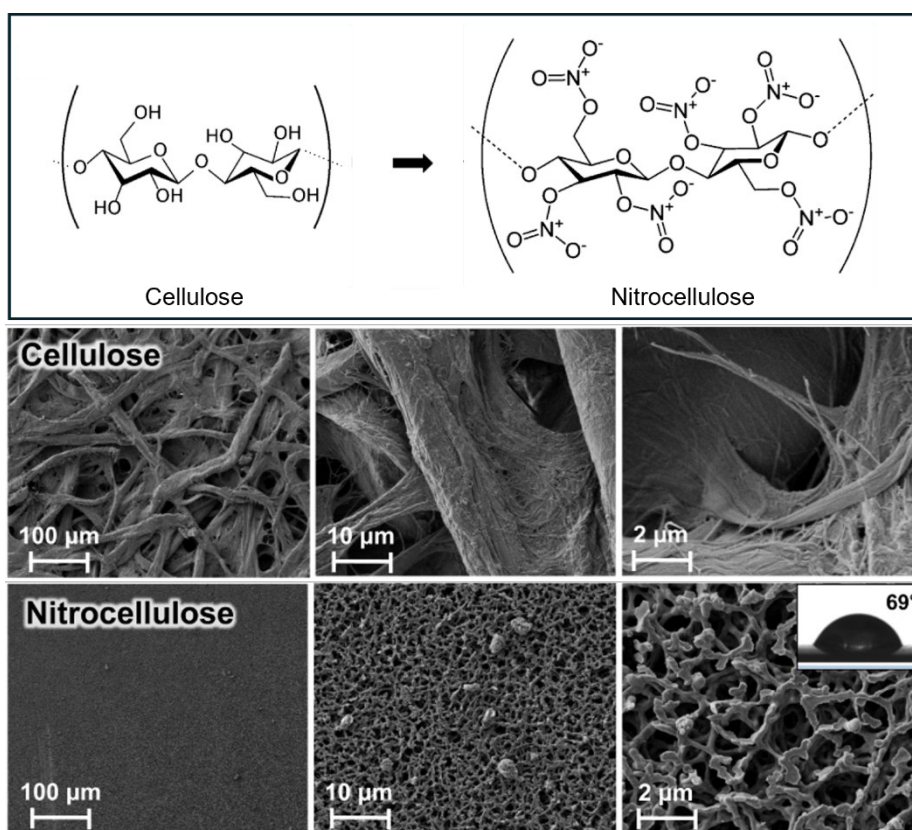


Figure 2.2. Chemical and physical structure of paper. Top row: chemical structures of cellulose and nitrocellulose, showing the nitration of cellulose through esterification with nitrating acid. Adapted with permissions from Ref. [57]; copyright 2013 Royal Society of Chemistry. Middle and bottom rows: scanning electron microscopy (SEM) images of chromatography paper Whatman no. 1 (middle) and nitrocellulose Whatman BA85 Protran (bottom). The inset illustrates the water contact angle on nitrocellulose. Adapted with permission from Ref. [58]; copyright 2014 IOP Science.

Physical properties influence fluid flow behavior, reagent loading capacity, and both the intensity and uniformity of colorimetric signals [8,46,59]. Key parameters include caliper or thickness, τ (m), basis weight, bw (g/m²), which is the mass of dry paper per square meter, porosity, defined as the ratio of the void volume to the total volume, pore size, and particle retention, which is the capacity of paper to retain particles of a certain size [8,56,57,60]. Porosity can be estimated from the thickness and basis weight. Given that the density of solid cellulose fibers is approximately 1540 kg/m³, the bulk density of a specific paper type can be calculated using $\rho_{\text{paper}} = \text{bw} / \tau$. For instance, Whatman No. 1 filter paper has a thickness of 180 μm and a basis weight of 87 g/m², resulting in a calculated bulk density of about 483 kg/m³. Using this value, the porosity (ϕ) is then calculated to be 0.69 using Equation 3.1.

$$\phi_{\text{air}} = 1 - \frac{\rho_{\text{paper}}}{\rho_{\text{fiber}}} \quad (3.1)$$

Moreover, the specific surface area of paper plays a significant role in determining the performance of printed sensors and biosensors, particularly in terms of reagent loading capacity, reactivity, and sensitivity [46]. The reagent loading capacity can be estimated by the product $\Gamma \cdot \sigma$, where Γ represents the maximum density of the immobilized reagent (in mg/m²), and σ is the specific surface area (in m²/g). For example, a biosensor with a low saturation coverage, Γ of 0.1 mg/m² deposited on a low-area paper such as glassine ($\sigma \approx 0.04$ m²/g) would have a loading capacity of just 0.004 mg/g. In contrast, a denser biosensor with a Γ value of 1 mg/m² applied to Whatman No. 1 ($\sigma \approx 9.5$ m²/g) could yield a loading capacity of 9.5 mg/g. Thus, depending on the substrate and the sensor, reagent uptake capacity can span several orders of magnitude [8]. If all other factors are held constant, surface area decreases nonlinearly with increasing pore size, increases linearly with thickness, and increases nonlinearly with porosity [46].

In addition to these properties, capillary flow rate plays a critical role in determining the duration and timing of assays. Capillary flow rate must be considered when designing μPADs , particularly with respect to the location of the sample introduction and detection zones [29]. Additionally, due to the random distribution of cellulose fibers, paper can exhibit variability in fluid transport and other aspects, which may affect assay reproducibility [8]. Therefore, it is essential to evaluate the compatibility of the paper substrate with the specific analytical method, particularly in terms of result reproducibility. Optical properties are also important, especially in colorimetric and fluorescence-based assays. For instance, many commercial papers (e.g., office paper) contain

optical brightening agents that enhance whiteness but can increase background fluorescence, compromising detection performance [8].

The electrical properties of paper are important in the design of ePADs and paper-based electrical sensors [61]. Historically, for over a century, paper has been used as an insulating material in capacitors and cables. Among the various electrical properties of paper, surface and volume resistivity are the most critical, followed by characteristics such as dielectric constant, dielectric loss factor, charging potential and decay rate, and dielectric breakdown strength. These properties are influenced by factors such as the paper substrate and coating, humidity, temperature, electric field, measurement frequency and morphology [55]. Notably, paper is hygroscopic and can absorb up to 10% of its weight in water at 80% relative humidity (RH), which significantly reduces its resistivity [55,61,62]. At 20-40% RH, typical surface and volume resistivities range from 10^{10} - $10^{15} \Omega \cdot \text{sq}^{-1}$ and 10^{10} - $10^{14} \Omega \cdot \text{cm}$, respectively [55,63]. As RH increases from 1% to 99%, cellulose resistivity may drop by a factor of 10^{12} to $10^4 \Omega \cdot \text{cm}$ [55,64]. Resistivity also decreases with temperature; for example, dry cellulose showed a five-order magnitude decrease when heated to 160 °C in vacuum [65]. The dielectric constant of dry paper is typically in the range of 1 to 4, lower than dry cellulose due to paper's porosity and the low dielectric constant of air [55,66,67]. When used as an insulating material, the paper's dielectric constant can be increased by filling pores with high dielectric constant materials such as oils and resins [55,67]. Table 2.1 summarizes some of the commonly used paper types in μ PADs and their characteristics.

Table 2.1. Commonly used papers in μ PADs and their characteristics.

Paper type/Ref.	Composition	Basis weight (g/m ²)	Typical thickness (μ m)	Particle retention/pore size (μ m) ^a	Wicking speed ^b	Filtration speed (s/100 mL)	Example applications in μ PADs
Filter paper grade 1 [59,68]	Cellulose (>98%)	88	180	11	NA	150	[59,69]
Filter paper grade 2 [68]	Cellulose (>98%)	103	190	8	NA	NA	[11,70]
Filter paper grade 3 [59,68]	Cellulose (>98%)	187	390	6	NA	2	[59,70]
Filter paper grade 4 [59,68]	Cellulose (>98%)	96	205	20-25	NA	37	[11,59]
Chromatography paper grade 1 [59,71]	Cellulose (pure)	87	180	11	130 (mm/30 min)	NA	[59,72]
Chromatography paper grade 2 [71]	Cellulose (pure)	NA	180	NA	115 (mm/30 min)	NA	[73,74]
Chromatography paper grade 3MM [59,71]	Cellulose (pure)	189	340	6	130 (mm/30 min)	NA	[59,75]
Chromatography paper grade 4 [59]	Cellulose (pure)	93	210	20	180 (mm/30 min)	NA	[11,59]
Nitrocellulose membrane (UniSart CN140) [76,77]	Cellulose nitrate	NA	120-160	8	90-150 (s/40 mm)	NA	[78,79]
Nitrocellulose membrane (Protran BA85) [80]	Cellulose nitrate (pure)	NA	NA	0.45	NA	NA	[81,82]

^a For cellulose papers, the values correspond to particle retention, whereas for nitrocellulose membranes they correspond to nominal pore size, as reported in the references.

^b Dimensions are reported in their original format as provided in the sources.

Paper possesses numerous advantages that make it ideal for POC/POU testing. It is low cost (~ 0.1 cent/dm²), widely available, and compatible with many printing and processing techniques. Paper is easy to store, stack, and transport, and is available in various thicknesses and porosities. Paper, and cellulose in particular, is protein and biomolecule friendly, and its surface is rich in organic functional groups that can be chemically modified to covalently or non-covalently bind analytes and reagents. Paper has a high surface-to-volume ratio that allows for efficient drying and storage of reagents and samples. These materials can be applied either manually or with automated high-speed printing systems. The white background of paper is well-suited for visual readouts in colorimetric assays, while its inherent chromatographic and filtration properties allow for the separation of analytes in complex biological samples such as blood or urine. Also, paper is biodegradable and can be easily burned. Most importantly, paper wicks fluids passively via capillary action, eliminating the need for pumps or external power sources and making it highly suitable for low-resource settings [8,52,54,55].

2.3.3. Fluid Flow in Paper Channels

One of the key advantages of paper in microfluidic applications is its ability to support passive capillary-driven flow, eliminating the need for external pumps or power sources. This property enables the autonomous transport of fluids through the fibrous structure of paper, making it especially attractive for low-resource settings.

The capillary flow rate refers to the speed at which a liquid travels through the length of a paper strip [46]. Accurate modeling of fluid behavior in paper-based systems is challenging due to various influential parameters. Despite that, the Lucas-Washburn equation is widely used as a theoretical foundation for understanding capillary flow in porous media such as paper. This equation describes how a liquid penetrates a porous structure under capillary action, based on a set of simplifying assumptions: 1) the liquid is Newtonian and incompressible, 2) the porous medium is idealized as an array of parallel cylindrical capillaries, 3) the flow is steady-state, laminar, and obeys the no-slip condition, 4) flow is one-dimensional within straight capillaries, 5) interconnectivity of the pores is not considered, 6) static contact angle and constant pore radius are assumed, 7) tortuosity is neglected; the actual convoluted pore path is replaced by a straight-line approximation, 8) capillary pressure is calculated using the Young-Laplace equation, which may introduce error at very small pore sizes [83,84].

Under these assumptions, the fluid flow follows the Hagen-Poiseuille law expressed in Equation 3.2:

$$\frac{dV}{dt} = \frac{\pi r^4 \Delta P}{8\mu l} \quad (3.2)$$

Where V is the volume of liquid, r is the effective pore radius within the paper matrix, μ is the dynamic viscosity of the liquid, and l is the distance traveled by the fluid. The volume V can be related to l using Equation 3.3. The pressure difference, ΔP , represents the net driving force and is defined as the difference between the capillary pressure (as described by the Young-Laplace equation) and the hydrostatic pressure, as given in Equation 3.4:

$$dV = \pi r^2 dl \quad (3.3)$$

$$\Delta P = \frac{2\gamma \cos \theta}{r} - \rho gh \quad (3.4)$$

In this equation, γ represents the surface tension of the liquid, θ is the liquid contact angle, ρ is the liquid density, and h is the height of the liquid. Assuming that the liquid is travelling in a horizontal direction, the hydrostatic pressure can be neglected. By substituting Equations 3.3 and 3.4 into Equation 3.2, equations 3.5 and 3.6 are obtained:

$$\frac{dl}{dt} = \frac{r^2}{8\mu l} \left(\frac{2\gamma \cos \theta}{r} \right) \quad (3.5)$$

$$l \frac{dl}{dt} = \frac{r\gamma \cos \theta}{4\mu} \quad (3.6)$$

Integrating Equation 3.6 with the initial condition $t = 0, l = 0$ gives the famous Lucas-Washburn equation (Equation 3.7):

$$l^2 = \frac{r\gamma \cos \theta}{2\mu} t \quad (3.7)$$

This equation demonstrates that the distance traveled by the liquid is proportional to the square root of time. As previously noted, it relies on several simplifying assumptions. However, it does not account for many other influential factors, such as paper permeability, tortuosity, flow orientation (horizontal vs. vertical), interactions with channel walls, ambient humidity and evaporation rate, channel geometry, and lamination effects [29,46,85]. Figure 2.3 illustrates a

typical fluid flow in paper-based microfluidic channels as well as its dependence on channel width and type of hydrophobic barrier. For common Whatman filter and chromatography (CHR) papers, the flow speed generally follows the order (from highest to lowest): Filter Grade 4 > Filter Grade 1 > CHR Grade 4 > Filter Grade 3 > CHR Grade 1 > CHR Grade 3MM [59]. It is also noteworthy that numerous research groups have proposed modifications to the Lucas-Washburn equation to account for more complex conditions, as extensively discussed in the literature [29,46,83].

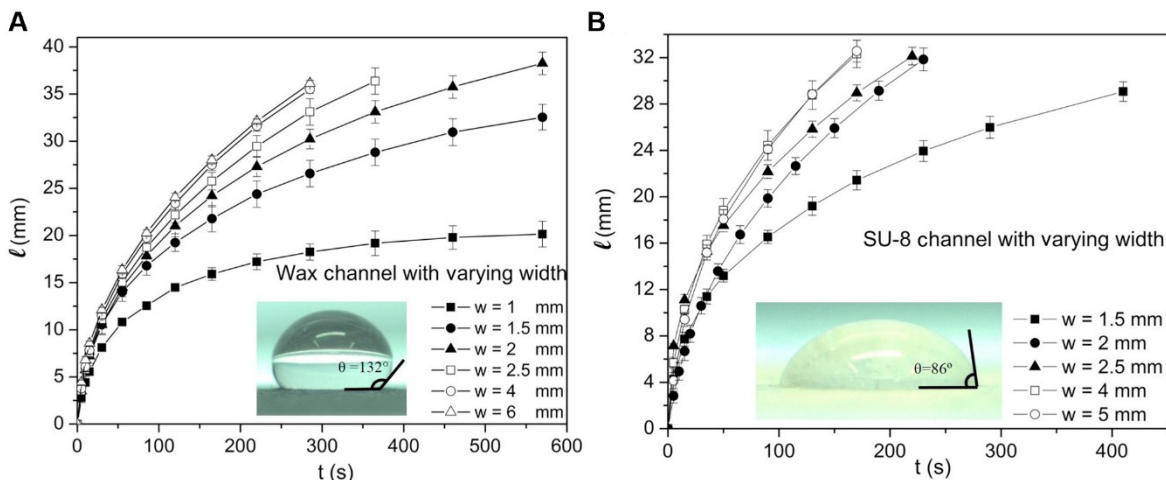


Figure 2.3. Fluid flow in paper-based channels. (A) Traveled length (l) vs time (t) for water flow in channels created with wax barrier (1-6 mm channel width); inset shows a water droplet on wax. (B) Water flow in channels formed with SU-8 barriers (1.5-5 mm channel width); inset shows a water droplet on SU-8. Adapted with permission from Ref. [85]; copyright 2016 Elsevier.

2.3.4. Fabrication

Once an appropriate paper substrate has been selected, the next critical step in developing μ PADs is the fabrication of the device. Fabrication involves several key aspects depending on the intended application, including the creation of microfluidic channels, device configuration (2D vs. 3D), lamination, fluid manipulation elements (e.g., valves and mixers), and reagent deposition [10,52,54,86,87]. This section focuses specifically on the methods used to create microfluidic channels within paper substrates.

The design of microfluidic channels typically begins with computer-generated patterns. These patterns can be created using commercial software such as Microsoft PowerPoint, AutoCAD, Adobe Illustrator, CorelDraw, or open-source software such as Inkscape. These designs define the hydrophilic pathways and channel walls (i.e., barriers) that direct fluid flow in a controlled manner.

The effectiveness of a channel fabrication technique is evaluated based on several criteria: channel and barrier resolution, fabrication speed, ease of prototyping, operational simplicity, accessibility of equipment and materials, compatibility with multilayered or 3D configurations, and chemical resistance of the fabricated barriers.

To define fluid pathways, two main strategies are used: (1) creating selective hydrophobic regions to form hydrophilic channels, and (2) physically cutting or etching the paper to shape the fluidic network. To change wettability a variety of techniques have been employed, including printing, drawing, masking, laser-induced chemical modification, and stamping and embossing. Alternatively, physical methods involve cutting/etching the paper using tools such as CO₂ laser cutters, knife plotters, or even scissors. The following sections provide a detailed discussion of some common fabrication methods, highlighting their principles, advantages, and limitations for μ PAD development.

Printing techniques:

Wax printing, introduced by the Whitesides group in 2009, quickly became the most popular fabrication method due to its simplicity and accessibility [14]. It uses Xerox solid ink printers to deposit molten wax onto paper. The process involves three basic steps: (1) designing the microfluidic layout, (2) printing the pattern onto paper, and (3) heating the printed paper on a hotplate or in an oven at 150 °C for 2 minutes. The heat melts the wax, allowing it to penetrate both laterally and vertically into the paper matrix (Figure 2.4A). Upon cooling, the wax solidifies to form hydrophobic barriers. Variations in temperature and heating duration have also been reported [88]. The method enables rapid prototyping of μ PADs, supports both 2D and 3D configurations, and achieves barrier and channel resolutions of $850 \pm 50 \mu\text{m}$ and $561 \pm 45 \mu\text{m}$, respectively, on chromatography paper [14]. Higher resolution can be achieved using nitrocellulose membrane ($60 \mu\text{m}$ for barrier and $100 \mu\text{m}$ for channel) [82], or through techniques such as double-sided printing [89], sodium periodate treatment [90], or pressure-assisted printing [91], though these introduce added complexity and equipment needs. Wax printing also enables creating fluid manipulation features such as valving [92–96] as well as anti-leakage structures [97]. However, wax barriers are only compatible with aqueous solutions and cannot withstand organic solvents. This can be mitigated by incorporating pullulan between two wax layers, but it lowers

resolution and adds steps [98]. Additional drawbacks include the risk of nozzle clogging and the discontinuation of Xerox solid ink printers in 2017, limiting equipment availability [10].

As an alternative to wax printing, thermal transfer printing was introduced by the Martinez group in 2022 as a newer and more accessible approach for μ PAD fabrication [72]. This method employs solid ink ribbons that melt onto the surface of the paper during printing. The fabrication process is similar to that of wax printing, consisting of two major steps: (1) printing the desired pattern and (2) heating the paper at 90 °C for 15 minutes to enable the hydrophobic material to reflow into the paper matrix. The technique achieves a minimum barrier width of $749 \pm 31 \mu\text{m}$ and a channel width of $627 \pm 2 \mu\text{m}$. A key advantage of thermal transfer printing is that the printers are more affordable and portable compared to wax printers. However, like wax-based barriers, those formed using this method are only effective with aqueous solutions and are not compatible with organic solvents.

Another approach for μ PAD fabrication is laser printing, which uses standard office laser printers [72,99–101]. As with wax and thermal transfer methods, the process involves printing the channel pattern onto paper and heating it to melt the toner, forming hydrophobic barriers. This technique achieves minimum functional barrier and channel widths of $\sim 400 \mu\text{m}$ and $411 \pm 29 \mu\text{m}$, respectively, as reported in different studies [100,101]. The barriers effectively contain aqueous solutions and can resist certain organic solvents, such as methanol, depending on the toner type [100]. Laser printers are widely available and less expensive than wax printers, making this method accessible. However, it typically requires higher temperatures and longer heating times, increasing the risk of paper pyrolysis. Although vacuum ovens can reduce this issue, they add equipment requirements. A key limitation of this technique is that barrier effectiveness varies significantly with printer and toner type, and not all combinations are compatible. Given the short lifecycle of many consumer laser printers, researchers often need to experiment with different models to identify suitable setups, which reduces the method's reliability [72].

Various 3D printing techniques have been used to create μ PADs [102]. The simplicity of the process and the characteristics of the resulting channels depend on the type of ink and 3D printing technology employed. The simplest approach involves fused deposition modeling (FDM) 3D printing of commercially available wax, polycaprolactone (PCL), or polylactic acid (PLA) filaments onto paper in the desired pattern, followed by heating to form hydrophobic barriers [103–105]. This method is straightforward and offers the advantage of being compatible with any type

of paper or substrate, unlike some other printing methods that are limited to specific paper types. Moreover, low-cost 3D printers are now widely available (under USD 300), making this technique accessible. However, the resolution is limited, with minimum reported barrier and channel widths of 0.4 mm and 2 mm, respectively [104,105], although finer features can be achieved using more complex approaches [106,107]. Careful selection of the filament is important, as some require higher temperatures and longer heating times, which may cause pyrolysis of the paper.

Inkjet printing is another method used for fabricating μ PADs, employing both specialized and standard desktop inkjet printers [108]. It typically requires custom-made inks formulated with suitable solvents and viscosities, and modifying printer cartridges to accommodate these inks. Two main approaches exist: (1) inkjet etching, where the paper is first rendered hydrophobic, e.g., with polystyrene in toluene, and then selectively etched by inkjet-printing solvents like toluene to form hydrophilic channels [109]; and (2) direct printing of hydrophobic materials, often followed by post-treatment [108,110–112]. Materials such as UV-curable inks (Figure 2.4B) [111], hydrophobic sol-gels [113], permanent marker ink [112], and alkyl ketene dimer (AKD) [110] have been used, with AKD favored for its low cost and commercial availability [10]. The technique allows for the barrier and channel resolutions of 425 ± 26 and 272 ± 19 μm , respectively [111], and depending on the ink, barriers can be resistant to various organic solvents, e.g., in the case of sol-gel inks [113]. A key advantage is the dual capability of printing both barriers and reagents in one step, enabling fully printed μ PADs. Reagents such as enzymes, chromogenic agents, and carbon dots have been successfully printed using this method [108,114]. However, printer type must be considered, as both thermal and piezoelectric inkjet printers can damage sensitive reagents [108]. While promising, inkjet printing is more complex than wax or laser printing due to the need for printer modification and custom ink formulation.

In addition to the methods discussed above, other printing techniques such as screen printing [115–118] and flexographic printing [119] have been reported, but they are less commonly used as they are more complex and labor-intensive for lab-scale fabrication and rapid prototyping.

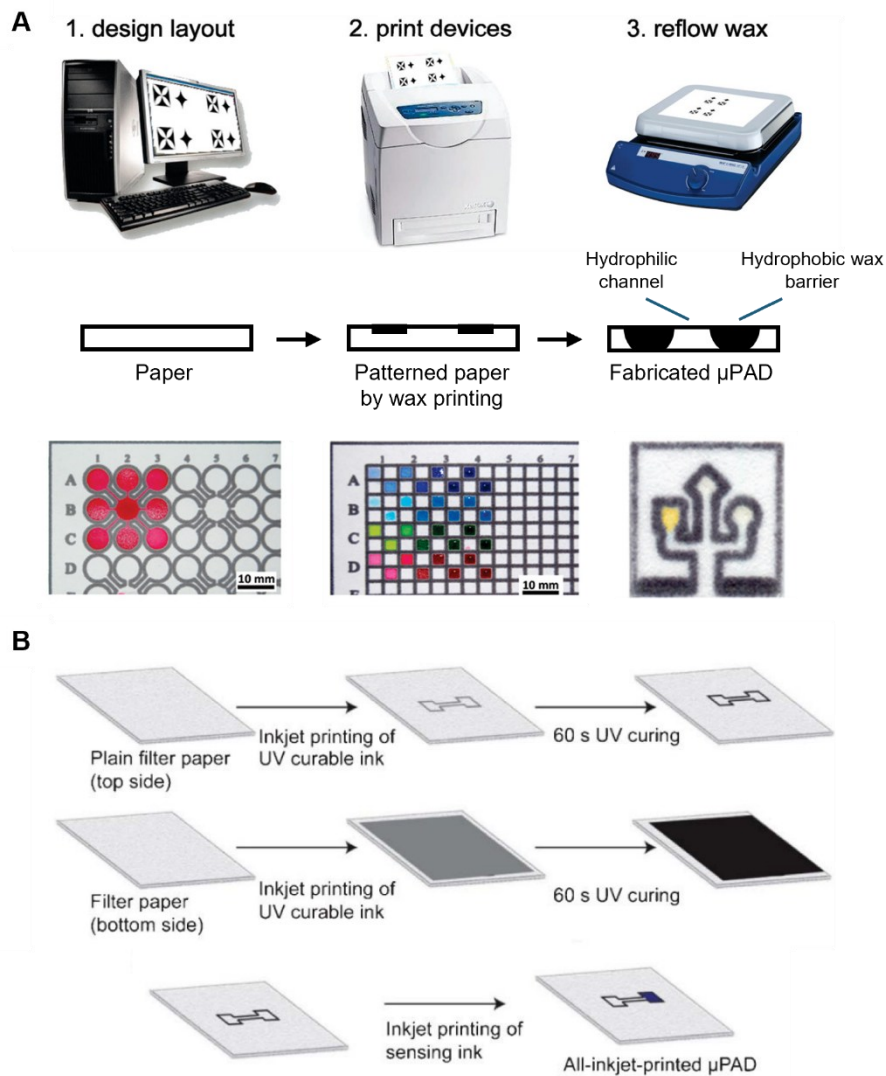


Figure 2.4. Examples of printing techniques for μ PAD fabrication. (A) Wax printing with representative devices, including a 96-well plate (left), a 384-well plate (middle), and a typical μ PAD device (right). Adapted with permission from Ref. [14]; copyright 2009 American Chemical Society. (B) All-inkjet printing of μ PADs and sensing reagents using UV-curable ink. Adapted with permission from Ref. [111]; copyright 2013 Royal Society of Chemistry.

Drawing-based techniques:

Drawing-based methods enable the simple fabrication of μ PADs by using common drawing tools like pens, markers, or crayons to apply hydrophobic materials onto paper. In its most basic form, this method enables single-step fabrication using commercially available items like permanent markers (Figure 2.5) [11,120–122] or correction pens [123]. Other approaches involve custom inks [124–127] or additional processing steps; for example, wax crayons require heating to reflow into

the paper [128], while 3D printing pens [129] and acrylate-based pens [130] may involve UV curing. The success of barrier formation depends on the pressure and speed applied during drawing, which influence the penetration of hydrophobic material into the paper matrix [54,121]. Depending on the hydrophobic material used, the resulting barriers can resist various organic solvents as well as acidic or basic solutions [123,129,130]. However, the resolution of drawing-based techniques is generally limited (barrier > 900 μm ; channel > 400 μm), making them less suitable for complex microfluidic designs [123,127,129,130]. Improved resolutions, down to 280 μm for barriers and $150 \pm 12.9 \mu\text{m}$ for channels, have been achieved by reducing paper thickness and using precision tools such as technical pens [124]. The drawing-based methods are particularly useful for low-cost, equipment-free rapid prototyping and educational demonstrations. For greater reproducibility and throughput, XY plotters can automate the drawing process [120–122,124,127,131].

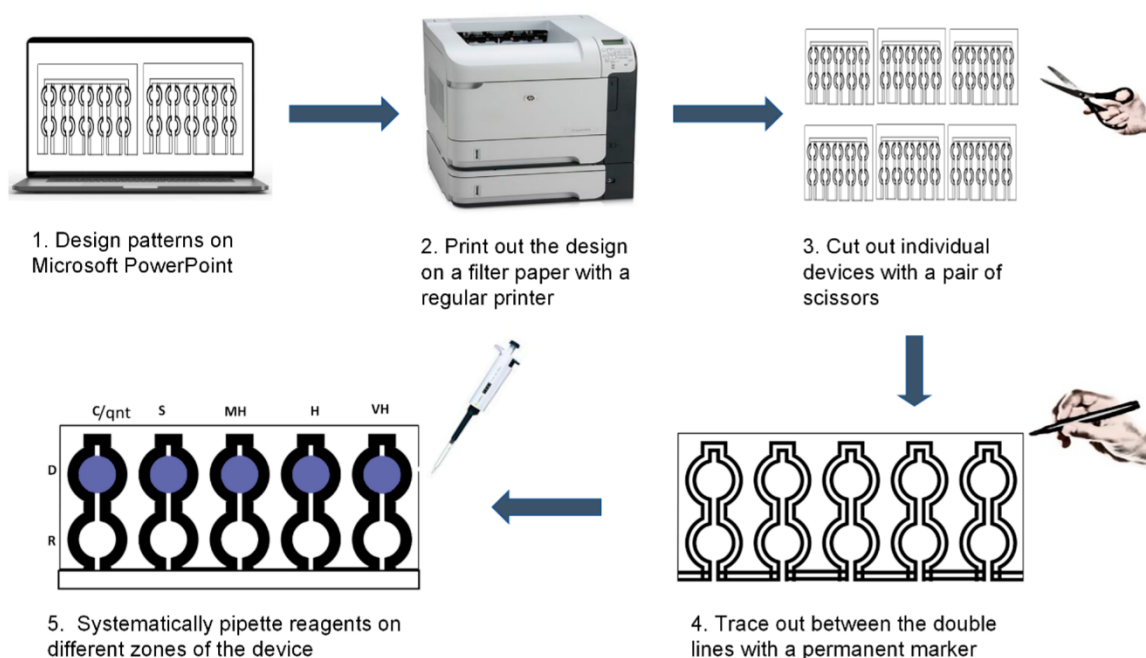


Figure 2.5. Example of μPAD fabrication by drawing with a permanent marker. Adapted with modifications from Ref. [11] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>).

Masking techniques:

Photolithography was the first technique used to fabricate μ PADs in 2007, given its established role in conventional PDMS-based microfluidics [9,54]. This method involves impregnating the paper with a photoresist, exposing it to UV light through a photomask to crosslink the desired regions, and then removing the unexposed areas with a solvent to define hydrophobic barriers (Figure 2.6A) [9,54,132,133]. A key advantage of photolithography is its high resolution, with reported channel widths as narrow as $180 \pm 20 \mu\text{m}$ and barriers as thin as $186 \pm 13 \mu\text{m}$ in different studies [132,133]. While early implementations were limited by the cost of commercial photoresists, the need for high-intensity UV sources, and the use of organic solvents, advancements have led to the development of low-cost and water-based photoresists that can be processed using inexpensive UV lamps or even sunlight [132,133]. Moreover, barriers formed via photolithography exhibit strong resistance to various organic solvents, including methanol and dimethylformamide (DMF). Despite these strengths, the method is more labor-intensive than printing techniques and is generally favored for applications requiring high-resolution features and complex microfluidic geometries.

Other mask-based fabrication techniques include plasma treatment and UV/O₃ treatment. In the plasma treatment, the entire paper is first rendered hydrophobic using chemicals such as AKD or octadecyltrichlorosilane (OTS). A physical mask is then placed over the paper, and plasma exposure, typically using a vacuum plasma device or a handheld corona generator, is applied to selectively convert the exposed areas back to hydrophilic (Figure 2.6B) [134–136]. To ensure precise patterning, proper adhesion of the mask to the paper is critical; otherwise, plasma gas can penetrate beneath the mask, leading to poorly defined or distorted channels [134]. One advantage of plasma treatment is that it enables the integration of functional units, such as switches, filters, and separators, into μ PADs [135]. However, the technique is generally labor-intensive, offers limited resolution (with minimum channel widths of around 1 mm), and is not suitable for generating arrays of isolated hydrophobic zones, thereby restricting the complexity of μ PAD designs [54]. Similarly, UV/O₃ treatment involves hydrophobizing the paper with OTS, followed by UV exposure through a quartz mask to define hydrophilic channels [137]. Although this method achieves high resolutions, $233 \pm 30 \mu\text{m}$ for channels and $137 \pm 21 \mu\text{m}$ for barriers, it remains time-consuming and labor-intensive.

Additionally, vapor phase polymerization [138–140] and chemical vapor deposition (CVD, Figure 2.6C) [141] techniques have been explored for fabrication. These methods involve vaporizing chemicals such as trichlorosilane (TCS) and depositing them into the paper matrix through a mask to modify surface wettability. An advantage is the ability to create UV-responsive polymers that enable switchable wettability for valving applications [139]. However, these techniques typically offer poor resolution and require specialized equipment such as vacuum chambers, limiting their accessibility.

Spraying is another technique used to fabricate μ PADs through masking strategies [142–145]. In its simplest form, a mask is placed over the paper, and a hydrophobic spray is applied to render the exposed areas hydrophobic [143]. Commercially available sprays, such as water-repellents [142,143], water wax [142], or spray paint [144,145] can serve as the hydrophobizing agents. Masks can be easily prepared using craft cutters on paperboard [143], laser cutting [145], or even laser printing toner on paper surface (Figure 2.6D) [142]. The success of this method depends heavily on the spraying time, angle, and distance from the paper. Proper bonding between the mask and the paper is crucial to prevent the spray from seeping underneath; this can be achieved by clamping the layers between a metal plate and a magnet [143]. This approach offers a low-cost, simple, and rapid way to prototype μ PADs. However, it suffers from key limitations such as uneven distribution of the hydrophobic material within the paper matrix, which leads to rough boundaries [142,143]. As a result, spraying is not suitable for applications that require high resolution or precisely defined microfluidic channels.

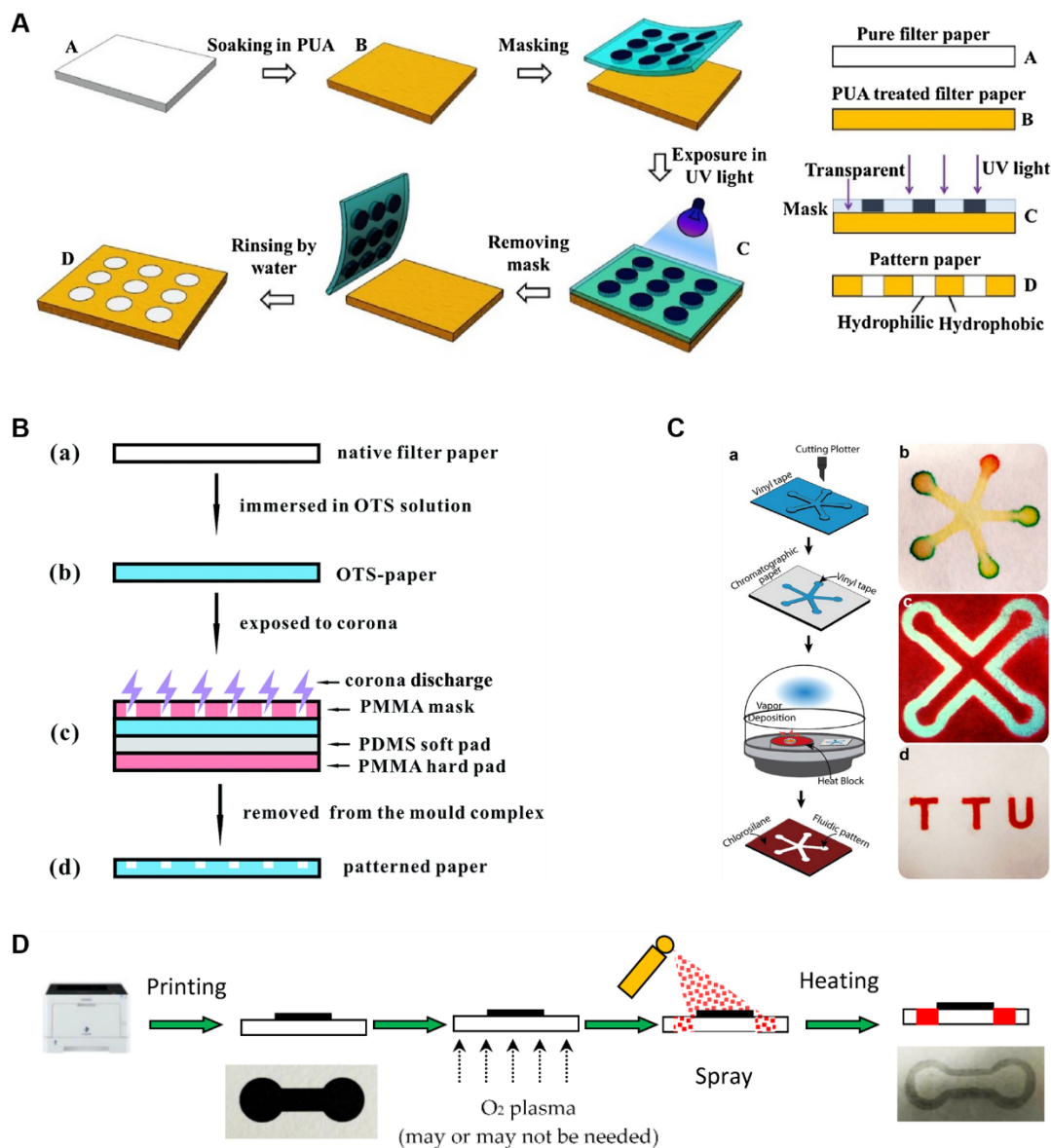


Figure 2.6. Example of masking techniques for μ PAD fabrication. (A) Photolithography using polyurethane acrylate (PUA) as a water-based photoresist. Adapted with permission from Ref. [133]; copyright 2020 Elsevier. (B) Fabrication by plasma treatment. Adapted with permission from Ref. [136]; copyright 2016 Royal Society of Chemistry. (C) Fabrication by chemical vapor deposition. Adapted from Ref. [141] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>). (D) Fabrication by spraying in combination with toner laser-printed masks. Adapted from Ref. [142] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>).

Laser-assisted chemical modification:

Some research groups have explored combining laser treatment with chemical modification to create hydrophilic channels. In one example, hydrophobic papers (e.g., parchment paper) were selectively treated using a CO₂ laser engraver. This process altered both the surface topography and chemical composition of the irradiated regions, significantly reducing the water contact angle and rendering the treated areas hydrophilic. These modified zones could effectively retain aqueous solutions on their surface, achieving high resolution with hydrophilic lines as narrow as $62 \pm 1 \mu\text{m}$ and separation gaps as small as $80 \mu\text{m}$. However, a key limitation of this approach is that while the treated areas can hold water, they do not support capillary-driven flow, which is essential for microfluidic operation. To enable fluid transport, an additional step involving treatment with silica particles was required to enhance capillarity [146]. Moreover, this method is only applicable to inherently hydrophobic papers and is ineffective on commonly used papers like filter or chromatography paper unless they are pre-modified to become hydrophobic.

In another approach, laser-induced photopolymerization was employed to create hydrophobic barriers on nitrocellulose membranes [147]. The process begins with pre-soaking the membrane in a light-sensitive photopolymer (DeSolute), followed by selective exposure to a 405 nm continuous-wave (c.w.) diode laser to cross-link the polymer in the desired regions. After exposure, the membrane is washed with toluene to remove the unpolymerized material (Figure 2.7A). This technique achieved remarkably high resolution, approximately $60 \mu\text{m}$ for barriers and $100 \mu\text{m}$ for channels on nitrocellulose membrane. Despite its high resolution, the method is limited by the need for specialized laser equipment and photopolymers, as well as a relatively labor-intensive workflow compared to printing-based techniques.

Additionally, laser heating of wax on paper can be used to create hydrophobic barriers [148,149]. In this method, a layer of wax is applied to the paper and selectively heated using laser irradiation, causing the wax to melt and penetrate into the paper structure, thereby forming the barriers (Figure 2.7B). This technique achieves a minimum barrier width of 1 mm and channel width of 0.3 mm in filter paper [149]. While the wax layer can be deposited using a wax printer, the discontinuation of such printers makes it challenging to produce a uniformly thick wax layer, which can complicate the fabrication process.

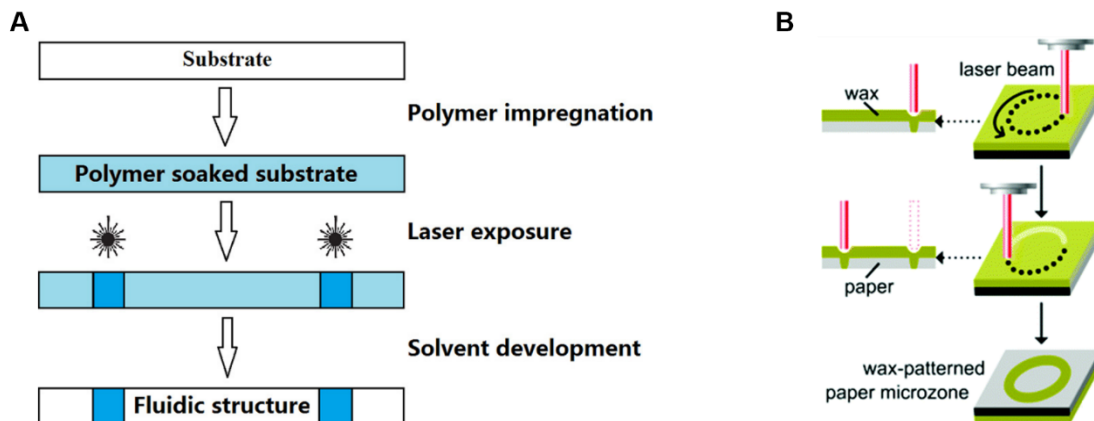


Figure 2.7. Example of μ PAD fabrication by laser-assisted-chemical modification. (A) Light-induced photo-polymerization using a 405nm continuous wave diode-laser. Adapted with permission from Ref. [147]; copyright 2015 AIP Publishing. (B) CO₂ laser-assisted reflow of wax in paper to create hydrophobic barriers. Adapted with permission from Ref. [148]; copyright 2017 Royal Society of Chemistry.

Stamping and embossing:

Various stamping methods have been developed for fabricating μ PADs, differing mainly in the stamp fabrication technique and the hydrophobic ink used. Depending on the ink, patterning can be achieved either in a single stamping step (Figure 2.8A) [150–153] or may require pre-heating the stamp or post-curing the paper [154–156]. Some approaches utilize commercially available hydrophobic inks [150,152,153], while others formulate custom inks such as PDMS-based solutions or molten paraffin [154–158]. Stamps are either outsourced [152,155] or fabricated in-house using methods like laser cutting [154,157], using flash stamp machine [151], photopolymerization [156], or 3D printing [153]. Reproducible patterning depends on applying appropriate pressure and contact time during stamping [155]. While the resolution is typically poor (barrier and channel widths $>700\ \mu\text{m}$), studies using laser-cut atom stamps have achieved resolutions as fine as $312\ \mu\text{m}$ for barriers and $328\ \mu\text{m}$ for channels [157]. Depending on the ink, the printed barriers can also withstand organic solvents, as seen with PDMS-based inks [156]. While the technique offers a low-cost and rapid option for device fabrication using pre-made stamps, it is limited by the need to fabricate a new stamp for each design and generally low resolution [54].

Another related method is embossing, which uses negative stamps or molds to imprint patterns onto paper (Figure 2.8B) [159,160]. However, this approach is more labor-intensive and complex than the previously discussed techniques.

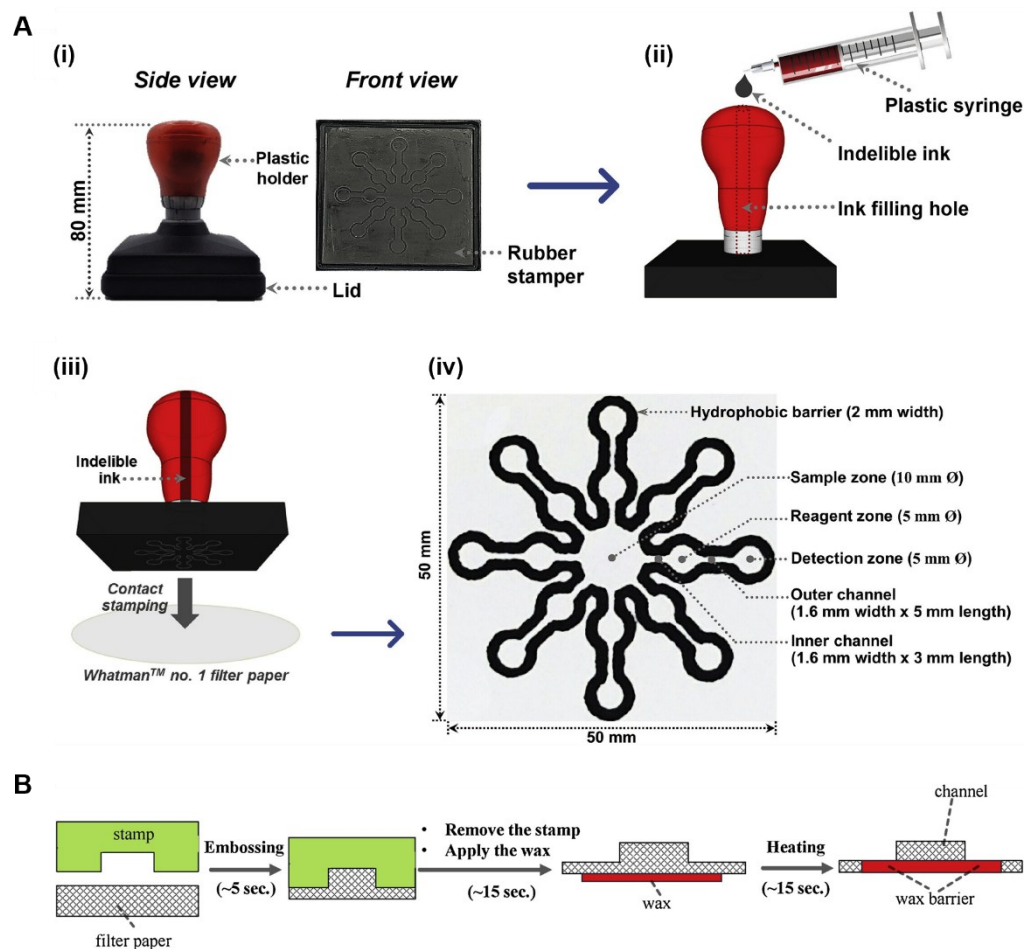


Figure 2.8. Examples of stamping and embossing methods for μ PAD fabrication. (A) Stamping with a commercial hydrophobic ink. Adapted with permission from Ref. [152]; copyright 2020 Elsevier. (B) Microembossing for μ PAD fabrication. Adapted with permission from Ref. [159]; copyright 2019 Elsevier.

Physical methods:

Physical methods for fabricating μ PADs rely on cutting/etching the paper to define fluidic paths. This can be achieved using tools such as knife/craft cutters [161–164], CO₂ laser cutters [59,81,165–172], femtosecond lasers [78,79], or even simple scissors or razor blades for basic designs [54,173]. Although patterned or cut paper can be used on its own (Figure 2.9A) [59,171,172], these devices are typically laminated to provide mechanical strength and improve handling [54].

Among cutting techniques, CO₂ laser cutting/etching is particularly attractive due to its high resolution, precise control, and reproducibility, especially as low-cost CO₂ laser cutters are now accessible for under USD 1000. Various laser-cutting/etching strategies have been proposed, differing primarily in the lamination materials and equipment used. Commercially backed nitrocellulose membranes can be employed [165,170], as well as custom lamination approaches such as aluminum and tape with a cold roller [81], Parafilm with a hot press machine [167] or thermal lamination [169], allowing adaptation based on available laboratory resources. In basic approaches, the paper is laminated on one side and placed in a laser cutter, where the barriers are cut through the paper while the backing lamination remains intact (Figure 2.9B). This technique enables the creation of barriers and channels as narrow as $39 \pm 15 \mu\text{m}$ and $128 \pm 30 \mu\text{m}$, respectively, in chromatography paper, and $24 \pm 3 \mu\text{m}$ channels in nitrocellulose membrane, the smallest features reported in the reviewed literature [81,166]. Depending on the lamination material, these devices can also resist organic solvents [167]. Additionally, laser etching enables the direct integration of fluid control elements, such as channel extenders, micro-cracks, and grooves, to accelerate or delay flow for sequential reagent delivery [170,174–178]. Other advantages include ease of prototyping and improved protection from contamination, along with more consistent fluid flow due to the lamination layer. However, a key limitation is that this approach is primarily suited for 2D device architectures due to the lamination layer and is less compatible with 3D μ PADs, although a recent study has demonstrated 3D configurations through careful layer engineering [169].

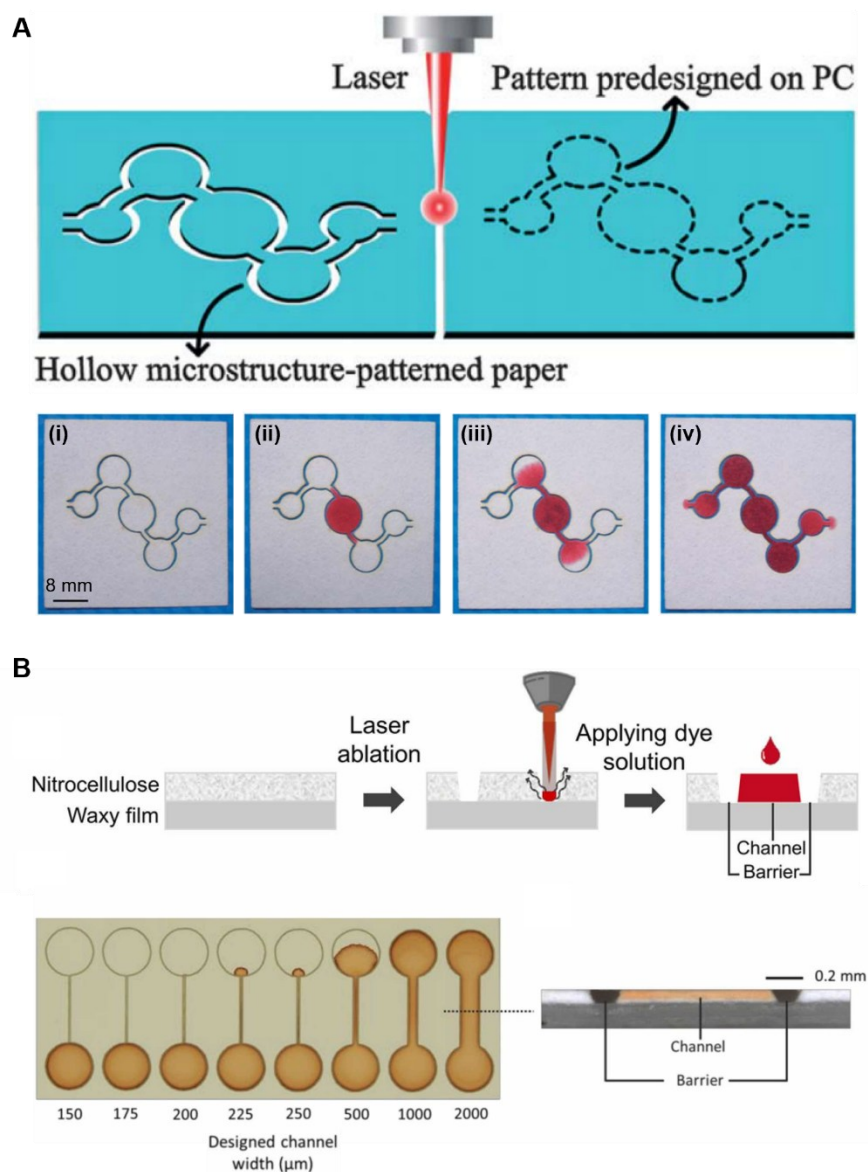


Figure 2.9. Examples of physical methods for μPAD fabrication. (A) Direct CO_2 laser cutting to create hollow patterns on paper and define hydrophilic channels. Adapted with permission from Ref. [171]; copyright 2013 Royal Society of Chemistry. (B) CO_2 laser etching on laminated paper. Adapted with permission from Ref. [169]; copyright 2023 Elsevier.

In summary, numerous methods are available for fabricating μPADs , and selecting the most suitable approach depends on the intended application and the available resources. Preferably, the chosen method should enable automated patterning from computer-designed layouts, rapid prototyping and fabrication, and high resolution. Printing and laser-etching techniques are particularly well-suited to these requirements. Further improvements in these techniques, particularly in terms of simplicity, accessibility, resolution, and eco-friendliness would be highly

beneficial. Drawing techniques are also effective for rapid prototyping and testing design concepts. Although a channel width of 1-5 mm is sufficient for most μ PAD applications [72], higher resolution offers several advantages: enabling the creation of more complex geometries and functional units (e.g., fluid control), facilitating the fabrication of smaller devices that consume less material and reagent (thereby reducing cost), allowing for smaller sample volumes in applications such as finger-prick testing, and supporting multiplex detection within a compact footprint [81]. Table 2.2 summarizes features of selected common fabrication methods studied in the literature.

Table 2.2. Selective summary of fabrication methods studied in the literature.

Method	Equipment	barrier/channel width (μ m)	Advantage	Disadvantage
<i>Printing</i>				
Wax printing [14,82,88]	Solid ink printer, oven or hot plate	850/561 (CH) 60/100 (NC)	Simple, rapid prototyping and fabrication, high throughput, allows integration of fluid control units	Discontinued printer, only works with aqueous solutions, not suitable for high-resolution applications
Thermal transfer printing [72]	Thermal transfer printer, oven or hot plate	749/627 (CH)	Low-cost and portable equipment, simple, rapid prototyping and fabrication	Only works with aqueous solutions, not suitable for high-resolution applications
Laser toner printing [72,99–101]	Laser printer, oven or hot plate	400/411 (FL)	Low-cost equipment, simple, rapid prototyping and fabrication, high throughput, resistant to some organic solvents	Barrier performance depends on printer/toner, may require high temperature which can cause pyrolysis, not suitable for high-resolution applications

FDM 3D printing [104,105]	FDM 3D printer, oven	400/2000 (CH)	Low-cost equipment, simple, can print on various papers and substrates	Low resolution, may require high temperature which can cause pyrolysis
Inkjet printing [108,111]	Inkjet printer	425/272 (FL)	Low-cost equipment, simple, rapid prototyping, high throughput, certain inks resistant to organic solvents, allows printing of sensing reagents	Requires printer modifications and custom ink preparation, may require equipment for post-printing treatment
Screen printing [116–118]	Screen printing setup	380/486 (FL)	Low-cost equipment, high throughput, works with different inks, works with different papers and substrates	Labor-intensive, may require large amounts of solvents to clean screen mesh, mask fabrication must be outsourced or requires extra equipment (e.g., CO ₂ laser cutter or craft cutter), not suitable for high- resolution applications
Flexographic printing [119]	Flexographic printer	400/NA (CH) NA/400 (CP)	High throughput, allows printing of sensing reagents	Expensive equipment, not suitable for high- resolution applications

<i>Drawing</i>				
Drawing [11,120,123,124,128,129]	Permanent marker, correction pen, technical pen, 3D pen, or wax crayon	280/150 (CH)	Low-cost and accessible equipment, simple, rapid prototyping, certain inks resistant to organic solvents	Generally low resolution, requires X-Y plotter for high throughput and reproducible fabrication
<i>Masking</i>				
Photolithography [132,133]	UV lamp or sunlight, hot plate or oven	186/248 (CH) 200/180 (FL)	High resolution, resistant to some organic solvents	Labor-intensive, requires extra equipment for mask fabrication like inkjet printers
Plasma treatment [54,134–136]	Vacuum plasma device or handheld corona generator	NA/NA	Allows integration of functional units like switches, filters, and separators	Low resolution, labor-intensive, may require special masks, difficult to create arrays of isolated hydrophobic zones
UV/O ₃ treatment [137]	UV lamp	137/233 (FL)	High resolution	Labor-intensive, time-consuming, requires equipment for mask fabrication
Vapor phase polymerization and chemical vapor deposition (CVD) [54,138–141]	Vacuum chambers	NA/500 (CH)	Allows integration of UV-responsive switches	Difficult to create arrays of isolated hydrophilic zones, requires extra equipment for mask fabrication, not suitable for high-resolution applications

Spraying [142–145]	Hydrophobic ink spray	NA/500 (FL)	Low-cost equipment, simple, rapid fabrication	Rough boundaries, difficult to create arrays of isolated hydrophilic zones, requires extra equipment for mask fabrication, not suitable for high- resolution applications
<i>Laser-assisted chemical modification</i>				
Laser modification of hydrophobic paper [146]	CO ₂ laser cutter	80/62 (PP)	High resolution, simple, rapid prototyping and fabrication	Only works on hydrophobic papers, requires extra modification with silica particles to enable capillary flow
Laser-induced photopolymerization [147]	405 nm continuous wave diode- laser	60/100 (NC)	High resolution	Requires special laser equipment and photopolymer, requires pre- and post- treatment of paper
Laser heating of wax [148,149]	CO ₂ laser cutter or diode laser	1000/300 (FL)	Simple, rapid prototyping and fabrication	Difficult to create a uniformly thick wax layer, not suitable for high-resolution applications
<i>Stamping and embossing</i>				
Stamping [150,152,153,156,157]	Stamp	312/328 (FL)	Simple, rapid fabrication, certain inks resist organic solvents	Generally low resolution, requires extra equipment to create stamp, a new stamp should be created for each new design

Embossing [159,160]	Molds	NA/2000 (FL)	Can create hollow open channels	Low resolution, requires extra equipment to create molds
<i>Physical methods</i>				
Cutting [59,171–173]	CO ₂ laser cutter, craft/knife cutter, scissors, or razor blade	400/500 (FL)	Low-cost and accessible equipment, simple, rapid prototyping	μPADs are not mechanically strong and are difficult to handle
Femtosecond laser etching of laminated paper [78,79]	Femtosecond laser	100/300 (NC)	High precision and resolution, smooth cuts and minimal thermal damage	Expensive equipment, may require extra equipment for lamination, difficult to integrate into 3D μPADs
CO ₂ laser etching of laminated paper [81,165–167,169]	CO ₂ laser cutter	39/128 (CH) NA/24 (NC)	High-resolution, simple, rapid prototyping and fabrication, allows integration of fluid control units, resists some organic solvents	May require extra equipment for paper lamination, difficult to integrate into 3D μPADs

Abbreviations: FDM, Fused Deposition Modeling; CH, chromatography paper; NC, nitrocellulose membrane; FL, filter paper; CP, cleanroom paper; PP, parchment paper.

2.3.5. Detection Methods

Various detection methods have been employed with μPADs, including colorimetric, electrochemical, fluorescence, chemiluminescence, electrochemiluminescence, and bioluminescence [10,52]. Each technique offers distinct advantages in terms of detection limit, accuracy, simplicity of design and application, assay time, and equipment requirements. The following subsections focus on two of the most widely used methods: colorimetric and electrochemical detection.

2.3.6. Colorimetric Detection

Colorimetric detection relies on a visible color change on μ PADs in response to the presence of the target analyte. This is the most widely used detection method due to its simplicity of signal readout with the naked eye or simple equipment, its ease of integration with multiplex assays, and its applicability for detecting a broad range of analytes. The white background of paper is particularly advantageous, providing high contrast between the assay signal and background, thereby improving the signal-to-noise ratio [10]. A variety of mechanisms can be employed to generate a color response on paper [10], including pH-responsive color change [179–182], metal-ligand complexation [183,184], coupling reactions [181,185], enzyme/nanozyme-chromogenic substrate interactions [186–189], nanoparticle-based visualization [190–194], and photopolymerization [195]. Signal readout can be performed in three modes: (1) qualitative (yes/no), (2) semi-quantitative (approximate concentration range), and (3) quantitative (more precise concentration determination). The first two modes can be assessed visually or with simple devices, whereas quantitative analysis requires external equipment such as flatbed scanners, smartphones, or portable transmittance-based reader [10].

One of the earliest and most common approaches to implementing colorimetric detection in μ PADs is the incorporation of a dedicated “detection zone,” where the colorimetric reaction takes place. In this design, the sample wicks from the sample zone to the detection zone, where the reaction occurs and a visible color change is produced [9,196]. For quantitative analysis, a calibration curve can be generated by measuring the color signal from samples of known concentration, which is then used to determine the concentration of unknown samples. Reflectance-based devices, such as smartphones [196,197] or low-cost desktop or portable flatbed scanners [196,198,199], can be used for this purpose, with the scanners being more robust and reliable due to consistent illumination and fixed imaging conditions [10,196]. In these methods, captured images are analyzed in color spaces such as red-green-blue (RGB) or cyan-magenta-yellow-key (CMYK) using software like Adobe Photoshop, ImageJ, or custom programs [10,15,198,199]. Alternatively, portable transmittance-based devices, which measure light passing through the thickness of paper (similar to a UV-Vis spectrophotometer), can achieve detection limits up to eight times lower than flatbed scanners [10,200,201]. Despite this, smartphone-based quantification remains popular due to their ubiquity and internet connectivity.

Although this approach to reagent deposition and device design is straightforward, several challenges remain. A key limitation is non-uniform color formation within the detection zone, which can compromise assay precision, accuracy, and overall reliability [15,52,202]. This issue arises from factors such as the intrinsic properties of the paper (e.g., heterogeneity of fiber structure), reagent wash off during liquid flow, and the functional stability of the reagents [15,52,202,203]. Another challenge is achieving reliable quantification using smartphones, as variations in ambient lighting, image-capture conditions (e.g., distance and angle), and image analysis can introduce significant errors [10,15,204]. Various strategies have been developed to address these limitations.

One such strategy involves improving color uniformity through careful selection of paper substrate and optimization of reagent volume. Evans et al. evaluated various paper types and reagent volumes for bienzymatic glucose detection, comparing the resulting color intensity and uniformity [59]. Across all paper types, increasing the reagent volume improved both color intensity and uniformity, with grade 1 papers, whether filter or chromatography, producing the highest color intensity. The increase in color intensity was attributed to the greater amount of reagent generating more colored product. Also, for a constant reagent volume, paper type significantly affected color uniformity due to differences in thickness and reagent retention capacity. For instance, with 0.5 μL of reagent solution, chromatography paper grade 1 (the thinnest substrate) exhibited the poorest uniformity, whereas with 1 μL , the same paper produced the best uniformity among all tested substrates (Figure 2.10A-i). Similar results were observed with an increased amount of deposited reagents for detecting human serum albumin using protein-sensitive reagents [109]. However, another study found that increasing the reagent volume for nitrite detection led to greater color gradients without a significant increase in color intensity [205]. These findings underscore the need to optimize paper type and reagent volume together to achieve both uniform and intense color signals in μPADs .

The geometrical design and configuration of μPADs can be tailored to improve color uniformity and intensity. While circular detection zones are commonly used, Abe et al. evaluated triangular and rectangular zones for detecting human serum albumin with protein-sensitive reagents and found that triangular zones improved both color uniformity and intensity. They also demonstrated that depositing reagents upstream of the detection zones enhanced color uniformity compared to placing them directly within the zones (Figure 2.10A-ii) [109]. However, the optimal shape of the

detection zone depends on the target analyte and reagents used; for example, another study reported that diamond-shaped zones improved glucose detection, whereas rectangular zones were selected for protein assays [196]. The ratio of sample volume to detection-zone size is another influential factor in color uniformity; smaller detection zones yielded more uniform color in glucose and lactate detection (Figure 2.10A-iii) [206]. In another study, positioning the detection zone and sample inlet at the same location, connected to a microchannel, improved glucose detection uniformity by preventing reagent accumulation at the edges and made the device sample-volume independent (Figure 2.10A-iv) [207]. Additionally, a central detection zone connected to six inlet zones for nitrite detection enhanced both color uniformity and intensity (Figure 2.10A-v) [208]. However, this approach is difficult to integrate in multiplex assays. Other studies have utilized 3D μ PADs to vertically deliver the sample or colorimetric indicator to the detection zone, thereby eliminating the elution effects associated with lateral flow [58,206,209–213]. In one example, Das et al. placed a glass fiber pad on top of filter paper containing dried glucose reagents. The sample was applied to the glass fiber, which acted as a distributor, and then transferred vertically to the filter paper beneath, resulting in a uniform color distribution for glucose detection (Figure 2.10A-vi) [214].

Another approach to improving color intensity and uniformity on paper is enhancing reagent stability. For example, a limitation of the Griess assay for nitrite detection is the poor stability of its reagents, sulfanilamide and N-naphthylethylenediamine, when mixed together. To address this and minimize degradation, Bhakta et al. incorporated an uptake zone before the detection zone to store sulfanilamide separately, enabling sequential reaction of the sample with the reagents (Figure 2.10B). This modified design significantly improved both color uniformity and intensity [185]. Other stabilizing strategies, such as adding sugars like trehalose [196] or pullulan [24], may also be effective.

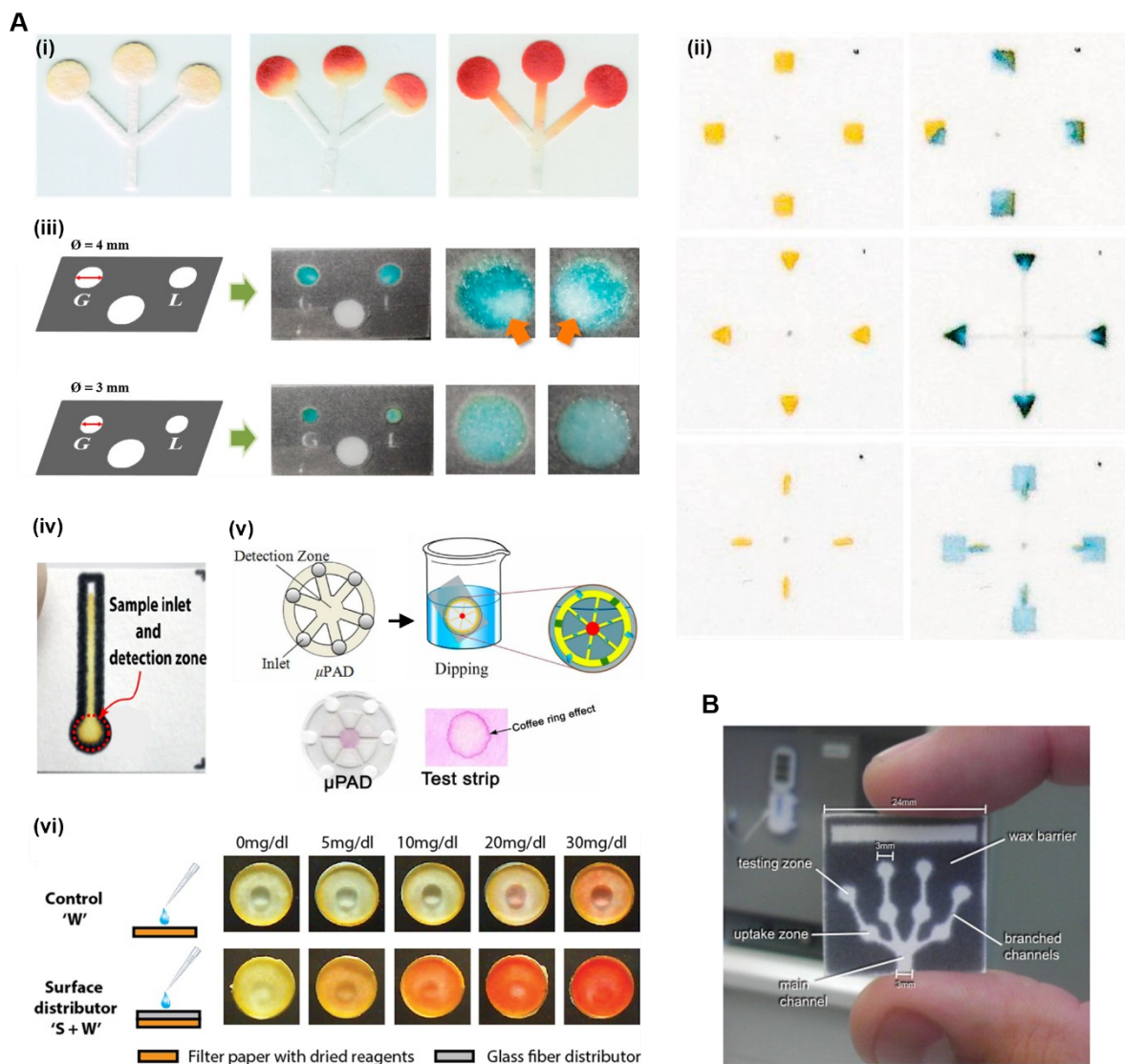


Figure 2.10. Design strategies to improve color uniformity and intensity on μ PADs. (A) Influence of paper substrate, reagent volume and placement, and device design. (A-i) Effect of reagent volume and paper substrate on glucose detection, with filter paper grade 3 with 0.5 μ L reagents (left), and chromatography paper grade 1 with 0.5 μ L (middle) and 1 μ L (right) reagents. Adapted with permission from Ref. [59]; copyright 2014 Royal Society of Chemistry. (A-ii) Effect of detection zone geometry and reagent placement on assays for human serum albumin (left: before sample addition; right: after sample addition). Top row: rectangular zones; middle row: triangular zones; bottom row: rectangular zones with reagents deposited upstream of the detection zone. Adapted with permission from Ref. [109]; copyright 2008 American Society of Chemistry. (A-iii) Effect of detection zone size with constant sample volume on glucose (left) and lactate (right) detection. Adapted with permission from Ref. [206]; copyright 2016 Elsevier. (A-iv) Placement of sample inlet and detection zones in the same place to detect glucose. Adapted with permission from Ref. [207]; copyright 2020 Springer Nature. (A-v) Multiple sample inlets with one detection zone for nitrite detection. Adapted with permission from Ref. [208]; copyright 2020 IOP Publishing. (A-vi) 3D μ PAD fabricated by stacking layers to enable vertical flow for glucose

detection. Adapted from Ref. [214] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>). (B) Separation of Griess reagents into distinct uptake and detection zones to improve stability and, in turn, enhance color formation for nitrite detection. Adapted with permission from Ref. [185]; copyright 2014 Elsevier.

Chemical treatment of paper to enhance reagent immobilization has also been explored for color intensity and uniformity. For example, Evans et al. treated filter paper with silica nanoparticles modified with 3-aminopropyltriethoxysilane to promote reagent adsorption and prevent elution during fluid flow. This approach improved both color uniformity and intensity for lactate, glucose, and glutamate detection (Figure 2.11A) [215]. In another study, Garcia et al. oxidized paper with sodium periodate followed by activation with EDC-NHS to enable enzyme immobilization. While this treatment improved the color uniformity of glucose and uric acid assays, it reduced color intensity, interfered with nitrite detection, and did not benefit complexometric assays [155]. In a subsequent study, the same group used chitosan to treat filter paper, which increased both color intensity and uniformity, and in turn detection limit, for glucose and uric acid detection (Figure 2.11B) [216]. Other treatments have also been reported such as poly(acrylic acid) [69] amine, carboxyl, and thiol treatment for heavy-metal detection via complexometric reactions [183]; poly-L-arginine for enzymatic detection of malathion [197]; Fe₃O₄ nanoparticles, multiwalled carbon nanotubes, and graphene oxide for glucose detection [217]; starch-gelatin [218] and chitosan-glutaraldehyde for glucose detection; and chitosan-glutaraldehyde-gold nanoparticles (AuNPs) for immunosensing of prostate-specific antigen [219].

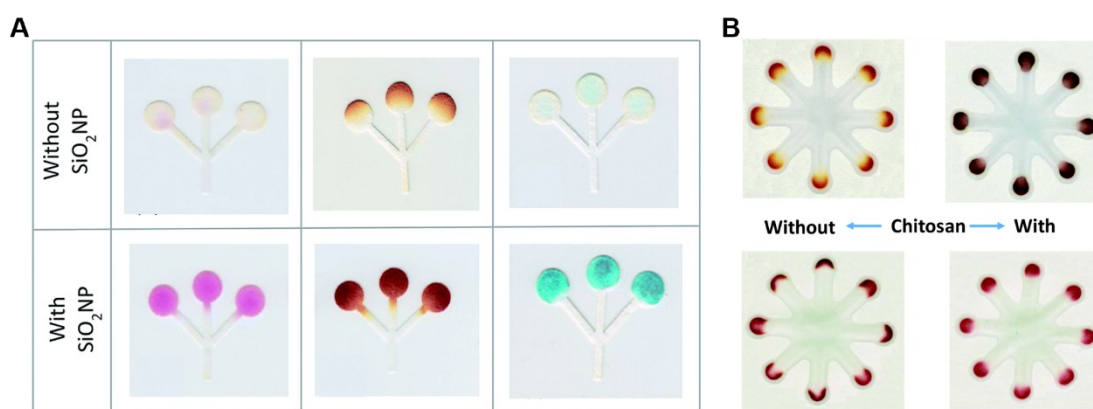


Figure 2.11. Examples of chemical modification to improve color uniformity and intensity. (A) Silica nanoparticles for lactate (left), glucose (middle), and glutamate (right). Adapted with permission from Ref. [215]; copyright 2014 Royal Society of Chemistry. (B) Chitosan modification to improve glucose (top) and uric acid (bottom) detection. Adapted with permission from Ref. [216]; copyright 2016 Royal Society of chemistry.

In addition to device design and modification, several strategies for smartphone-based image capture and analysis have been proposed to improve quantification. A simple approach to reduce the impact of color gradients is to use the entire detection zone as the region of interest, rather than selecting a specific spot, as manual selection can introduce errors [204,220]. Another method, colorimetric absorbance mapping, quantifies the analyte concentration in each pixel of the detection zone based on absorbance and integrates the values across the entire area, making quantification independent of the shape of the color response [221]. To correct for environmental lighting effects, a control zone can be incorporated into the μ PAD design, enabling comparison between the test and control zones to minimize ambient light interference (Figure 2.12A-i) [197]. Optimizing the color space for image analysis has been shown to enhance overall sensing performance and reduce the impact of ambient lighting [10,15,222]. Additionally, portable and smartphone-mountable light boxes have been developed to provide consistent illumination and fixed shooting conditions, and can be fabricated by 3D printing (Figure 2.12A-ii) [102,223–227]. Also, more recently, machine learning algorithms have been developed to improve the robustness of smartphone quantification under varying lighting conditions, smartphone models, and shooting angles (Figure 2.12A-iii) [228–230].

Beyond the aforementioned techniques, various innovative μ PAD designs and reagent-deposition strategies have been developed to overcome issues with color uniformity and enable result interpretation without analyzing color intensity [10,231]. Distance-based assays, for example, quantify results by measuring the length of the colored region formed in a channel (Figure 2.12B-i) [232,233], while radius- or diameter-based assays estimate analyte concentration from the diameter of the area that undergoes a color change in a circular μ PAD [234]. In signal-counting assays, the target concentration is determined by counting the number of colored regions formed on the paper within a set time [235,236]. Time-based assays, on the other hand, estimate analyte concentration based on the time required for color to develop [235]. In the text-based assays, analytical results are displayed directly on the paper in the form of text (Figure 2.12B-ii) [237,238]. Smartphone integration has also been achieved with QR-code assays for qualitative and semi-quantitative analysis. In these systems, the color development forms a QR-code pattern, allowing the code to be scanned by a smartphone and the results rapidly uploaded for epidemiological purposes [239,240].

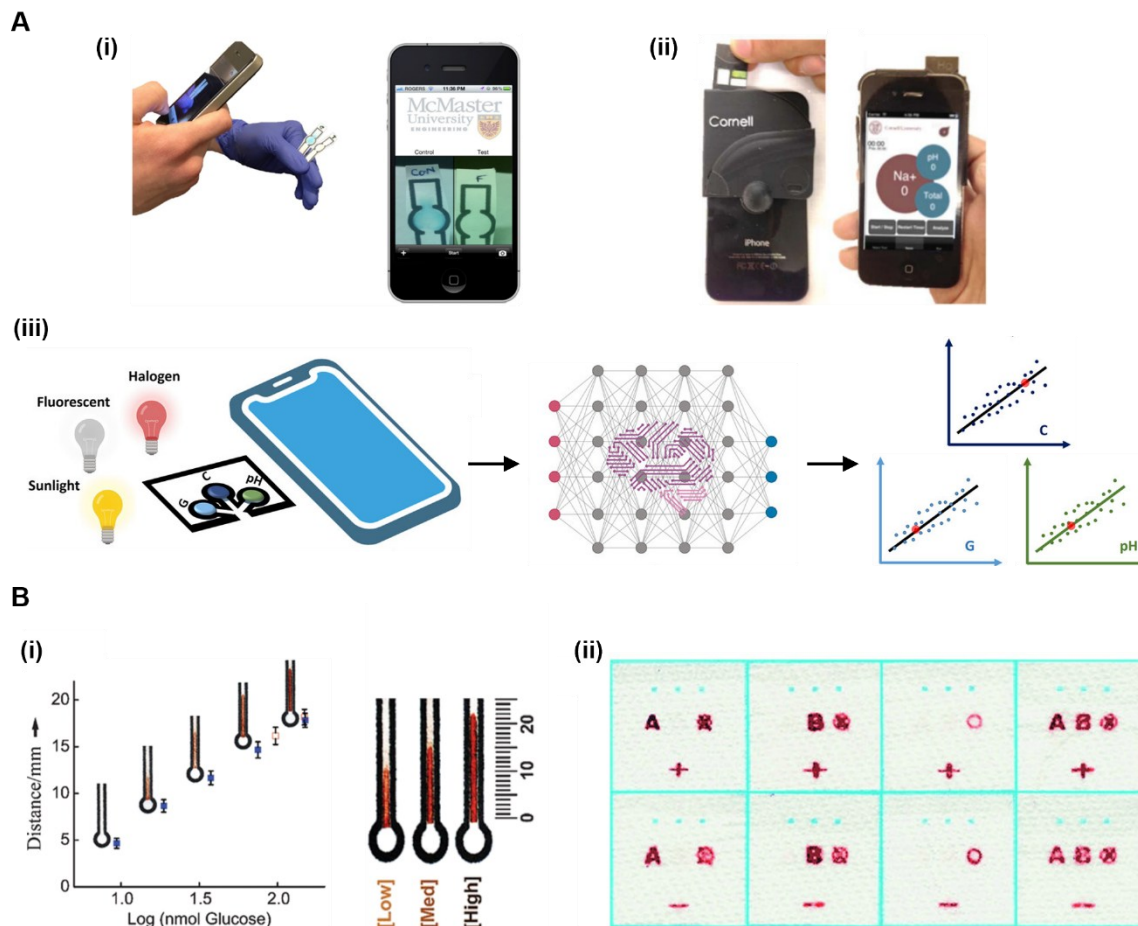


Figure 2.12. Smartphone quantification and innovative reagent decoration. (A) Example of strategies to improve smartphone-based quantification. (A-i) Incorporation of a control zone to mitigate lighting effects for pesticide (malathion) detection. Adapted with permission from Ref. [197]; copyright 2015 Elsevier. (A-ii) Smartphone-mountable light box for consistent lighting to detect pH. Adapted with permission from Ref. [224]; copyright 2013 Royal Society of Chemistry. (A-iii) Artificial intelligence (AI)-assisted quantification to address lighting variations and smartphone brand differences for detecting glucose, cholesterol and pH. Adapted with modifications from Ref. [228] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>). (B) Examples of reagent decoration enabling interpretations beyond color intensity. (B-i) Distance-based detection of glucose. Adapted with permission from Ref. [233]; copyright 2013 Royal Society of Chemistry. (B-ii) Text-based detection for blood typing. Adapted with permission from Ref. [237]; copyright 2012 Wiley.

Overall, colorimetric detection remains highly attractive for POC/POU applications due to its operational simplicity and compatibility with smartphones. However, its relatively low signal intensity can lead to false negatives, underscoring the need for further research to enhance signal strength and detection limits [241]. For improving color uniformity, various strategies are available, with the optimal choice depending on the specific application and reagents used.

Continued research aimed at integrating these improvements directly into the μ PAD fabrication step will simplify device production and enhance their scalability and overall applicability. Reliable smartphone-based quantification remains a challenge, but advancements in machine learning and image analysis hold promise for addressing this issue. Finally, innovative color-formation methods can expand the applicability of colorimetric techniques for field-based testing and equipment-free readouts.

2.3.7. Electrochemical Detection

Electrochemical paper-based analytical devices (ePADs) were first reported by Henry's group in 2009 [19] and have since grown rapidly, with wide applications from diagnostics to food safety and environmental monitoring [16,17,242]. Leveraging the advantages of both paper and electrochemical sensing, these devices offer low cost, capillary-driven microfluidics, potential for multiplex detection, low detection limits, rapid analysis, insensitivity to ambient light and sample color, and straightforward integration with digital technologies [16–18,53]. By definition, ePADs include electrochemical sensors fabricated either on paper capable of capillary microfluidics or on non-capillary paper as a low-cost, environmentally friendly alternative to plastic strips [18,243]. In terms of cost, ePADs can be fabricated for as little as ~USD 0.014 per strip, compared to USD 0.5-1.0 for commercial electrochemical strips [244], particularly since paper is among the least expensive substrates available (0.1 cent/dm² versus 2 cent/dm² for polyethylene terephthalate, PET) [55]. Also, with the advent of portable, smartphone-compatible potentiostats as well as battery-free near-field communication (NFC) potentiostats, available for as little as ~USD 10, electrochemical sensors have become an increasingly attractive platform for POC/POU applications [53,245,246].

Electrochemical detection usually relies on either a single redox reaction or a sequence of redox reactions, whose extent depend on the analyte concentration. Depending on the assay and detection mode, the reaction may produce a measurable current (amperometric), generate a measurable potential or charge accumulation (potentiometric), or alter the conductivity of the medium between electrodes (conductometric), all of which can be detected using a potentiostat [16,247–249]. However, over time, electrochemical sensors have expanded to include non-redox-based approaches, such as impedometric detection [16,249]. From a design perspective, electrochemical sensors conventionally employ three electrodes: a working electrode, a reference electrode, and a

counter electrode. The working electrode is the key component for detection, as it determines the device's functionality and analytical performance. The reference electrode is positioned as close as possible to the working electrode to minimize voltage drop caused by solution resistance. The counter electrode is typically made larger than the working electrode to ensure unrestricted current flow within the circuit [10].

The choice of electrode material and its fabrication method on paper are critical to ePAD performance. Carbon and noble metals are most commonly used, each with advantages and limitations. Metallic electrodes, such as gold, silver, and platinum, offer high conductivity and strong electrochemical activity but often have narrower potential windows due to their electrocatalytic activity toward water splitting [10,250–253]. Carbon electrodes are more affordable and provide wider potential windows for detecting a broader range of analytes [10,19,254,255]. Hybrid designs combining metals or metal oxides with carbon are also common to enhance performance of the electrodes, with carbon serving as the substrate and the metal component as a coating or embedded modifier [10,256–259]. Numerous fabrication methods have been developed to integrate electrodes onto paper substrates, including screen printing [19,20,260], stencil printing [261,262], drawing [122,263–265], inkjet printing [266,267], laser scribing and pyrolysis [22,268,269], sputtering [270,271], and microwire placement [251,272]. Several of these approaches are discussed in more details below.

Screen printing. Screen printing, the first technique used to fabricate ePADs, remains one of the most common methods. Also, this method is already widely employed for the mass production of electrochemical strips, known as screen-printed electrodes (SPEs) [16,17]. A typical setup consists of a screen-printing table, a mesh screen, often made of silk or nylon, and a squeegee. The desired mask is designed using computer software and mounted on the mesh; the ink is then loaded onto the mesh and transferred to the substrate with the squeegee (Figure 2.13A) [243,273]. Among the parameters influencing screen printing, mesh count (threads per inch) and thread diameter are particularly important; higher mesh counts and smaller diameters improve resolution, allowing smaller and more precise patterns to be printed [274,275]. In addition, appropriate squeegee speed and pressure requires consideration of ink viscosity, as these parameters govern both ink transfer efficiency and the consistency and reproducibility of printing [275]. Screen printing offers good resolution for most applications, rapid fabrication, and, when automated, high reproducibility of 1-2% [16,17,276]. It is compatible with various substrates and ink compositions, many of which

are commercially available, and can incorporate functional sensing elements such as catalysts or nanoparticles directly into the ink to avoid post-fabrication steps [16,19,277–279]. Depending on the ink, post-printing thermal curing may be needed [16,254]. Limitations include the need to outsource mask fabrication, often costing USD 50-100 and adding time to production, as well as the labor intensiveness of the process and the need for cleaning of the mesh, typically with organic solvents.

Stencil printing. Stencil printing is similar to screen printing but does not use a mesh screen. Instead, a stencil is created from materials such as transparency sheets using a CO₂ laser cutter or craft cutter. The stencil is then placed directly on the paper substrate, and the ink is applied with a squeegee (Figure 2.13B) [261,262,280,281]. Of note, stencil printing is sometimes mistakenly referred to as screen printing, likely because both techniques use a mask and a squeegee for patterning. However, the key distinction is that in stencil printing, the pattern is defined by a cut-out stencil placed directly on the substrate, whereas in screen printing, the ink passes through a patterned mesh. Stencil-printing is among the most cost-effective fabrication methods, allowing simple and rapid prototyping; however, to achieve clean boundaries, the ink typically needs to be more viscous [16]. Compared to screen printing, stencil printing offers less control over ink transfer, lower reproducibility, and slower fabrication speed [16,17,276].

Drawing. Several freehand drawing techniques have been developed for ePAD fabrication using tools such as pencils [265,282,283], ballpoint pens with custom inks [263], and 3D printing pens with commercially available conductive filaments [264]. Pencil drawing is the simplest approach, requiring only a graphite pencil, preferably with higher grades (e.g., 5B or 6B) for improved conductivity, with simple stencils or rulers aiding in consistent patterning (Figure 2.13C) [16,17,122]. A key factor in this method is controlling the applied pressure during drawing, as variations can lead to inconsistencies in material deposition and device performance [16]. For greater reproducibility and higher throughput, XY plotters can also be employed [122]. While this method is currently unsuitable for mass production, it provides an equipment-free option for rapid prototyping and generates minimal or no waste compared to other fabrication techniques [16].

Inkjet printing. Inkjet printing is a versatile technique for fabricating ePADs, employing both standard office printers and specialized printing systems. In this approach, conductive ink is loaded into the printer cartridge and printed onto paper to form electrodes (Figure 2.13D). Ink viscosity

is a critical parameter, typically lower than that used in screen printing, to ensure compatibility with the printer and prevent clogging printing system [16,17]. A wide range of inks have been successfully used to produce ePADs, including silver nanoparticle, carbon [266], AuNPs [267,284], and carbon nanotube [285]. Inkjet printing offers high resolution, rapid prototyping, and low material waste, with the added potential to enable all-in-one fabrication of electrodes, hydrophobic barriers, and sensing reagents [16,17]. For example, da Costa et al. demonstrated disposable ePADs for dopamine sensing, in which both carbon nanotube electrodes and mineral spirit oil hydrophobic barriers were fabricated via inkjet printing [285]. In another study, inkjet printing was employed to deposit graphene and silver nanoparticle electrodes, along with potassium chloride, iron chloride, and ion-selective membranes, onto wax-patterned paper for ion sensing. However, multiple intermediate procedures between printing steps made the overall fabrication process more complex [286]. Also, another drawback of inkjet printing is that inks are often dilute, and printing may need to be repeated several times to achieve sufficient conductivity, which slows the overall process [17,285].

Laser scribing and pyrolysis. Laser scribing and pyrolysis involves the in-situ carbonization of paper using a CO₂ laser cutter [17,18]. The first application of this technique was reported by de Araujo et al. in 2017, in which a CO₂ laser was used to create porous conductive electrodes on paperboard for the detection of ascorbic acid and caffeic acid [287]. However, for other paper types, such as filter or chromatography paper, pretreatment with a fire retardant was necessary to prevent complete burning [17,22], with reported examples include sodium tetraborate [288], phosphate-based retardants [269,289], ammonium sulfamate [22], and ammonium sulfate (Figure 2.13E) [289]. The characteristics of the resulting electrodes are highly dependent on laser parameters, including power, speed, pulses per inch, and focal distance [22,289,290]. Laser scribing can also be applied to modify chemicals already deposited on paper to enhance electrode properties [258], and it works with various other substrates, such as polyimide tape, to produce hybrid paper-based devices [269,290–292]. A key advantage of this technique is that the resulting electrodes can be hydrophilic, allowing capillary-driven fluid flow through them and expanding the design possibilities for ePADs [22]. It also offers high resolution, rapid prototyping, minimal waste, and scalability for mass production; however, it is limited by the need for fire retardants and relatively low control over electrode composition [17,18].

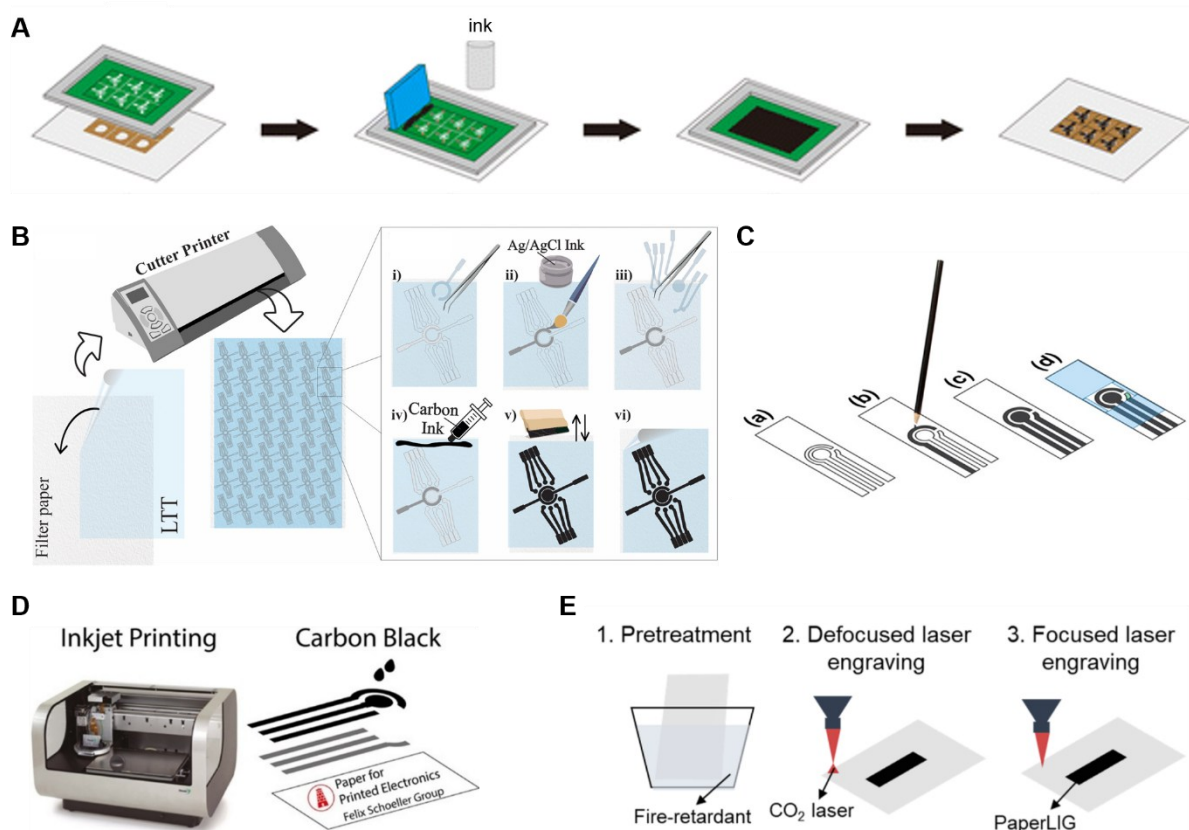


Figure 2.13. Electrode fabrication on paper. (A) Screen printing. Adapted with permission from Ref. [273]; copyright 2016 Springer Nature. (B) Stencil printing using low tack transfer tape (LTT) as a stencil. Adapted with permission from Ref. [262]; copyright 2019 Elsevier. (C) Pencil drawing. Adapted with permission from Ref. [282]; copyright 2018 Wiley. (D) Inkjet printing. Adapted with permission from Ref. [266]; copyright 2018 Elsevier. (E) Laser scribing on paper modified with fire retardant. Adapted with modifications from Ref. [289] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>).

While various fabrication methods have been explored, ink deposition techniques such as screen printing remain the most common. A major limitation of these methods is that typical inks, such as carbon and silver, often contain hydrophobic components that hinder capillary flow in paper. This issue not only limits the applicability of ePADs but also reduces liquid-electrode contact, thereby diminishing their sensitivity and overall performance [18,21,22]. Although, according to the studied literature, no direct solution to this issue has been reported, several engineering strategies have been developed to enhance ePAD performance.

One approach involved creating 3D paper wells by wax infusion, allowing the sample to remain on top of the electrodes rather than being wicked into the paper pores (Figure 2.14A) [293]. This

design increased the electroactive surface area by ~ 2.7 -fold and improved sensor-to-sensor consistency, attributed to enhanced liquid access to the electrode sidewalls and more uniform electrode roughness on the top surface. The devices were successfully used for pH, glucose, and dopamine detection. However, wax infusion eliminated capillary flow, restricting the system to spot testing and thereby limiting the applicability of such ePADs. In another study, 3D ePADs were fabricated by stencil-printing the working and reference electrodes on one side of the paper and the counter electrode on the other (Figure 2.14B) [294]. This configuration reduced the gap between the working and counter electrodes and allowed greater flexibility in electrode geometry design, improving electrochemical reactions. As a result, signal intensity increased by 2.9-fold, as indicated by peak currents in cyclic voltammetry. The device was successfully applied to detect clozapine in human serum. Nonetheless, the need for two-side printing complicates fabrication, and the dual-sided electrode arrangement is incompatible with many common portable potentiostats.

Furthermore, an origami-style 3D ePAD was developed in which the electrodes were sandwiched between two layers of paper by folding the device (Figure 2.14C) [295]. This configuration resulted in a twofold increase in sensitivity for serotonin detection, although the underlying mechanisms and key influencing parameters were not thoroughly examined [296]. The design, however, was limited to spot testing and was incompatible with lateral flow operation. In contrast, when a similar sandwiching strategy was applied for *E. coli* detection, the sensitivity decreased compared to a conventional 2D ePAD [297]. These contrasting findings underscore the need for further investigation into the sandwiching approach to clarify the conditions under which it can enhance electrochemical performance.

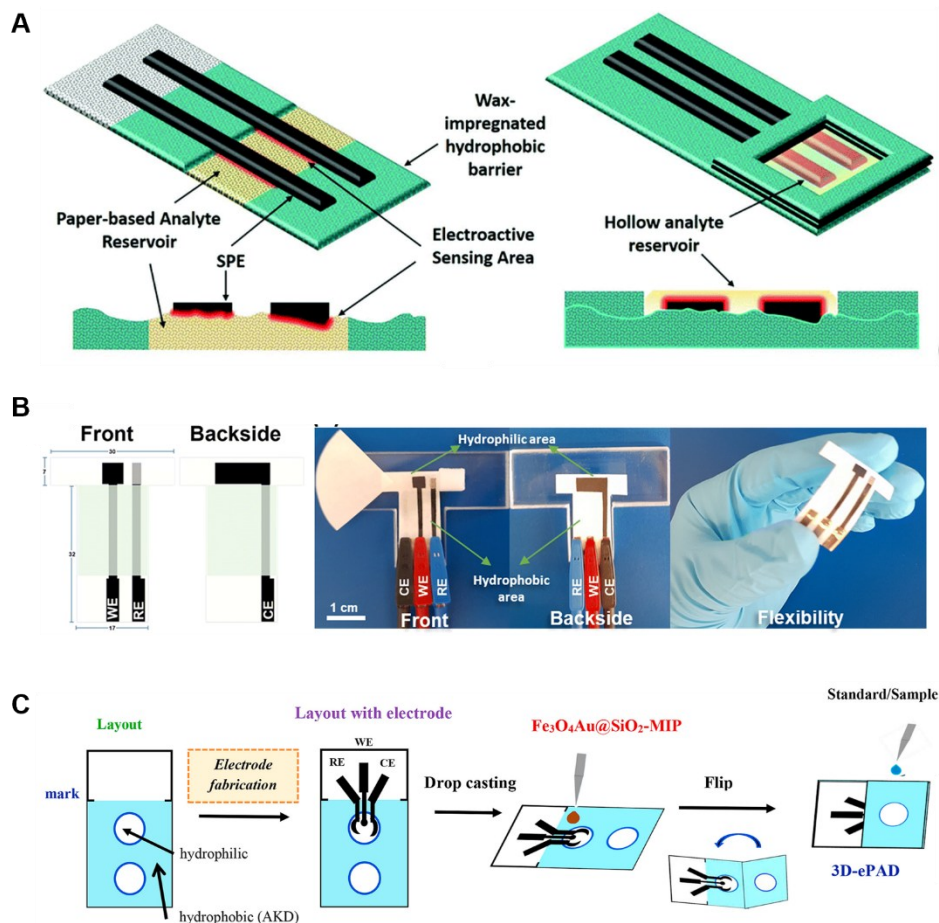


Figure 2.14. Engineering strategies to enhance signal on ink-deposited ePADs. (A) Creation of a 3D well by wax-infused paper (right) compared to a 2D ePAD (left). Adapted with permission from Ref. [293]; copyright 2018 Royal Society of Chemistry. (B) Electrode patterning on both sides of paper. Adapted with modifications from Ref. [294] under a Creative Commons Attribution license (CC BY 3.0, <https://creativecommons.org/licenses/by/3.0>). (C) Origami-based 3D ePAD with electrodes sandwiched between two paper layers. Adapted with permission from Ref. [295]; copyright 2019 Elsevier.

In addition to the methods described above, several other strategies can help address the hydrophobicity of inks or enhance the analytical performance of ePADs. Nanomaterials have been widely employed to enhance the sensitivity and detection limits of ePADs, owing to their high surface-to-volume ratio and excellent electrical conductivity. Both metal nanomaterials (e.g., gold, silver, platinum) and non-metal nanomaterials (e.g., carbon nanotubes, graphene) have been utilized for this purpose. Incorporation into ePADs can be achieved through various approaches, including direct addition to the ink, in situ synthesis, electrogeneration, or drop-casting [298–300]. Additional approaches include careful selection or combination of paper substrates, controlling

fluid flow within the channels, and employing fabrication techniques other than ink deposition to create flow-through electrodes, thereby improving device performance [22,253,301].

Overall, the development of ePADs has been driven by the need for low-cost, portable, and sensitive analytical platforms that leverage both the microfluidic capabilities of paper and the versatility of electrochemical detection. A variety of fabrication techniques enable diverse electrode designs, each with specific advantages and limitations in terms of resolution, reproducibility, cost, and scalability. Despite these advances, key challenges remain, including the hydrophobicity of conventional conductive inks, limited device-to-device reproducibility and consistency, and the long-term stability of reagents stored on paper [16,18,53]. Continued research into optimizing fabrication methods, improving stability, and integrating novel materials will be critical for advancing ePADs toward broader, real-world POC/POU applications.

2.4. Tablet-Based Assays

In this section, a brief history of tablet-based assays is first presented. Then, major advancements in the field are discussed, categorized by fabrication technologies, namely powder compression and polysaccharide encapsulation.

2.4.1. A Brief History of Tablet-Based Assays

Tablet-based assays have a longstanding history and represent one of the earliest platforms for POC/POU testing in the modern scientific era. Based on the reviewed literature, their origins can be traced back to before 1920, when methylene blue tablets were employed in Denmark for the colorimetric assessment of milk's sanitary quality [302]. By the 1930s, additional tablet-based methods had been developed for field analysis of milk pasteurization [303].

A major breakthrough occurred in 1941, when the Ames Company introduced Clinitest, a tablet-based assay for semi-quantitative detection of reducing substances in urine, mainly intended for glucose estimation and diabetes management. This test, based on the copper reduction method, enabled glucose measurement with simple, user-friendly steps in less than a minute [304,305]. The success of Clinitest spurred the release of several other commercial assays in the following years, including Acetest for acetone detection and the Bumintest for albumin measurement in urine (Figure 2.15A) [306,307].

However, the introduction of paper-based dipstick tests by Ames in 1956 to further simplify detection [42], along with the emergence of LFAs [29,46,47] and, later, microfluidic technologies [10,49], led to a decline in interest in tablet-based assays. Nevertheless, such assays continued to be manufactured for specific applications, e.g., Lovibond Company's tablet-based products for water monitoring.

In 2014, a landmark study by Jahanshahi-Anbuhi et al. revitalized academic interest in tablet-based assays. Departing from the conventional powder compression method, the authors developed reagent tablets by encapsulating sensing reagents in the polysaccharide pullulan and forming tablets through drop-casting. These tablets were successfully applied to pesticide detection in water using the indoxyl acetate (IDA)-acetylcholinesterase (AChE) colorimetric method (Figure 2.15B). Notably, pullulan encapsulation extended reagent stability from less than one week to at least two months, addressing a longstanding limitation in the field [24]. Following this work, tablet-based assays have regained attention in the research community, with studies demonstrating their applicability across diverse areas, from diagnostics to soil monitoring, and incorporating a variety of detection mechanisms.

A

Save time on Urine tests with . . .

CLINITEST and ACETEST

TRADE MARK

Reagent Tablets for the detection of
GLYCOSURIA **KETONURIA**

Both tests performed together in one minute!

'Clinitest' tablets have been widely used and prescribed since 1947. Many valuable hours have been saved. Following successful clinical trials, the makers of 'CLINITEST' have produced 'Acetest' reagent tablets for the detection of Ketonuria. Reliable routine sugar and acetone tests can now be carried out *together* in one minute!

'CLINITEST'

No external heating. No measuring of reagents.

Approved by the Medical Advisory Committee of the Diabetic Association.

Available under the N.H.S. on Form E.C.10.

(Basic Drug Tariff Prices: Set 6/8d. complete. Refill bottles of 36 Tablets 2/4d.)

'CLINITEST' HOSPITAL EQUIPMENT

An invaluable time-saver in wards and clinics. Write for details and hospital prices.

The advantages of **'ACETEST'**

A single tablet provides all the reagents required. Low cost permits use as a screening procedure or as a routine for diabetic patients. No danger of false positives with normal urine. No caustic reagents.

- 1 Put 1 drop of urine on tablet.
- 2 Take reading at 30 seconds. Compare tablet to colour chart provided.
- 3 Record results as negative, trace, moderate or strongly positive.

Available under the N.H.S. on Form E.C.10. Basic Drug Tariff price 3/10 per bottle of 100 tablets (with colour scale).

REFERENCES: *Lancet*, April 17th 1954, pp. 801/804 and July 10th 1954, p. 95 *Med. Ill.*, May 1954, p. 289 *Med. World*, Oct. 1954, pp. 373/376

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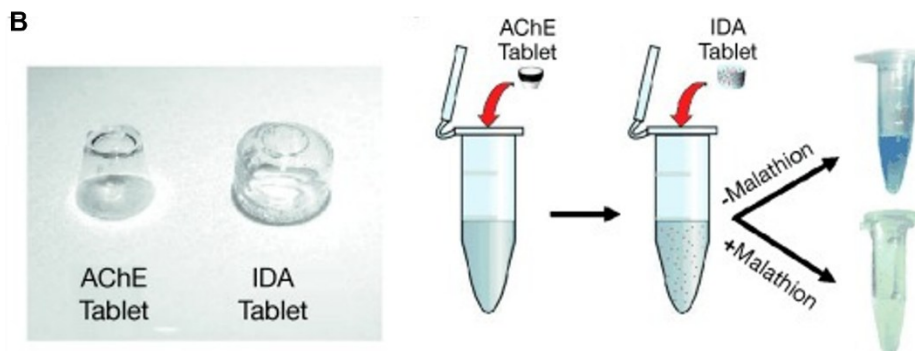


Figure 2.15. Key milestones in tablet-based assays. (A) Advertisement of early tablet-based assays for glucose and acetone detection in urine by Ames Company, published in the British Medical Journal in 1955. Adapted from Ref. [306]. (B) First polysaccharide-based tablets, reported by Jahanshahi-Anbuhi et al. in 2014 for malathion detection in water. Adapted with permission from Ref. [24]; copyright 2014 Wiley.

2.4.2. Tablets via Powder Compression

Powder compression is the traditional method for producing tablets, where powders are weighed, blended, and then compacted into tablet form. This well-established technique is highly scalable with standard industrial equipment and offers the flexibility to incorporate a wide range of chemical components into the formulation [25,308,309].

One of the earliest examples of tablet-based assays prepared by powder compression was the Clinitest, introduced by the Ames Company for glucose estimation in urine [304]. This test relied on the copper reduction method to detect total reducing substances, including glucose, and the tablets were formulated with copper sulfate, sodium hydroxide, citric acid, and sodium bicarbonate. Upon dissolution in diluted urine, exothermic heat was generated both from sodium hydroxide dissolution and its partial neutralization by citric acid, while the effervescence from the citric acid-sodium bicarbonate reaction promoted mixing. Together, these conditions facilitated the reduction of copper ions, producing visible color changes ranging from green to yellow to orange cuprous oxide. The procedure was straightforward: five drops of urine were added to a test tube, followed by ten drops of tap water and one tablet. The reaction was complete in less than a minute, and the results were interpreted semi-quantitatively by comparing the developed color with a reference color chart [304,310]. However, the method also had drawbacks, including potential overestimation of glucose due to interference from other reducing substances, the caustic nature of the tablets that made handling difficult, and their limited stability once the package was opened [304,311]. A similar tablet-based assay was reported for detecting total protein in urine using the salicylsulphonic acid test. In this method, filtered urine was placed in a test tube, followed by addition of a reagent tablet, and the resulting turbidity was correlated with protein concentration [312].

Another type of tablet-based assay was developed to detect ketone bodies in urine to aid diabetes management. These assays relied on the nitroprusside reaction, producing a purple-magenta color upon interaction with ketone compounds such as acetone and acetoacetic acid [313,314]. A prominent example was the Acetest tablet by the Ames Company, formulated with sodium nitroprusside, glycine, disodium phosphate, and lactose. A key feature of these assays was their simplicity. The tablet was placed on a clean white surface, and a drop of urine was added directly onto it. Within 30 seconds, the tablet changed color according to analyte concentration, which was compared against a reference chart for semi-quantitative interpretation [315,316]. This eliminated the need for test tubes, making the workflow even simpler than earlier glucose tablets. However, limitations included potential interference from highly pigmented urine and other urinary constituents that could compromise accuracy [317].

Tablet-based assays were also combined with filter mats to improve performance by pre-concentrating analytes. One notable case was the Ictotest, also created by Ames Company to detect

bilirubin in urine. Each tablet contained 0.2 mg *p*-nitrobenzene diazonium *p*-toluene sulphonate, 100 mg sulphosalicylic acid, 10-20 mg sodium bicarbonate, and 15-25 mg boric acid, supplied with a 20 × 20 × 4 mm filter mat composed of asbestos and cellulose fibers. The procedure consisted of soaking the mat with five drops of urine, placing a tablet on the moist area, and adding one drop of water, followed by a second drop to carry the reagents into the mat. Results were read within 30 seconds, with the speed and intensity of purple color development correlating with bilirubin concentration [318,319]. In this system, the mat concentrated bilirubin, while the diazo dye was solubilized by water and carried into the mat with the aid of effervescence from sulphosalicylic acid and sodium bicarbonate, with boric acid serving as a vehicle [320]. A major drawback of this test was the use of asbestos, which is highly toxic and has been banned in many countries [321]. Alternative substrates such as filter papers and cellulose waddings produced inferior results compared to the original mat, with the exception of Franklin's barium chloride-impregnated plaster [319]. Nonetheless, other studies demonstrated the use of tablet-based assays with Whatman Grade 1 filter paper for detecting analytes such as blood in urine [322] and stool [323].

In recent years, bioreagents have been incorporated into tablet formulations to enable more complex assays. In 2017, Udugama et al. demonstrated this by lyophilizing reagents with trehalose, a cryoprotectant that preserved their stability [25]. The lyophilized reagents were then blended with mannitol as an inert bulking agent, croscarmellose sodium to promote rapid disintegration (<30 seconds), and sodium stearyl fumarate to lubricate the press for smooth tablet ejection (Figure 2.16A). Using this approach, proteins, enzymes, nucleic acids, and quantum dots were stabilized in tablet form without loss of function for at least four weeks at room temperature. The tablets were also cost effective, at USD 0.16 for 100 tablets. To demonstrate practical utility, the authors developed a tablet-based assay for detecting hepatitis B virus (HBV) in serum. Three color-coded tablets were created for the multistep assay: one for recombinase polymerase amplification (RPA), one containing quantum dot barcodes, and one with a fluorescent reporter probe. A handheld device was also designed to excite the fluorescent agents and capture images with a smartphone, allowing clear differentiation between healthy and HBV-positive samples (Figure 2.16B) [25]. This work established the feasibility of using powder-compressed tablets with bioreagents and extended their application to complex immunoassays and nucleic acid amplification tests as well as fluorometric assays. However, for the detection, the workflow still required multiple steps

between tablet uses, and each tablet first had to be dissolved in solution and aliquots of these solutions were then used in the assay, adding procedural complexity.

Building on the previous work, the same group developed multi-layered tablets for sequential reagent release, enabling automation of multi-step assays [324]. Each tablet contained three layers: a top layer with the first reagent, a barrier layer to control incubation time, and a core layer with the second reagent. Upon immersion in solution, the top layer disintegrated to release the first reagent, followed by a barrier-controlled delay, after which the core layer released the second reagent (Figure 2.16C). The top and core layers were designed to dissolve rapidly using hydrophilic polymers such as microcrystalline cellulose and poly(vinylpyrrolidone) or small amounts of effervescent agents. The barrier layer's disintegration time (0-83 min) was tuned by adjusting the ratio of microcrystalline cellulose (hydrophilic) to hydroxypropyl cellulose (hydrophobic) and the compression force. These tablets were successfully applied to the stepwise detection of the *tpp47* gene from *Treponema pallidum* (syphilis) using quantum dot barcodes and secondary fluorophores, and to nitrite detection in water via sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride reactions [324]. With an estimated cost of USD 0.13, the tablets offered customizable incubation times and the potential for additional layers. However, the fabrication process remained labor-intensive, involving multiple steps that may hinder scalability.

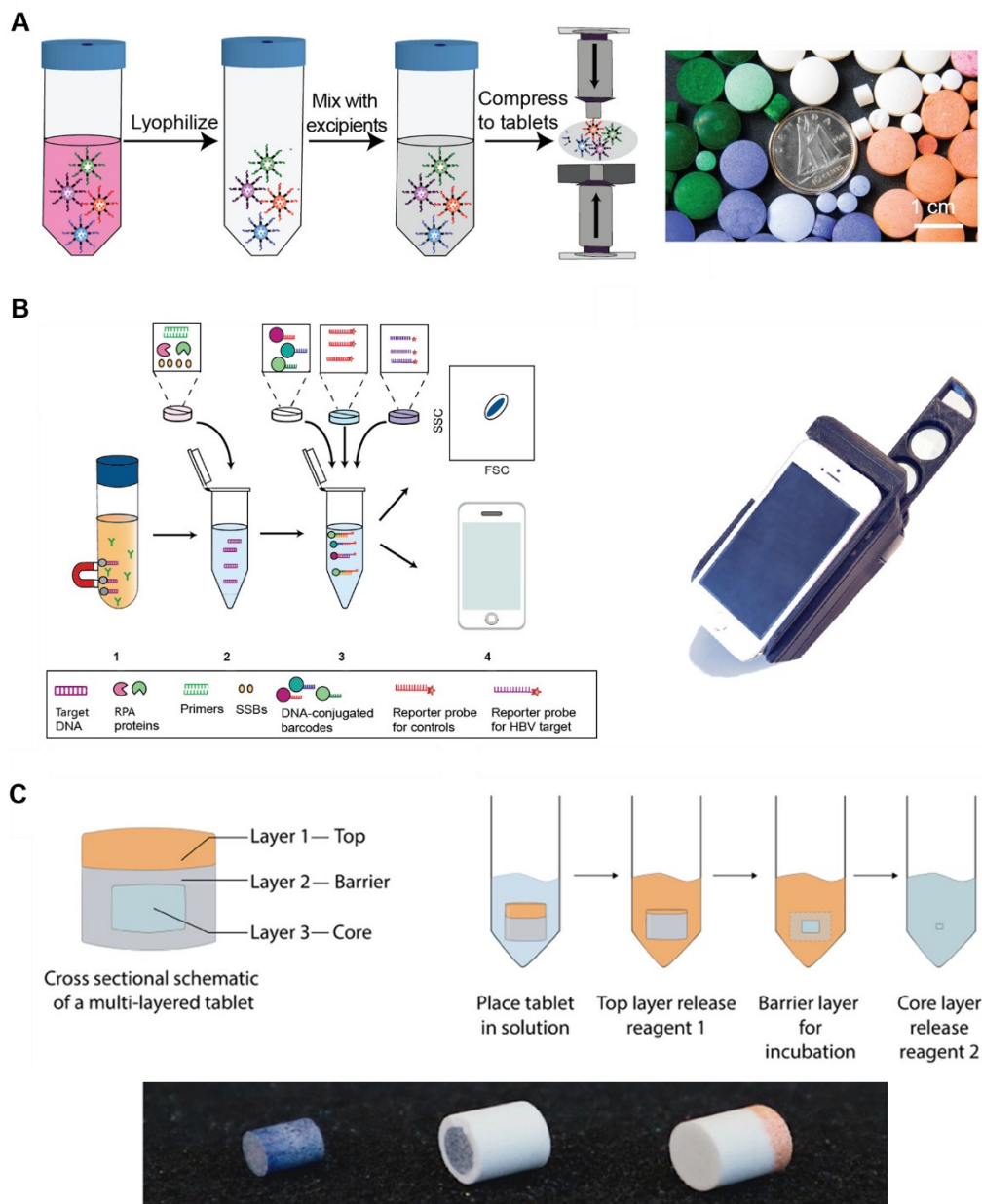


Figure 2.16. Tablets fabricated by powder compression. (A) Fabrication by lyophilizing reagents and then compressing them into tablet form. (B) Schematic of HBV detection in serum using nucleic acid amplification with color-coded tablets and smartphone fluorescence imaging. Panels (A) and (B) adapted with permission from Ref. [25]; copyright 2017 American Chemical Society. (C) Multi-layered tablets for sequential release of reagents. Adapted with permission from Ref. [324]; copyright 2022 American Chemical Society.

Overall, powder compression has proven to be a versatile and effective strategy for developing tablet-based assays. This technique allows incorporation of various powdered reagents in desired quantities and the addition of functional elements such as effervescent agents for rapid disintegration and mixing [325], or exothermic chemicals for equipment-free heating [304,326]. Advances such as multi-layered tablets for sequential reactions and integration with smartphone imaging have further broadened their applicability. Nonetheless, challenges remain in incorporating bioreagents like enzymes, which are often required in small amounts and are prone to instability. Although lyophilization has enabled their inclusion, it is an expensive and time-consuming process, which can increase manufacturing costs and limit applicability in resource-limited settings [327]. Innovations in reagent stabilization and simplified manufacturing could overcome these barriers, further advancing the potential of powder-compressed tablets as low-cost, portable platforms for POC/POU applications.

2.4.3. Tablets via Polysaccharide Encapsulation

Tablet fabrication via polysaccharide encapsulation typically involves preparing a solution with the desired concentration of reagents, dissolving film-forming polysaccharides such as pullulan or dextran in the same solution, and then drop-casting aliquots to form solid tablets containing premeasured quantities of reagents (Figure 2.17A). This process is straightforward and scalable, enabling low-cost tablet fabrication.

The first demonstration of this process was reported by Jahanshahi-Anbuhi et al. for detecting the pesticide malathion in water using the reaction between IDA and AChE enzyme [24]. To prepare the tablets, separate solutions of IDA and AChE were mixed with pullulan solution (10-12 wt%) and cast into polypropylene molds, then dried to form solid tablets. For detection, an AChE tablet was first added to the sample and incubated for 5 minutes, followed by the addition of an IDA tablet. In the presence of malathion, AChE activity was inhibited, producing a reduced color intensity from the IDA reaction that correlated with malathion concentration. Encapsulation in pullulan not only simplified fabrication but also markedly improved reagent stability. Both IDA and AChE retained activity for at least two months at room temperature, whereas their unencapsulated counterparts lost activity within a week. A similar stabilization effect was observed for Taq DNA polymerase, commonly used in DNA amplification. Thermal stability was also enhanced: while unencapsulated AChE lost activity after 30 minutes at 50 °C, encapsulated AChE

retained 90% activity even after 30 minutes at 90 °C [24]. Despite these benefits, reliance on molds made the process relatively labor-intensive and time-consuming.

To further simplify fabrication and demonstrate the versatility of polysaccharide encapsulation, the same group developed an all-in-one tablet for the luciferase/luciferin assay system for adenosine triphosphate (ATP) detection [23]. This assay is particularly challenging due to the labile nature of its reagents. To prepare the tablets, all required components, including luciferase, luciferin, MgSO₄, MgCO₃, dithiothreitol, EDTA, coenzyme A, tricine, and buffer were combined in solution with 10 wt% pullulan. Aliquots of this mixture were then pipetted directly onto PET sheets and dried, forming tablets without the need for molds and thereby greatly simplifying fabrication. Using these tablets, ATP was detected by luminescence with a low detection limit of 5.6 pM, and they were also successfully applied to detect ATP from lysed *E. coli* cells with a detection limit of 3×10^3 cells/mL, comparable to a highly sensitive commercial assay kit. The tablets retained reagent activity for at least one month at room temperature, while the fabrication method was highly cost-effective, with ~USD 1 per 100 tablets.

In recent years, the application of polysaccharide-based tablets has expanded to a range of detection mechanisms and applications, primarily through our group's work. Notably, Sadiq et al. reported the first polysaccharide tablets encapsulating AuNPs, enabling aggregation-based sensing through their plasmonic properties [328]. Dextran was used to encapsulate AuNPs into tablets, which exhibited outstanding stability for a minimum of 9 months and pH-responsive behavior [329]. In follow-up studies, dextran-AuNP tablets were applied for hypochlorite detection in water [330] as well as hydrogen peroxide [331] and uric acid [27] in urine. The reducing nature of polysaccharides also allowed AuNP synthesis from gold salts and preparation of tablet solution to be achieved in a single pot at room temperature, greatly simplifying fabrication (Figure 2.17B) [27]. However, a key limitation was that the dextran formed a strong protective layer around the nanoparticles, which made aggregation difficult and limited their utility in certain detection scenarios.

Beyond their plasmonic properties, the nanozyme activity of AuNPs was also exploited to create tablet-based sensors. In one study, pullulan was used to encapsulate AuNPs into tablet form. Together with glucose oxidase (GOx) and the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB), these tablets enabled glucose detection in diluted saliva via their

peroxidase-like activity, producing a glucose-dependent grey-to-blue color change [26]. Pullulan-AuNP tablets were also applied for cysteamine detection in serum [332]. Moreover, by combining plasmonic and nanozyme functions, dual-functional dextran-AuNP tablets were developed for both uric acid detection via aggregation and glucose sensing via peroxidase-like activity [27]. However, a notable limitation was workflow complexity: since TMB could not be encapsulated in polysaccharides, it had to be used in solution format in a separate microtube, increasing assay steps [26,332]. Other applications from our group include pullulan tablets with Griess reagents for nitrite detection in soil and water [333], dual-enzyme dextran tablets containing GOx and horseradish peroxidase (HRP) for glucose detection in urine [334], and europium tablets for AuNP-based phosphate detection in water [335].

Combinatorial use of sugars has also been explored in tablet-based assays, as mixtures of stabilizers can provide superior protection compared to single-sugar formulations [336]. A recent study demonstrated this with pullulan-trehalose blends to encapsulate bacteriophages (phages) in tablet form [337]. Phages are viruses that specifically infect bacteria and hold great promise as therapies against antibiotic-resistant infections. However, effective phages must be screened against target bacteria prior to use, a process that is hampered by poor stability of both phages and assay reagents at room temperature, the need for cold-chain storage, and labor-intensive, multi-step assay procedures. To address these challenges, phages were co-formulated with luciferin-luciferase ATP assay reagents into tablets containing 10% pullulan and 0.5 M trehalose. By incorporating a distinct phage into each tablet, a solid-phase phage library was created for rapid screening against bacteria. The workflow was greatly simplified: a bacterial suspension was added to each tablet, and if the phage was effective, bacterial lysis released ATP that was simultaneously detected via luminescence within 30-120 minutes (Figure 2.17C). Importantly, the tablets maintained stability for up to four weeks at room temperature under vacuum [337]. These findings underscore the potential of tablet-based formats to not only enhance stability but also streamline phage screening into a simplified, high-throughput, one-pot detection strategy.

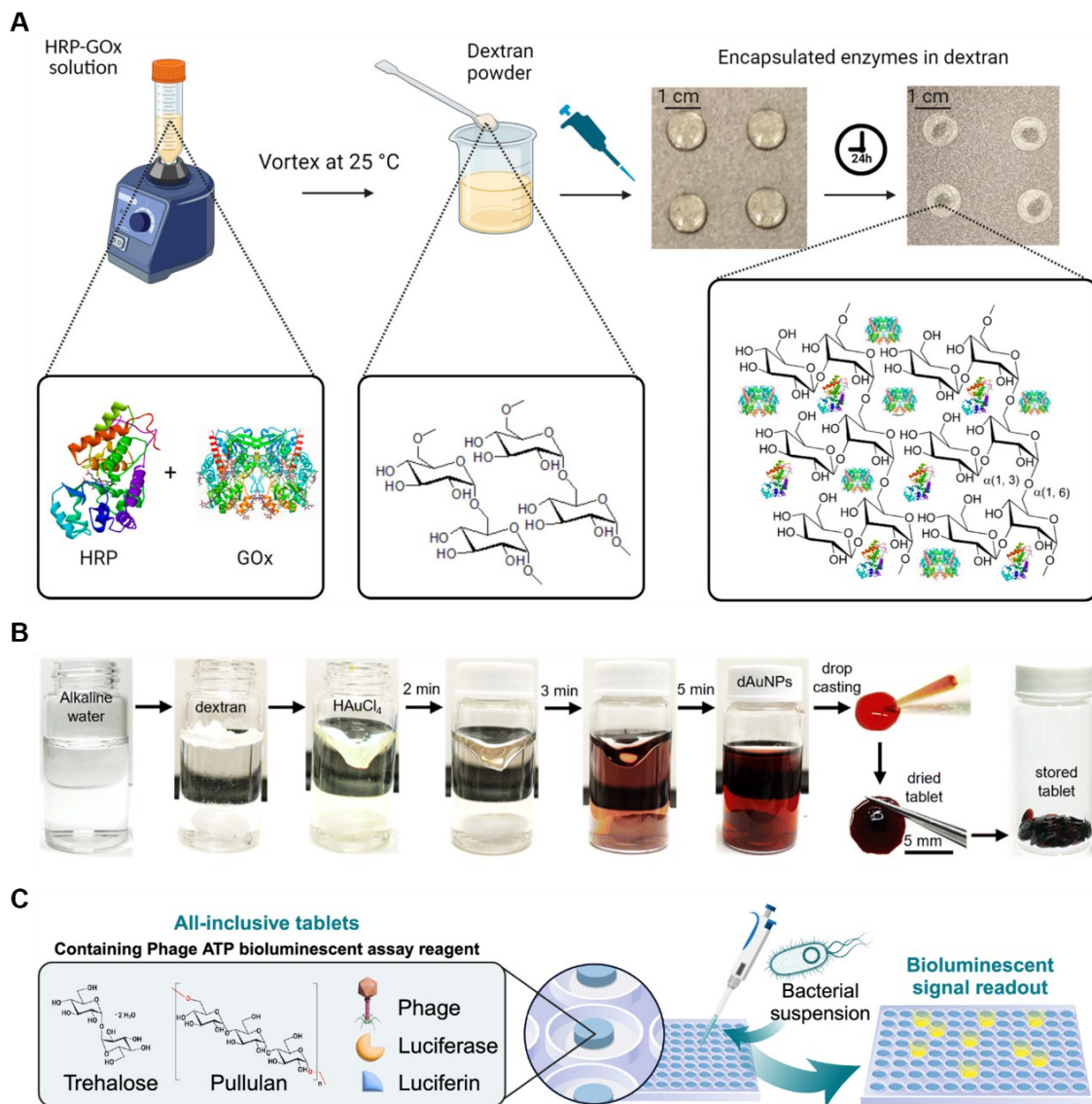


Figure 2.17. Tablets fabricated by polysaccharide encapsulation. (A) Typical procedure for tablet fabrication by polysaccharide encapsulation; example of HRP-GOx encapsulated in dextran for glucose detection in urine. Adapted from Ref. [334] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>). (B) One-pot synthesis of gold nanoparticles with dextran at room temperature followed by tablet fabrication. Adapted from Ref. [27] under a Creative Commons Attribution license (CC BY 3.0, <https://creativecommons.org/licenses/by/3.0>). (C) All-inclusive pullulan-trehalose tablets for phage-mediated ATP release from bacteria and subsequent bioluminescent detection. Adapted from Ref. [337] under a Creative Commons Attribution license (CC BY 4.0, <https://creativecommons.org/licenses/by/4.0>).

In summary, polysaccharide-based tablets offer a low-cost and straightforward approach for developing tablet-based assays. They are particularly advantageous for bioassays, where only small amounts of reagents are required, and reagent instability poses a major challenge. The stabilizing effect of polysaccharides enables the creation of room-temperature-stable assays, eliminating the need for cold-chain storage, especially valuable in low-resource settings. Nevertheless, several limitations remain. First, not all assay reagents can be effectively encapsulated in polysaccharide matrices, requiring some components to be added separately in the assay and thereby increasing procedural complexity. Second, the loading capacity of polysaccharide tablets is limited, sometimes necessitating the use of multiple tablets per assay. In addition, complete dissolution of the tablets may require vigorous shaking or vortexing, adding difficulty to the assay procedure. Looking forward, exploring a broader range of polysaccharides and their derivatives, particularly those with diverse molecular weights and properties, may help overcome these challenges and expand the inclusivity and applicability of tablet-based assay platforms.

2.5. Conclusions

Both μ PADs and tablet-based assays have witnessed significant progress in recent years, yet further advancements are needed to accelerate research and broaden their accessibility and applicability. For μ PADs, simplifying and improving channel and electrode fabrication, enhancing color formation in colorimetric assays, and boosting signal strength and reproducibility in electrochemical assays remain key goals. For tablet-based assays, increasing dissolution speed and extending applicability to more complex samples are particularly important.

When comparing the two platforms, each is best suited for specific applications. From the perspective of assay simplicity for end-users, dip-and-read formats are among the most attractive: users simply dip the device in the sample and read the results, without requiring pipettes, samplers, or graduated tubes. In this context, μ PADs excel due to their intrinsic capillary action. Their other major features include minimal sample requirements (e.g., for finger-prick tests), ease of multiplexing, flexibility for integration into wearable devices, and potential for automated multi-step assays. Nonetheless, μ PADs may face challenges such as reagent instability on paper and limited mixing efficiency within paper networks. Tablet-based assays can address these challenges by conducting reactions in free solution, which facilitates efficient mixing; employing

polysaccharide matrices with strong stabilizing effects on labile reagents; and providing a practical route to portable assays when reagents are difficult to immobilize on paper. Beyond POC/POU applications, tablet-based assays also streamline conventional laboratory workflows by providing pre-measured reagents in solid form and integrate seamlessly with equipment such as 96-well plates and UV-Vis spectrophotometers.

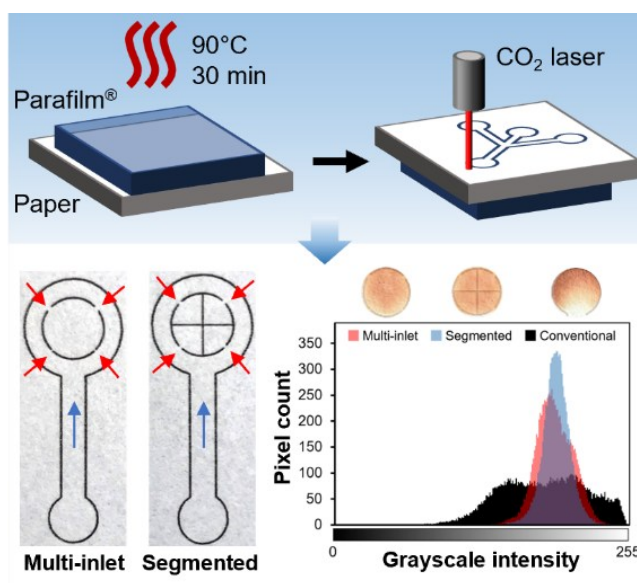
Taken together, these platforms offer a wide range of possibilities for developing low-cost, portable, and user-friendly devices for POC/POU applications, with significant potential to advance public health and safety.

Chapter 3

Engineered Detection Zone to Enhance Color Uniformity on Paper Microfluidics Fabricated via Parafilm®-Heating-Laser-Cutting

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3.1. Abstract

In this article, we first report a simple fabrication technique to create Parafilm®-laminated microfluidic paper-based analytical devices (μPADs) with a high resolution and then introduce novel engineered detection zones (D-zones) to enhance color uniformity without any chemical modifications. The Parafilm®-heating-laser cutting fabrication method comprises of three steps: 1) flattening a Parafilm® layer on paper, 2) heating in an oven at 90 °C for 30 min, and 3) CO₂ laser ablation of the patterns. This method enabled laminated μPADs with a minimum barrier width of $172 \pm 15 \mu\text{m}$, with resistance to various organic solvents and surfactant solutions. Using this technique, μPADs with two types of engineered D-zones were fabricated. The multi-inlet D-zone had four micro-inlets ($<500 \mu\text{m}$ width), enabling sample entrance into the D-zone from four directions simultaneously towards the center. The segmented D-zone had four segments each with its own micro-inlet allowing for separate reactions to occur in miniaturized segments simultaneously. Using an enzymatic glucose assay for the colorimetric reaction, the multi-inlet and segmented D-zones significantly decreased color gradient from 28.77 ± 1.49 to 12.35 ± 1.18 and $8.95 \pm 0.98\%$, respectively, compared to the conventional devices. We further used the μPADs with segmented D-zones for the detection of glucose in artificial urine in the clinically relevant range of 2-50 mM, where $R^2 > 0.99$ of the fitted curve showed an excellent correlation between the generated signals and the glucose concentrations. Also, compared to the conventional devices, the engineered D-zones showed improved precision and reproducibility and a better linear range. Finally, spiked real human urine samples were tested, demonstrating the applicability of the engineered devices for real-world applications. Overall, with a simple fabrication method and a novel design for color uniformity, this paper contributes to the advancement of μPADs and paves the way towards commercialization.

3.2. Introduction

Microfluidic paper-based analytical devices (μ PADs) have been reported in a wide range of applications from point-of-care disease diagnostics [3,338] and food assessment [114,339,340] to environmental monitoring [11,12,341] and forensics [342]. Historically, paper microfluidics were introduced in 1949 by Müller and Clegg [36,343,344], but they became popular after works of Whitesides' group initiated in 2007 [9,14,196]. Paper is a low-cost, biodegradable, and ubiquitous material allowing for pump-less fluid transfer by capillary action, making μ PADs a great candidate to meet the World Health Organization (WHO)'s ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end users) for point-of-care diagnostic devices [8,10,345,346].

Fabrication is a major step in developing efficient μ PADs that should allow for creating complex geometries, be reproducible, scalable, and easy-to-perform, and give robust μ PADs resistant to various liquids. So far, a variety of fabrication techniques have been reported [54], each with their own advantages and disadvantages, including photolithography [9], wax printing [14], screen printing [115,117], inkjet and laser printing [8,100,110,347], embossing [159,348], pen and marker drawing [125,349], and several methods incorporating laser cutting/heating [59,81,146,148,149,165–167,171]. Among these techniques, wax printing has been the most common one owing to its simplicity and rapid process. However, wax printing has a relatively low resolution, and Xerox Corporation, the main producer of wax printers, has discontinued the production of this type of printers, stimulating researchers to look for suitable alternatives [54,350].

Laser cutting/heating techniques are a promising alternative to wax printing as they allow for high-resolution fabrication of computer-designed patterns with simple procedures, especially considering that relatively low-cost laser engravers are now available. Methods using laser cutting/heating include simple paper cutting [59,171], selective melting of hydrophobic sheets into paper structure [148,149], modification of hydrophobic papers [146], and applying cutting/etching with double or single-sided laminated papers [81,164–168,351]. The former three methods either produce μ PADs with low mechanical strength that are difficult to handle, decrease the fabrication resolution, or require post-processing of the fabricated channels, which can be labor intensive. The etching of laminated papers is an effective strategy to develop paper-based devices with enhanced

mechanical strength, especially useful for the devices with low channel widths [165]. Also, lamination minimizes contamination of reagents upon contact with other surfaces and enables more consistent liquid flow within the channels as lamination prevents random contact of paper with different surfaces that may alter the flow behavior within the paper substrate [87]. Various substances have been used for lamination such as polyester [165], aluminum [81,166], and Parafilm[®] [167]. Parafilm[®] is an attractive choice as it is a low-cost, commercially available, and extensively used material in laboratories with resistance to various alkaline, acidic, and saline solutions as well as organic solvents. While Parafilm[®]-laminated μ PADs were reported in 2018 [167], the procedure required extra equipment such as a hot press machine or weights, which makes the procedure difficult to perform, leaving the gap for simpler approaches with more accessible equipment.

Aside from fabrication methods, various detection techniques have been employed on μ PADs among which colorimetric method is the most common one as it allows for equipment-free readout with naked eye or smartphones that is in line with the simplicity concept for μ PADs. However, one major challenge associated with colorimetric methods is the non-uniform distribution of the generated color on the μ PADs, which may cause errors in reading or quantification [15,57,203,241,352]. Strategies reported to address this challenge include chemical modification of the detection zone, image-processing, and modification of the μ PAD design [15]. It was observed that While chemical modification strategies (e.g., by silica nanoparticles [215] and chitosan [216]) can improve color uniformity significantly, it needs to be compatible with the assay reagents and adds to the procedural steps of the μ PAD preparation. Image-processing techniques (e.g., color mapping [221] and machine learning [229]) mitigate errors in the quantification step but do not prevent the source of the error, i.e., non-uniform color distribution, in the first place. Among design modifications for 2D μ PADs, it was reported that the diamond-shape detection zones partially improved color uniformity for glucose assay compared to the typical circle ones [196]. In another study, adding an uptake zone before the detection zone resulted in improved color uniformity for nitrite detection [185]. However, this strategy only works when the stability of the mixed reagents is the issue and is not efficient when particle movement is the cause of the color gradient.

In this paper, we first introduce a simplified method for the fabrication of Parafilm[®]-laminated μ PADs via Parafilm[®]-heating-laser-cutting (PHLC) that only requires a typical oven besides a

laser engraver machine (Figure 3.1). The fabricated channels were completely characterized in terms of minimum barrier width to contain the liquid, minimum channel width with wicking ability, resistance to various solvents and detergents, and wicking speed. Next, we introduce two novel engineered detection zones (D-zone), namely multi-inlet and segmented D-zones and investigate their effectiveness in improving the color uniformity of glucose assay compared to the conventional μ PADs. Glucose assay was chosen for the colorimetric reaction as its non-uniform color distribution is well-documented and studied [203,229]. To the best of our knowledge, this is the first time that the concept of engineered detection zone is introduced for 2D μ PADs to enhance color uniformity through controlled sample delivery into the D-zone. Finally, we show the applicability of the engineered devices in the detection of glucose in complex matrices using both artificial and real human urine samples.

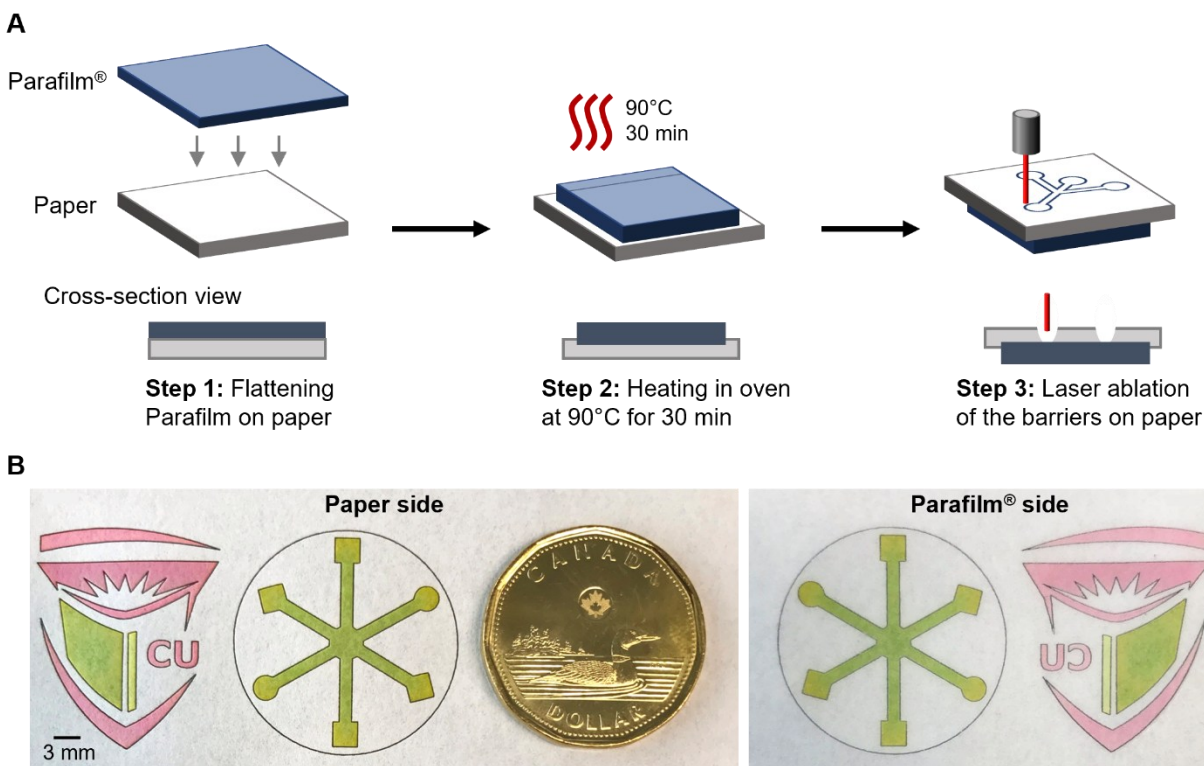


Figure 3.1. Parafilm®-heating-laser-cutting (PHLC) fabrication of paper microfluidics. (A) Schematic of the fabrication of the devices. First, a Parafilm® layer is placed on the paper and flattened with a stick to avoid any bubbles between the layers. Then, the paper with Parafilm® is treated at 90°C for 30 min in an oven, resulting in the laminated paper. The Parafilm® layer contracts with increase in temperature resulting in higher thickness and stiffness, and also the formation of the paper-Parafilm® double layer gives a structure that is mechanically stronger than either of paper and Parafilm® alone. Finally, the hydrophobic barrier design is ablated on the paper

side to create paper microfluidics. (B) Photos of different fabricated patterns, highlighting the versatility of the method in creating channels with different sizes and shapes.

3.3. Material and Methods

3.3.1. Chemicals and Materials

All chemicals and materials were used as received. Whatman grade 1 filter paper (Cat. No. WHA1001917), Whatman grade 4 filter paper (Cat. No. WHA1004917), Whatman grade 4 chromatography paper (Cat. No. WHA3004917), hexadecyltrimethylammonium bromide (CTAB, Cat. No. H6269), sodium dodecyl sulfate (SDS, Cat. No. 75746), methanol (Cat. No. 34860), 2-propanol (Cat. No. 190764), toluene (Cat. No. 179418), sodium phosphate monobasic monohydrate (Cat. No. S9638), sodium phosphate dibasic (Cat. No. S9763), glucose oxidase from *Aspergillus niger* (GOx, Cat. No. G7141), horseradish peroxidase (HRP, Cat. No. P8250), glucose (Cat. No. G8270), potassium iodide (KI, Cat. No. 221945), and HPLC water (Cat. No. 270733) were purchased from Sigma-Aldrich, Canada. Dimethyl sulfoxide (DMSO, Cat. No. D128-1) and acetone (Cat. No. A18P-4) were purchased from Fisher Scientific, Canada. Artificial urine (Cat. No. BZ104) was purchased from Biochemazone, USA, Parafilm[®] M (Amcort PLC, Cat. No. PM-996) was purchased from central chemical store at Concordia University, Canada, Neon food colors (Clubhouse) were purchased from a local Walmart store, Canada, and carbon steel trays (Betty Crocker) were purchased from a local store, Canada. Real urine samples were obtained from a healthy volunteer and were used without any further processing. This study was conducted with the approval of Concordia University's Institutional Review Board with the approval number of SC5823 and BioPermit number of B-SJA-22-01. Also, written and oral informed consent was obtained from the volunteer.

3.3.2. Fabrication of Microfluidic Paper-Based Analytical Devices (μ PADs)

The fabrication of μ PADs was done by creating Parafilm[®]-laminated papers via thermal bonding and then laser ablation of the patterns on the paper as shown in Figure 3.1. Thus, we call the method Parafilm[®]-heating-laser-cutting (PHLC). First, a Parafilm[®] layer (10 $\times$ 15 cm) was placed on the Whatman grade 1 filter paper (12 $\times$ 17 cm) on a carbon steel tray and gently flattened by a stick (e.g., wooden stirrer or glass rod) to avoid bubble entrapment in the lamination step. Then, the carbon steel tray was put in a typical laboratory oven (Thermo Scientific, Cat. No. 11-475-152)

without forced air convection at 90°C for 30 min. The tray was used to avoid non-uniform heating due to the metal rods in the oven. The melted Parafilm[®] was dragged towards the paper by gravity, resulting in thermal bonding and lamination of the paper. Of note, with increase in the temperature, the Parafilm[®] contracted and increased in thickness, which made it stiffer than the bare Parafilm[®] sheet. In addition, the bonding between the paper and Parafilm[®] gave a double layer structure that was mechanically stronger than paper and Parafilm[®] alone. The laminated papers were then taken out of the oven and cooled in the room temperature (~2 min), followed by laser ablation of the designed patterns on the paper side using a CO₂ laser cutter (Universal Laser Systems, VLS 2.30DT with a 2.0 lens; see Table 3.S3 for the details of laser settings). The patterns were designed with the open-source software Inkscape 1.1.2. In order to find the optimum conditions for the lamination, various temperatures (80-140°C), duration of heating (5-60 min), and cooling conditions (room temperature and slow cooling in the oven) were evaluated (Figure 3.S1).

3.3.3. Characterization of the Devices

The thickness of the laminated papers as well as the water contact angle of pristine Parafilm[®] sheet and Parafilm layer after laser ablation of paper were assessed by a micrometer (Mitutoyo, Cat. No. 293-832-30) and Dropometer (Droplet Lab), respectively. For the assessment of the water contact angle of the Parafilm[®] after laser ablation, an area of 2.5 × 2.5 cm paper was completely ablated from the Parafilm[®] laminated paper, and then the water contact angle was measured on the ablated area on the Parafilm[®] layer. The cross-sectional structure of the μ PADs was analyzed by an optical microscope (Westover Scientific). Also, the channels were characterized in terms of minimum barrier width, hydrophilic channel width, and flow speed using color dye solutions in HPLC water. The measurements of the width of the barriers and channels were done by taking photos and subsequently analyzing by ImageJ. For the flow speed measurements, the μ PADs were vertically dipped in a dye aqueous solution, and the flow was video-recorded for 10 min, followed by ImageJ analysis. At the time of the measurements for the flow speed, a relative humidity of ~35% and temperature of 22 °C was recorded by a thermo-hygrometer (Govee Inc.).

3.3.4. Engineered Detection Zones and Glucose Assay

Glucose assay experiments were done on the μ PADs with multi-inlet, segmented, and conventional D-zones (Figure 3.2). In the conventional D-zone, sample enters the D-zone from

one direction (bottom) that may cause leaching of the chromogenic agents and their accumulation in the edges of the D-zone, resulting in non-uniform color distribution. In contrast, to improve the distribution of the chromogenic agents in the D-zone, we designed the multi-inlet and segmented D-zones such that both of them had four inlets that allowed for sample entrance from four directions simultaneously towards the center of the D-zone, avoiding color accumulation in one edge of the D-zone. To have a fair comparison, each device had an equal circular sample zone with a designed diameter of 4 mm, a transfer zone of 2 mm width and 10 mm length, and a circular D-zone with a designed diameter of 5 mm. In addition, the multi-inlet D-zone contained two micro-inlets with the designed width of 200 μm at the bottom and two micro-inlets with the designed width of 500 μm at the top. For the segmented D-zone, the bottom micro-inlets had a designed width of 200 μm , while the top ones had a designed width of 400 μm . These micro-inlets were created by laser ablating the arcs around the D-zone and leaving short sections of paper with the desired width as micro-inlets. Both engineered D-zones were optimized with regard to micro-inlet width, number, and place to give the best color uniformity and reproducibility.

Glucose assay method was based on previous reports with some modifications [196,203] (Figure 3.2). First, a solution containing GOx (120 U/ml) and HRP (30 U/ml) was prepared in 0.1 M phosphate buffer at pH 6. The phosphate buffer was a water-based solution of 13.69 mM sodium phosphate dibasic and 86.31 mM sodium phosphate monobasic monohydrate. A 1.5 μL aliquot of the enzyme solution was added to the center of the D-zone of the μPADs with multi-inlet and conventional D-zones, while for the ones with the segmented D-zone, an aliquot of 0.37 μL was added to each segment separately. After 5 min drying at room temperature, a 0.56 μL aliquot of 1.8 M KI solution in water was introduced to the center of the D-zone of the μPADs with multi-inlet and conventional D-zones, while for the ones with the segmented D-zone, 0.14 μL was added to each segment separately and let dry at room temperature for 5 min. Of note, it is important that the KI solution does not pass through the inlets of the D-zones when introduced since KI moves with the liquid meniscus and thus most of the KI would leave the zone. To initiate the assay, 10 μL of the glucose samples were gently added to the sample zone of each device, and images were captured for analysis after ~ 30 min when the color was fixed. All glucose assays were done with artificial urine.

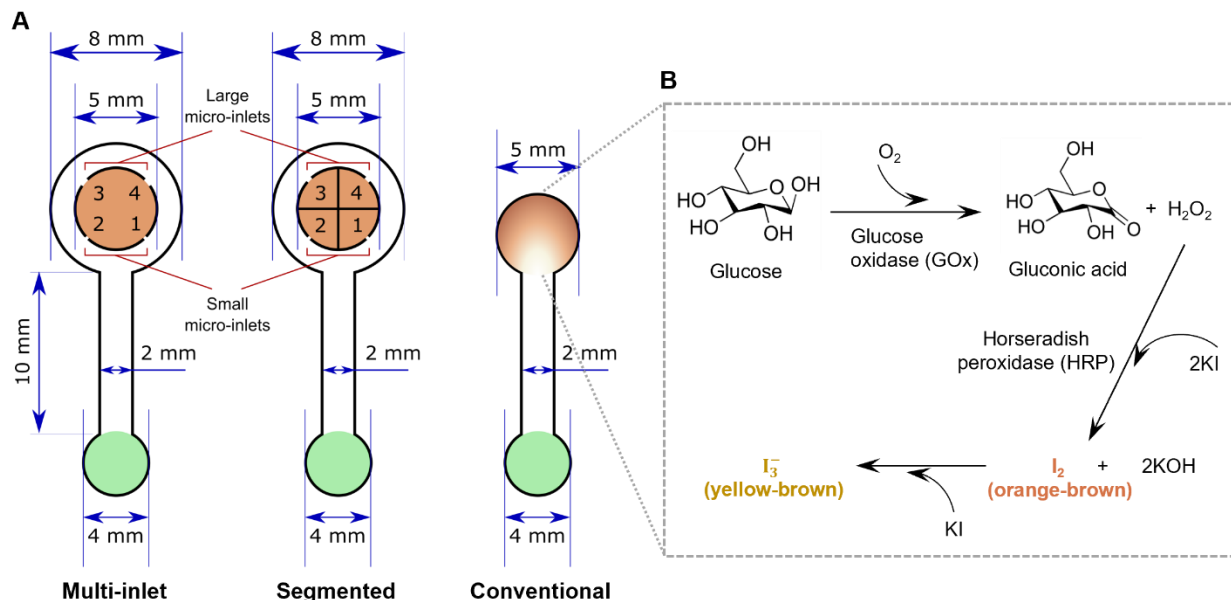


Figure 3.2. Design of the μ PADs with engineered detection zones (D-zones) besides the conventional μ PAD used in this study. (A) The multi-inlet D-zone had four micro-inlets with a designed width of 200 μ m (1 and 2) at the bottom and 500 μ m (3 and 4) at the top, allowing for sample entrance into the D-zone simultaneously from all four directions. The segmented D-zone had four segments each with its own micro-inlet with 200 μ m (1 and 2) and 400 μ m (3 and 4) width. The segmented D-zone allows for separate reactions to occur simultaneously in the miniaturized segments with one time sample introduction. The brown area shows the detection zone (D-zone) in each design, where assay reagents are deposited, and the color change is expected. The green area shows the sample zone, where the sample is introduced to initiate the assay. (B) The reaction profile of the enzymatic glucose assay with glucose oxidase (GOx), horseradish peroxidase (HRP), and potassium iodide (KI) that occurs in the D-zone of each device. Glucose oxidizes to gluconic acid in the presence of oxygen and GOx and generates hydrogen peroxide. This hydrogen peroxide reacts with KI in the presence of HRP, resulting in potassium hydroxide and iodine which is orange-brown in color. Also, iodine reacts with the excess KI and gives yellow-brown triiodide in an equilibrium reaction.

3.3.5. Image Analysis

All the devices were scanned by an office scanner (Canon, Pixma TS5020), and the images were analyzed by ImageJ software using data of unweighted grayscale color intensity. Regarding investigations on the color uniformity, for the μ PADs with the multi-inlet and conventional D-zones, the whole D-zone was selected and analyzed, while for the segmented ones, only the four segments were selected excluding the hydrophobic barriers between the segments. For the calculation of the color gradient percentage, the equation $(SD/127.5) \times 100$ was used. In this equation, SD is the measured standard deviation of the color intensity in the selected area. Also,

127.5 is the maximum possible standard deviation that can happen in an area because, by definition, the minimum and maximum color intensity of a pixel are 0 and 255, respectively, that results in 127.5 to be the maximum standard deviation. The formula gives a value of 0 for an ideal case of uniform color distribution (no color gradient) and 100 for the completely non-uniform color distribution (maximum color gradient) in a given area.

For the investigations on the analytical performance of the devices, the whole D-zone of the segmented D-zones were selected for image analysis including the hydrophobic barriers. Similarly, for the conventional devices, the whole D-zone was selected for analysis. Limit of detection (LOD) was calculated by using $(3.3\sigma)/S$ where σ is the standard deviation of the y-intercept of the linear calibration curve and S is the slope of the linear calibration curve [183,353].

Also, two-tail *t*-student tests were used to investigate the statistically significant difference between two sets of data, where *p*-values less than 0.05 indicate the statistically significant difference. All analysis was preformed by MS Excel 2019.

3.4. Results and Discussion

3.4.1. Fabrication of the Devices

The μ PADs were fabricated via the PHLC method by first laminating the filter papers by thermal bonding of Parafilm[®] in an oven and then laser ablation of the patterns on the paper side (Figure 3.1). In order to get a robust bonding and uniform lamination, we optimized the process in terms of temperature (80-140 °C), duration of heating (5-60 min), and cooling (at room temperature or slow cooling in the oven). We found 90 °C and 30 min with cooling at room temperature (~2 min) gave proper lamination with a margin of 1 cm on the Parafilm[®] layer (9.5×12 cm), while other conditions either resulted in weak bonding, gave non-uniform lamination with wrinkles, or were time-consuming (Figure 3.S1). We also observed that the thickness of the laminated papers ($328\pm10\ \mu\text{m}$) was higher than that of the stacked Parafilm[®] and paper ($301\pm1\ \mu\text{m}$) as the Parafilm[®] shrinks and increases in thickness when heated. Laser settings were optimized in terms of speed and power. High power and low speed can cut through both paper and Parafilm[®], while low power and high speed can result in partial ablation of the paper and leakage of the liquid in the channels. The details of laser settings used in this study is demonstrated in Table 3.S3. For the experiments of this study, Whatman grade 1 filter paper was chosen as the paper substrate, but the PHLC

method was successfully employed to fabricate μ PADs on other paper types such as grade 4 filter paper and chromatography paper as well (Figure 3.S2).

Water contact angle was assessed on the pristine Parafilm[®] sheet and was compared with the water contact angle of Parafilm[®] layer after laser ablation of the paper to investigate if laser ablation has any effect on the hydrophobicity of the Parafilm[®] (Figure 3.3A). A water contact angle of $108 \pm 5^\circ$ was found for the pristine Parafilm[®] sheet, while the water contact angle of Parafilm[®] after laser ablation of the paper was $109 \pm 5^\circ$. These results indicate that the hydrophobicity of Parafilm[®] after laser ablation of paper was similar to that of the pristine Parafilm[®] sheet. It is noteworthy that laser ablation may alter surface roughness, and in turn, surface wettability characteristics which process is dependent on the laser ablation settings and the substance being ablated [354,355]. However, our results suggested that the surface wettability of Parafilm[®] did not significantly change after ablation possibly due to the fact that we used laser settings such that the laser only cuts the paper with minimal impacts on Parafilm[®]. Next, as shown in Figure 3.3B, the cross-section of a sample μ PAD was studied by optical microscopy indicating the double-layer structure of the laminated devices. Also, it was observed that the laser ablation is not uniform through the depth of the paper, and the formed hydrophilic channel has a slightly lower width at the top, i.e., the microchannel roof.

Finally, the designed widths of the channels were compared with the actual widths after fabrication to assess the accuracy of the laser cutting process (Figure 3.3C). Channels with different widths were fabricated both in the horizontal and vertical direction. The fitted linear curve with an $R^2 > 0.99$ indicated a good agreement between the designed width of the channels compared to the fabricated ones. Of note, the vertical channels had an 18-120 μm higher measured width compared to that of the horizontal channels with the same designed width, probably due to the elliptical shape of the laser beam focus. While at higher channel widths such a difference may be negligible, this difference can cause errors in flow patterns when working with narrow channels and should be considered in the fabrication of the devices.

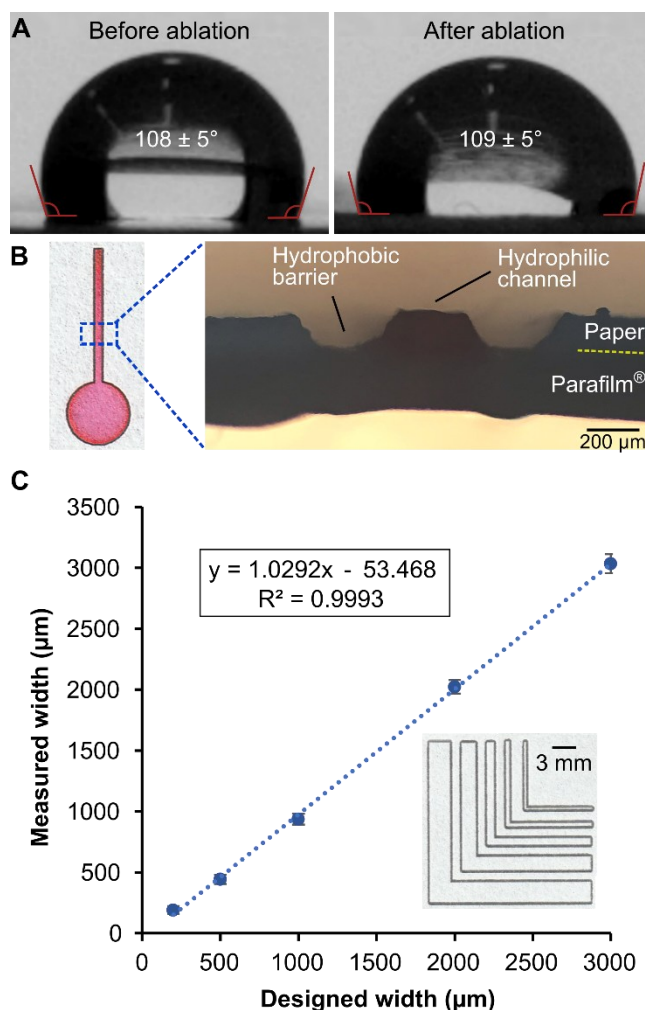


Figure 3.3. Parafilm® wettability and evaluation of laser-cutting accuracy. (A) Comparison between the water contact angle of unmodified Parafilm® (left) with $108 \pm 5^\circ$ and Parafilm® after laser cutting of the paper (right) with $109 \pm 5^\circ$ ($n=3$). (B) Top view and cross-sectional microscopic image of a sample channel fabricated via the PHLC method. (C) Assessment of the accuracy of the laser cutter in the fabrication of channels with different widths including the designed widths of 200, 500, 1000, 2000, and 3000 μm . Each data point is the mean $\pm$ standard deviation of the replications ($n=3$, some error bars are hidden behind the data points; too small to visualize).

3.4.2. Characterization of the μPADs

After optimization of the fabrication process, the μPADs were characterized in terms of minimum barrier width, minimum hydrophilic channel width, resistance to various solvents and solutions, and flow speed. These characteristics are important features of μPADs that determine the applicability of the devices in different conditions and sensing approaches.

Figure 3.4A shows the fabricated devices with different widths of hydrophobic barriers. The minimum barrier width that could completely contain the red dye aqueous solution inside the devices was $172 \pm 15 \mu\text{m}$. This value is much less than the minimum barrier width of some of the other common methods such as wax printing ($467 \pm 33 \mu\text{m}$), photolithography ($248 \mu\text{m}$), screen printing ($248 \mu\text{m}$), and laser ($400 \mu\text{m}$) and inkjet ($302 \mu\text{m}$) printing, indicating the high resolution of the method [54]. Only a few other methods have shown better or similar resolution ranging from 39 to $137 \mu\text{m}$, including other laser cutting methods [117,166,167] as well as UV-lithography [137] and direct-write photopolymerization [147]. Also, it is noteworthy that the resolution can further be enhanced by using laser cutters with higher resolution or lenses with smaller focal spot size. Next, we analyzed the minimum channel width that allowed for liquid wicking for at least 1 cm. As shown in Figure 3.4B, the minimum channel width with efficient wicking ability was $269 \pm 32 \mu\text{m}$ below which either the channel did not wick the liquid or the wicking was slower than the evaporation rate. These results are consistent with reports in the literature stating that, depending on the paper type, there is a threshold for the channel width below which there would be no observable wicking behavior [81].

We tested the resistance of the μPADs in containing different types of solvents and solutions without leakage. As shown in Figure 3.4C, the μPADs with the barrier width of $\sim 200 \mu\text{m}$ effectively contained 1 Wt.% solution of SDS and CTAB surfactants, which concentration was above the critical micelle concentration for each of them [113]. The devices were also resistant against organic solvents including dimethyl sulfoxide (DMSO), acetone, and methanol. Of note, as a comparison, wax printed channels cannot hold any of the mentioned solvents and solutions, indicating the wider range of applicability for the μPADs proposed in this study [14,113,167]. Also, the devices kept their resistance against these solvents and solutions for a minimum of 2 months when stored in open air at ambient conditions, demonstrating that the attachment between paper and Parafilm[®] remains robust over time (Figure 3.S3). However, leakage was observed with other organic solvents such as 2-propanol and toluene. In the case of 2-propanol, the solvent readily passed through the barriers, but after its evaporation, the channels were functional. As proposed before, this characteristic can be useful for washing purposes in some bioassays [14].

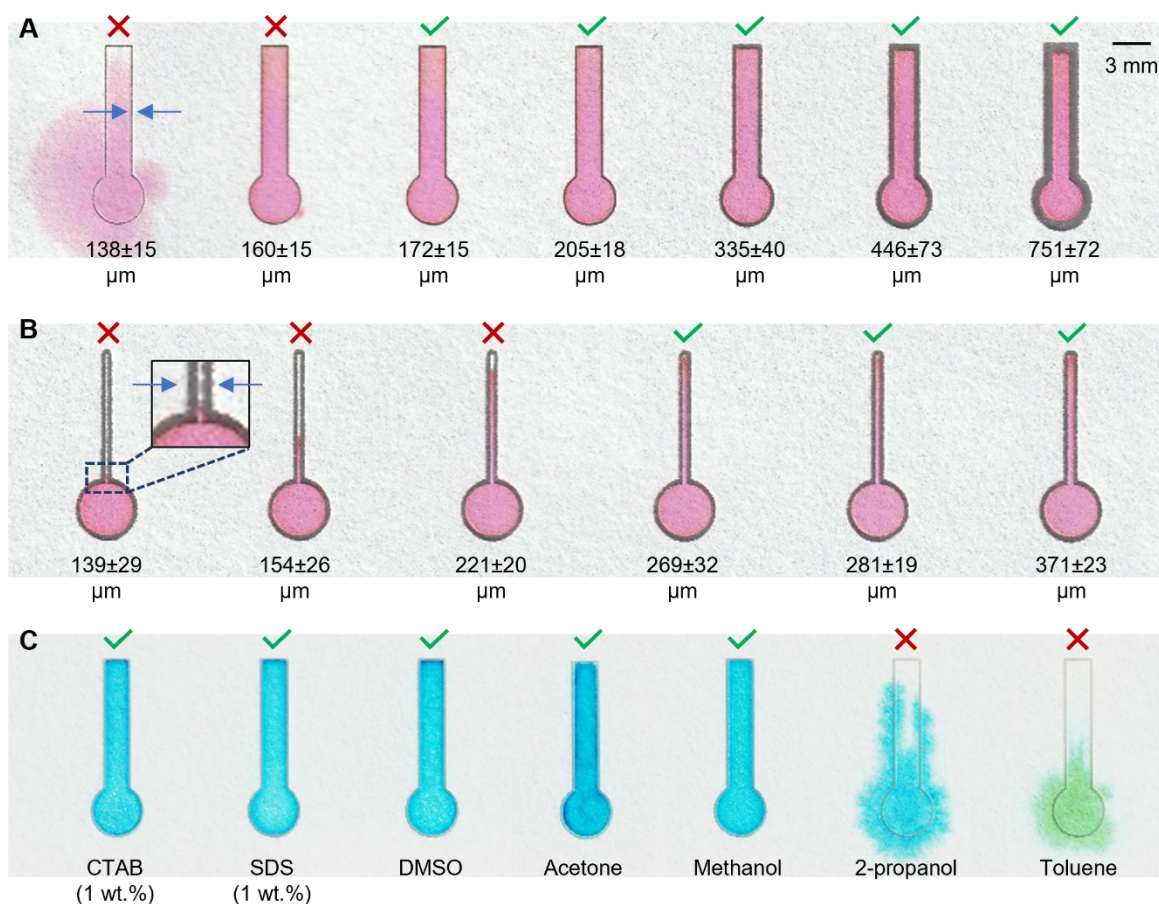


Figure 3.4. Characterization of the PHLC fabricated μ PADs. (A) Typical μ PADs with a reservoir (4 mm diameter) and a straight channel (1 cm) were fabricated with different barrier widths and tested with 8 μL of red dye aqueous solution. The minimum barrier width required to effectively avoid leakage throughout the devices was $172 \pm 15 \mu\text{m}$ ($n=7$). (B) Devices with different widths of hydrophilic channels were fabricated and tested with 5 μL of red dye aqueous solution. The minimum width of a channel capable of wicking the color dye solution for at least 1 cm was $269 \pm 32 \mu\text{m}$ ($n=7$). (C) Barrier resistance towards different solvents and solutions throughout the devices, tested with 8 μL of different samples ($n=10$).

Next, the wicking velocity of liquid in the μ PADs with different channel widths was investigated to understand how the width of the channels influences the wicking velocity. μ PADs with the channel widths of 0.5, 1, and 2 mm were dipped in a blue dye aqueous solution vertically, and the travelled distance of the liquid was recorded in 10 min. As shown in Figure 3.5, increasing the channel width resulted in the increase of the flow velocity, with the highest velocity of $3.16 \pm 0.29 \text{ mm/min}$ for the 2 mm channel and the lowest velocity of $2.29 \pm 0.18 \text{ mm/min}$ for the 0.5 mm channel. These findings support reports in the literature that wicking velocity has a direct relationship with the channel width of paper-based devices [59,81]. Of note, Lucas-Washburn

equation is frequently used as the predicting model for wicking behavior in paper-based microfluidics [87]: $L = \sqrt{\frac{\gamma D t \cos\theta}{4\eta}}$, where L is the distance travelled by the liquid fore front in the channel, γ is the surface tension of the liquid, D is average pore size of the substrate, t is the total travel time, θ is the contact angle between the liquid and the substrate, and η is the liquid dynamic viscosity. Nonetheless, the model does not account for channel width, indicating the need for further investigations for more accurate models such as the work of Castro et al. [81,356].

For the experiments in the next sections, 172 μm barrier was used for the outline of all devices, while the barriers inside the segmented D-zones were $\sim 150 \mu\text{m}$. We used the barriers inside the segmented D-zones at minimum possible thickness so that these barriers occupy as less space as possible in the D-zones and more space remains for the color formation. It should be noted that since the liquid flow at the D-zone was slowed and only a portion of the liquid was delivered to the D-zone, the $\sim 150 \mu\text{m}$ barrier thickness was strong enough to keep the liquid from penetrating to other sections in the D-zone.

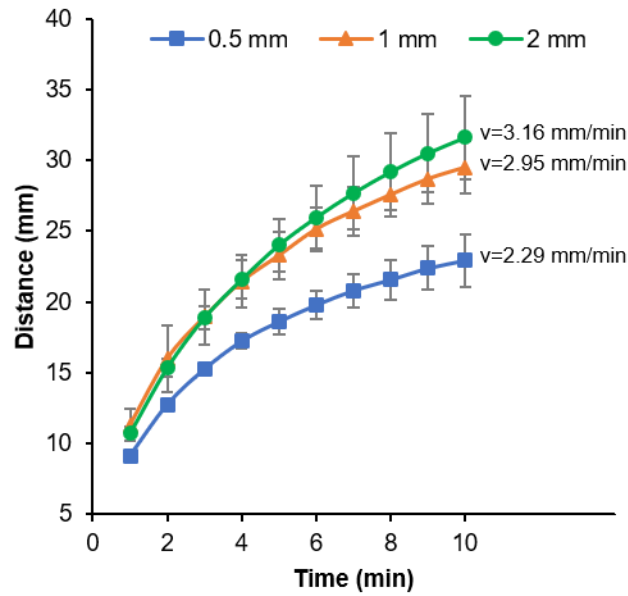


Figure 3.5. Effect of channel width on the wicking velocity. Channels with 0.5, 1, and 2 mm width were fabricated via the PHLC method and dipped in color dye aqueous solution vertically. The travelled distance of the liquid inside the channels was recorded within 10 min. The velocities were calculated based on the distance traveled after 10 min. Each data point is the mean $\pm$ standard deviation of the replications ($n=3$).

3.4.3. Engineered Detection Zones

After successful fabrication of the μ PADs via Parafilm[®]-heating-laser-cutting and investigation of the fluid behavior in the channels, here, we introduce the novel engineered detection zones for the enhancement of color uniformity. Colorimetric μ PADs have a spot where color change occurs, and we call it here the detection zone (D-zone). Conventionally, in 2D μ PADs, the D-zones have a single liquid entrance, where the sample enters into the D-zone and mixes with the pre-deposited reagents, resulting in a color change. Nonetheless, sample entrance from one direction may cause the “wash-off” and leaching of the molecules towards the edges of the D-zone, resulting in non-uniform color formation. We hypothesized that if the sample could enter from different directions simultaneously towards the center of the D-zone, a more uniform color distribution would be achieved.

Consequently, as shown in Figure 3.2 and 3.6, we introduced two engineered D-zones: multi-inlet and segmented D-zones. The multi-inlet D-zone had four micro-inlets, enabling sample introduction from four directions towards the center of the D-zone. The segmented D-zones also had four micro-inlets but with four separate segments, allowing for simultaneous reactions in each segment. In both designs, the bottom micro-inlets had a narrower width than the ones at the top, and thus, the liquid flow was slower through the bottom micro-inlets, allowing for almost simultaneous liquid entrance from four directions towards the center of the D-zone ([Movie 3.S1](#)). In addition to the micro-inlet width, the number of the micro-inlets, their place around the D-zone, and sample volume were other influential parameters that were optimized to give the best color uniformity.

To test if the engineered D-zones are able to provide better color uniformity, we used enzymatic glucose assay with potassium iodide as the model reaction because a number of studies have reported non-uniform color distribution of this assay [203,229]. As shown in Figure 3.6C, after the entrance of the glucose sample into the D-zone of the multi-inlet device, a cross-shaped strong brown color was formed at the center of the D-zone after 5 min. The color gradually spread throughout the D-zone, giving an almost uniform color after ~15 min. Similarly, in the segmented D-zone, an arc-shaped brown color was formed in each segment directed towards the center of the D-zone, which spread in each segment through time and gave a uniform color distribution (Figure 3.6D). However, in the conventional D-zone, a dark brown color was formed at the top of the D-

zone and gradually spread towards the bottom but ended in a non-uniform distribution of color, with the top being more intense. Of note, the devices with multi-inlet and segmented D-zones showed lower assay duration due to having a higher surface area by including the surrounding area of D-zone, which in turn led to a higher evaporation rate and faster termination of the reaction. The assay duration for conventional, segmented, and multi-inlet devices were 30 ± 1.7 , 27.7 ± 0.6 , and 23.7 ± 1.5 min, respectively, which are in accordance with those reported in the literature for glucose bioassays, with variable assay time up to 30 min [59,159,196,207,215,216,218,357]. Further improvement of the design to minimize the required sample and reduce the sample uptake time towards the D-zone can shorten the assay duration, which is desired according to the ASSURED criteria. Next, some leakage can be observed around the engineered D-zones, which is because we used an excess amount of reagents in the D-zones to make sure that the formed color will occupy the whole D-zone, and the leakage has no influence on the analytical performance of the devices (see section 3.4).

It appears that the reason for better color uniformity in the engineered D-zones roots in the lower travelling distance for the colored products of the reactions, i.e., iodine and triiodide (Figure 3.2). It is well documented that the generation of color gradient in this glucose assay in the conventional devices is due to the free movement of yellow-brown iodine and triiodide molecules with the liquid meniscus [203]. Thus, in the multi-inlet and segmented D-zones, initially, four liquid menisci were formed, resulting in the formation of four arc-shaped colors that met each other near the center of the D-zone. Then, through time, the color spread throughout the whole D-zone. Same scenario happened for the conventional D-zone, but as the initial color was formed at the top of the D-zone, the molecules had a higher traveling distance towards the bottom of the D-zone compared to that of the multi-inlet and segmented ones, resulting in a high color gradient from top to bottom.

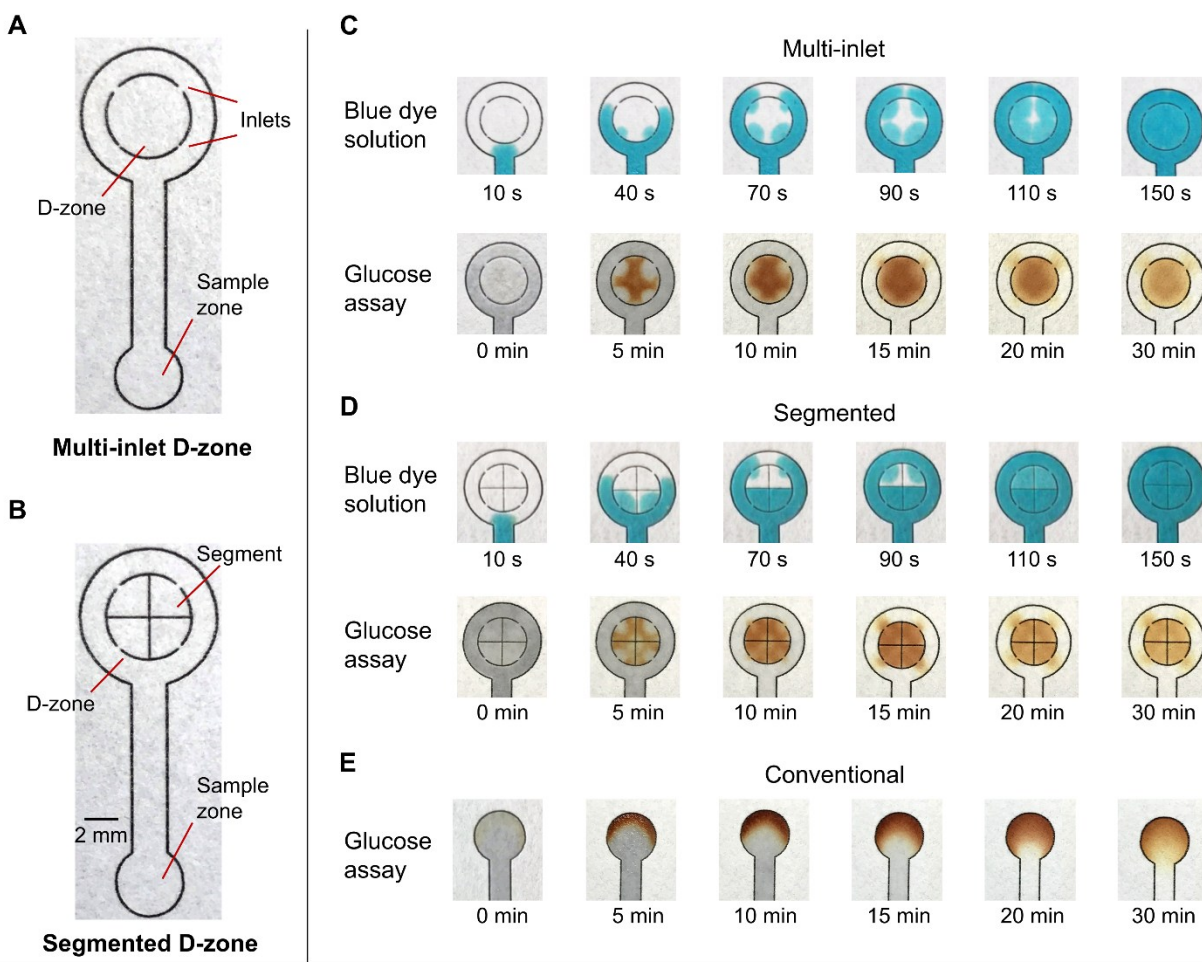


Figure 3.6. Engineered detection zones (D-zone). (A) Actual image of a μ PAD with multi-inlet D-zone, containing four micro-inlets for simultaneous sample entrance into the D-zone. (B) Actual image of a μ PAD with segmented D-zone, allowing four separate reactions occur simultaneously in the D-zone. (C) Time-lapse image of the liquid entrance into the multi-inlet D-zone (top) and glucose assay color formation over time (bottom), which reached to a fixed color after 23.7 ± 1.5 min ($n=3$). (D) Time-lapse image of the liquid entrance into the segmented D-zone (top) and glucose assay color formation over time (bottom), which reached to a fixed color after 27.7 ± 0.6 min ($n=3$). (E) Time-lapse image of the glucose assay color formation in a conventional D-zone, which reached to a fixed color after 30 ± 1.7 min ($n=3$). For all devices, 10 μ L sample of 10 mM glucose in artificial urine was used.

We further assessed the color distribution in each of the D-zones quantitatively. A 10 μ L sample of 10 mM glucose was added to each device, followed by scanning and analysis by ImageJ after ~ 30 min of reaction. As shown in Figure 3.7A, the histogram plot of the pixel count versus color intensity of the D-zones was the narrowest for the segmented one among the three types of D-zones, indicating less dispersion of color intensity values throughout the D-zone and better color uniformity. The Box and Whisker plot was also depicted to quantify the color distribution (Figure

3.7B). The interquartile range (IQR) obtained for the segmented D-zone was 14, while for the multi-inlet and conventional D-zones values of 22 and 58 were obtained, respectively. The IQR is a representative of the statistical spread of data, showing the range in which the middle 50% of the data is dispersed. Also, mean color intensities of different selected areas on the detection zones showed much lower variation for the segmented and multi-inlet D-zones compared to the conventional devices (Figure 3.S4). However, better average color intensity was observed for the conventional D-zone with a mean intensity of 181.17 ± 2.33 compared to 190.41 ± 1.13 and 191.93 ± 3.64 for the multi-inlet and segmented D-zones, respectively. The higher color intensity of the conventional D-zone was probably due to the lower surface area of the μ PADs with conventional design that led to lower liquid evaporation rate, and in turn, more time for the reaction to occur as well as accumulation of chromogenic agents near the edges with the liquid flow that resulted in higher color intensities. The percentage of color gradient was assessed in each of the D-zones, which is proportional to the standard deviation of the color intensity inside the D-zone. As shown in Figure 3.7C, both multi-inlet and segmented D-zones showed significantly lower color gradient compared to the conventional D-zone with the values of 12.35 ± 1.18 , 8.95 ± 0.98 , and 28.77 ± 1.49 for the multi-inlet, segmented, and conventional D-zones, respectively. These values translate to $\sim 57\%$ and $\sim 69\%$ decrease in color gradient for the multi-inlet and segmented D-zone, respectively, compared to the conventional one.

Non-uniform color distribution has been considered as one of the major challenges of colorimetric assays on μ PADs, especially for semi-quantitative analysis with naked eyes and quantitative analysis with imaging devices such as smart phones [15,57,203,241,352]. Heterogenous color formation has been reported to adversely impact accuracy, precision and reproducibility, and reliability of the results for different assays (e.g., glucose and nitrite) [155,181,215,351]. Also, color heterogeneity can generate uncertainty in the results interpretation as the results can be affected by the selection of the area to be analyzed for color intensity [204]. Various efforts have been made to address this problem, including proper choice of the substrate [59], chemical treatment of the substrate [155,183,215–218], applying image processing techniques [221,229,358], and design of the μ PADs [185,196,209,210,214,359]. While these methods have achieved important improvements, they suffer from limitations such as increased labor-intensiveness and complexity of procedures, limited enhancement in color uniformity, or limited applicability of the method to only a specific assay. Regarding the design of the μ PADs, a few

studies have improved the color uniformity by complex multi-layer design of 3D μ PADs and controlling the fluid transport into the detection zones [209,210,214,359]. Nonetheless, to the best of our knowledge, the present work is the first study that the design of the D-zone on 2D μ PADs is manipulated to get uniform color through controlled sample entrance into the D-zone. With this strategy, no chemical modification is needed, and the design can be implemented into the device in the fabrication step, without the need for post-processing.

Altogether, both multi-inlet and segmented D-zones showed significant improvements in color uniformity compared to the conventional one. Choosing between the multi-inlet and segmented D-zones, we found that although the signal saturation time is longer for the segmented D-zone (~28 min vs ~24 min), the segmented D-zone gives a more uniform color with better reproducibility, improving the reliability of the results. The multi-inlet D-zone was susceptible to batch-to-batch variations of micro-inlets, which could influence the pattern of the liquid entrance into the D-zone, and in turn color formation, but this problem was solved in the segmented ones. Thus, we chose the μ PADs with segmented D-zones for the investigations on analytical performance. We also tested the storability of the devices with segmented D-zones, and the results showed that storing the devices in open air at ambient conditions for a minimum of 2 months did not affect the obtained color intensity and gradient when compared to the newly fabricated devices (Figure 3.S3), making them suitable for practical applications.

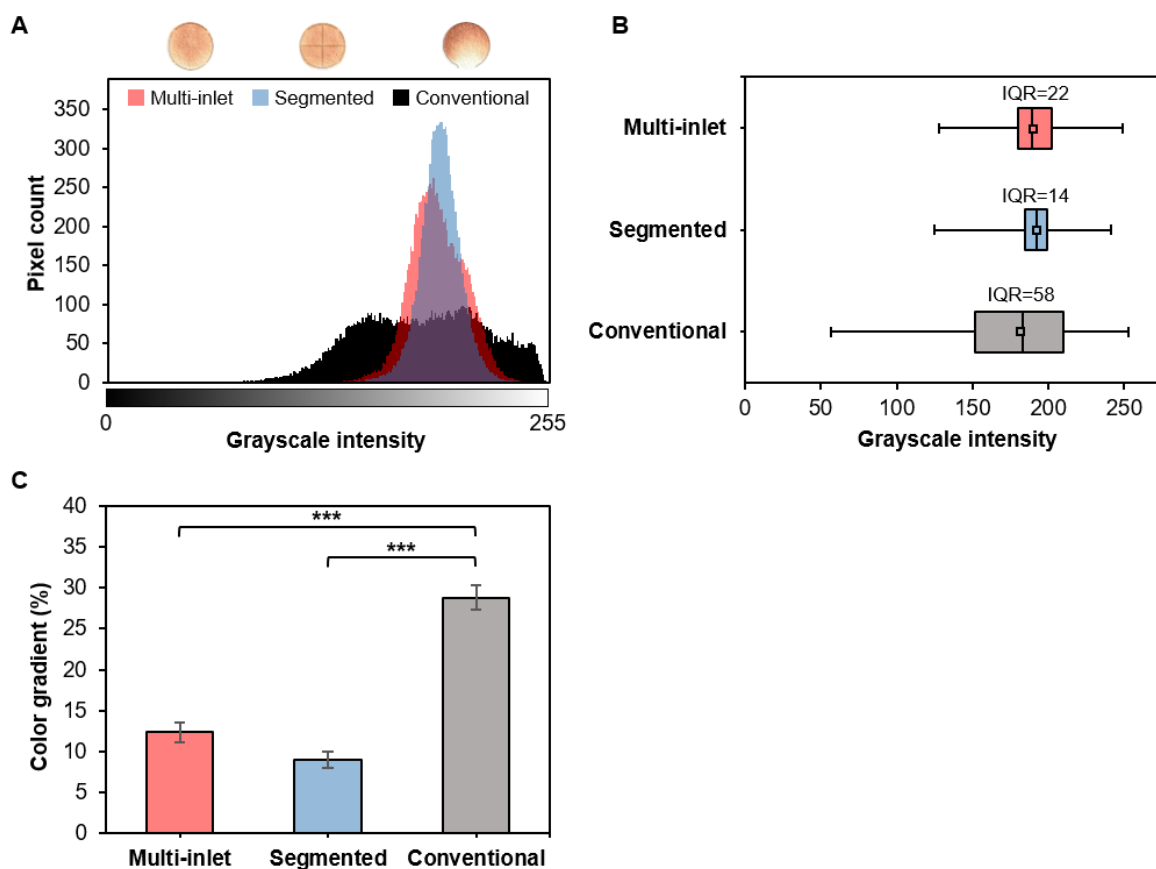


Figure 3.7. Comparison of the color distribution between the multi-inlet, segmented, and conventional detection zones. (A) Histogram of grayscale intensities, showing the number of the pixels counted for each intensity in the whole detection zone for various D-zones demonstrated on the top. The histogram demonstrated that the segmented D-zone had the lowest color distribution and variation throughout the D-zone. (B) Box and Whisker plot of the data for each D-zone, showing the spread of the color intensities in the entire D-zone, quantitatively. Square signifies the mean color intensity values, line signifies median values, box signifies the interquartile range (IQR), and the left and right lines show the minimum and maximum grayscale intensities, respectively. (C) Comparison of the color gradients between different D-zones calculated by the standard deviation of the color intensity in the whole detection zone. The segmented D-zone showed the lowest color gradient of 8.95 ± 0.98 , followed by 12.35 ± 1.18 for the multi-inlet and $28.77 \pm 1.49\%$ for the conventional D-zone. Each data is the mean of 5 replications (three asterisks imply statistically significant difference with a p-value < 0.001).

3.4.4. Analytical Performance

We proved that the engineered D-zones significantly improve color uniformity, with the segmented D-zone being the superior one. Here, we further investigate the analytical performance of the μ PADs with the segmented D-zones in glucose detection in artificial and real urine samples to understand their practicality for actual testing.

Glucose samples in the concentration range of 2-50 mM were prepared and tested with the μ PADs to create the calibration curve. As shown in Figure 3.8, the signal correlated well with the concentration of glucose in the entire concentration range when using the Hill function as a typical saturation curve, giving an $R^2 > 0.99$. The μ PADs also gave a linear response in the range of 0-10 mM, again with an $R^2 > 0.99$. Using the linear calibration curve, an LOD of 1.65 mM was calculated, which is similar to some other reports in the literature [138,159,360]. It should be noted that in comparison with conventional devices, the LOD was increased (1.26 mM for conventional), which is mainly because the distribution of color in the entire D-zone resulted in the decrease in color intensity, and in turn, increase in the LOD. Similar observation has been reported by de Tarso Garcia et al. for glucose assay with enhanced color uniformity by chemical modification of paper [155]. Despite that, the segmented D-zone showed improved precision and reproducibility with RSD of 11 independent datapoints being in the range of 0.36-6.75%, enhanced linear range, which is the range where the sensor has its best performance, from 0-6 to 0-10 mM, as well as shorter assay duration (see Figure 3.S5 & Table 3.S2). Also, the analytical performance of the segmented D-zones is suitable for applications in the clinical range as the LOD is below the postprandial glucose levels in urine for normal (2.78-5.55 mM) and diabetic people (above 5.55 mM) [361]. Changing the concentration of the reagents, sample volume, and using techniques such as standard addition can further improve the color intensity and LOD of the device.

Finally, to assess the applicability of the engineered devices for real-world applications, real human urine samples were spiked with 5.5, 7.5, and 9.5 mM glucose and tested with the μ PADs with the segmented D-zones. As shown in Figure 3.8 with the blue diamonds, the results of the measured intensities for the blank sample and the spiked samples followed the trend in the calibration curves, with the calculated recovery values of 92 ± 11 , 101 ± 7 , and $111 \pm 6\%$ for the 5.5, 7.5, and 9.5 mM spiked samples, respectively. Also, great precision was observed for the real samples with the RSD values for all data points being less than 3% for three replicates. These results show the applicability of the devices with engineered D-zones for complex matrices in real-world applications.

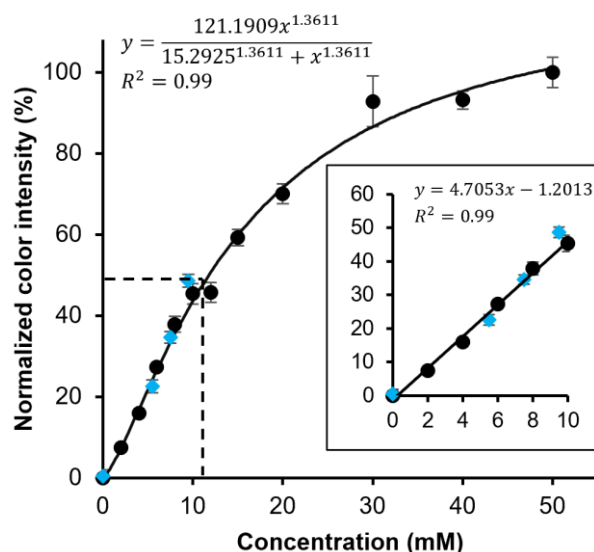


Figure 3.8. Analytical performance of the PHLC fabricated paper-based devices with segmented D-zone to detect glucose in artificial and real human urine samples. The calibration curve was obtained by testing the device with the known concentrations from 2 to 50 mM glucose in artificial urine. A Hill function was fit to the whole data with an $R^2 > 0.99$, showing the compatibility of the data with a typical saturation model. The inset shows the linear range from 0 to 10 mM, which was fitted to a linear curve with an $R^2 > 0.99$. The blue diamonds show the results of the tests of real human samples, including the blank sample and the spiked samples with 5.5, 7.5, and 9.5 mM glucose concentration. Each data point is the mean $\pm$ standard deviation of the replications ($n=3$).

3.5. Conclusions

In this paper, we first reported a simplified technique to fabricate Parafilm[®]-laminated μ PADs requiring only a typical laboratory oven in addition to a CO₂ laser cutter. Being simple and allowing for high-resolution computer-designed μ PADs, this method can be a promising alternative to the discontinued wax printers, considering that low-cost laser cutters are now available. The method allowed for fabrication of μ PADs with a minimum barrier width of 172 ± 15 μ m, with resistance to various organic solvents (DMSO, methanol, acetone) as well as surfactant solutions (SDS and CTAB). Furthermore, we introduced two novel engineered D-zones, namely multi-inlet and segmented D-zones to enhance the color uniformity of glucose assay as the example colorimetric reaction without any chemical modifications on paper. With these new designs, the color gradient in the glucose assay decreased significantly by 57 and 69% in the multi-inlet and segmented D-zones, respectively. We showed the analytical performance of the μ PADs with the segmented D-zone in the detection of glucose in artificial urine for the clinically relevant

range of 2 to 50 mM, with an $R^2 > 0.99$ for the fitted curves. The segmented D-zone also demonstrated improved precision and reproducibility and a better linear range compared to the conventional devices. Finally, we used the engineered devices to detect glucose in spiked real human urine, demonstrating their applicability for complex matrices in real-world applications.

Despite the advantages mentioned here, there are limitations associated with the fabrication method and engineered D-zones. 1) While the lamination provides mechanical strength and protection against contamination, it may hinder straightforward fabrication of 3D μ PADs. 2) In order for the engineered D-zones to work properly, the fabrication should be consistent in terms of lamination and micro-inlet creation, otherwise there might be alterations in flow speeds, resulting in loss of reproducibility. Using laser cutters with higher resolutions or lenses with a smaller focal spot size as well as precise controlling of the lamination steps can address these challenges. Aside from these limitations, the segmented D-zone provides a promising platform for multiplex assays with less required sample volumes and less occupied space that can further be investigated. Also, the engineered D-zones might work for any assay with color gradient due to the movement of the assay reagents. Overall, the methods provided here will simplify μ PAD fabrication and enhance color uniformity for colorimetric assays that will be important for acceleration towards commercialization.

3.6. Supporting Information

Optimization of the Lamination Process

We optimized the lamination process to get a robust and uniform lamination. All lamination processes were done with a 12×17 cm paper and 10×15 cm Parafilm[®] layer (before melting). We noticed that the time and temperature played an important role in lamination because we did not use any pressing for the lamination, and the process was only dependant on melting of Parafilm[®] and its partial infusion and binding to the paper through gravity. In addition to time and temperature, the cooling process (room temperature or slow cooling in the oven) was important for the uniformity of lamination.

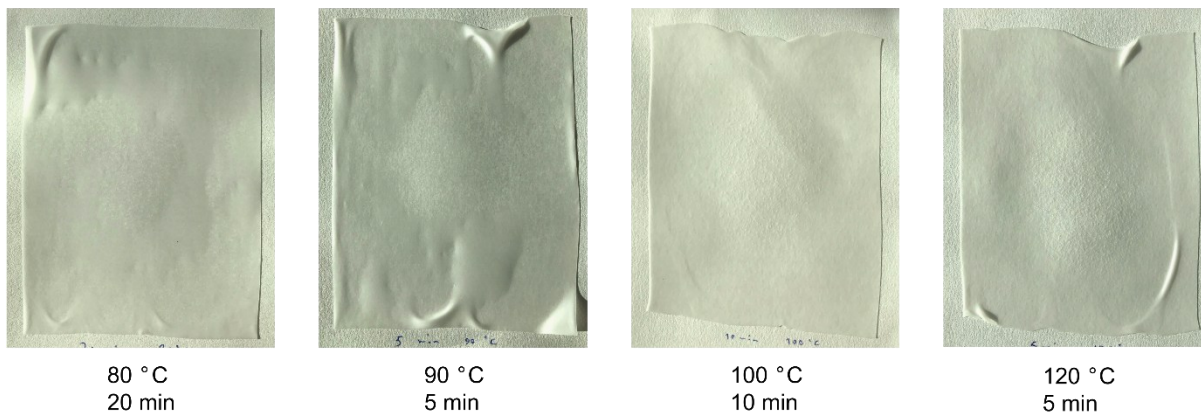
For example, 80 °C for 20 min was not enough to completely melt the Parafilm[®] into the paper and give a robust attachment as can be seen with the bubbles below the Parafilm[®] in Figure 3.S1A. Similar results can be seen for 90 °C for 5. Increasing the duration of heating at 90 °C to 30 min resulted in a complete melting and eventually strong attachment of Parafilm[®] to the paper (Figure 3.S1B). We did not observe any difference in the laminated papers when higher duration of heating up to 60 min was applied at 90 °C with those laminated in 30 min at the same temperature. Hence, we found 30 min to be the proper duration at 90 °C as faster fabrication is preferred. However, for temperatures below 90 °C, even with the increased duration up to 60 min, the attachment was not efficient.

In addition, increasing the temperature accelerated the melting process and resulted in a more robust attachment in a shorter time, but when the laminated paper was taken out of the oven for cooling at room temperature, the final lamination was not uniform. This non-uniformity is mainly attributed to the faster solidifying of the melted Parafilm[®] from the edges that can result in non-uniform lamination and bulges, especially when the temperature difference between the melted Parafilm[®] and cooling environment is high, as can be seen for the cases of 100 and 120 °C. Ideally, the cooling should be done in a homogenous manner to avoid heterogenous solidification of the melted Parafilm[®], and the best results were achieved when cooling was done naturally and slowly in the oven by turning off the oven after heating. However, for the laminated papers at 90 °C for 30 min, a proper lamination was observed when cooled at room temperature with a margin of 1 cm on the Parafilm[®] layer. Thus, 90 °C for 30 min with cooling at room temperature was chosen

as the optimum condition for the lamination process as cooling at room temperature was much faster (~ 2 min) than natural cooling in the oven (~ 1 hr).

A

Failed lamination conditions



B

Successful lamination

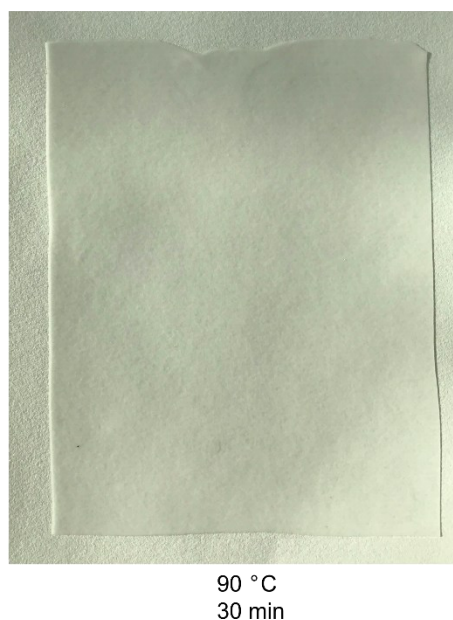


Figure 3.S1. Optimization of the lamination process. (A) Examples of failed laminated conditions which resulted in either weak bonding or non-uniform lamination. (B) A successful lamination at 90 °C for 30 min, cooled at room temperature (~ 2 min). All lamination processes were done with a 12×17 cm paper and 10×15 cm Parafilm[®] layer (before melting). The Parafilm[®] layer shrank after the process and became $\sim 9.5 \times 12$ cm.

Fabrication of μ PADs on Different Paper Types

To investigate if the Parafilm[®]-heating-laser-cutting (PHLC) method work on different paper types with different characteristics, we tested the method on Whatman filter paper grade 4 and Whatman chromatography paper grade 4, in addition to Whatman filter paper grade 1. Different characteristics of these paper types are summarized in Table 3.S1. As shown in Figure 3.S2, μ PADs were successfully fabricated on different paper types.

Table 3.S1. Some characteristics of different paper types used in this study [59].

Characteristic	Filter paper grade 1	Filter paper grade 4	Chromatography paper grade 4
Weight (g/m ²)	88	96	93
Thickness (μ m)	180	205	210
Pore size (μ m)	11	25	20

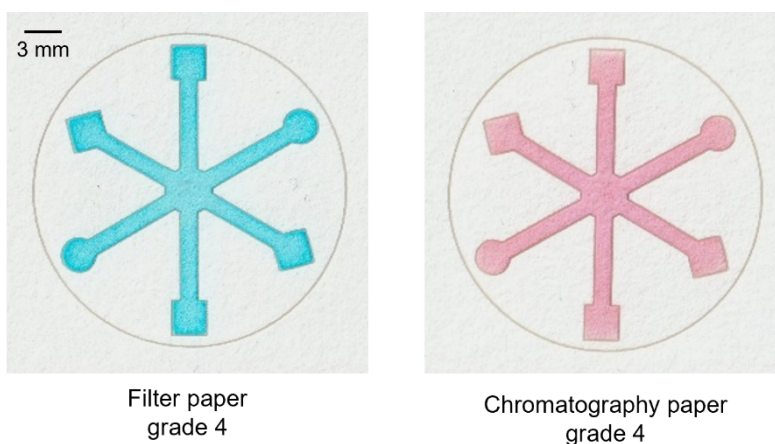


Figure 3.S2. Fabrication of μ PADs via Parafilm[®]-heating-laser-cutting (PHLC) method on Whatman filter paper grade 4 (left) and Whatman chromatography paper grade 4 (right), showing the versatility of the method with different paper types.

Effect of Storage on the Fabricated μ PADs

We investigated the effect of storage on the fabricated μ PADs to make sure the attachment between the Parafilm[®] and paper remains robust through time and the devices remain appropriately functional, especially since no external pressure was used for the lamination process in the fabrication. We assessed the effect of storage on the performance of the fabricated μ PADs with regard to their resistance to various solvents and solutions as well as color intensity and gradient. The μ PADs were stored in open air at ambient conditions for 2 months (at ~ 22 °C and 35-80% relative humidity). As shown in Figure 3.S3A, the devices remained resistant to different solvents and solutions after the storage period. For the experiments on the color intensity and gradient, the assay reagents were added to the newly fabricated and stored devices before the test. No significant difference was observed for the color intensity and color gradient values between the newly built devices and the stored ones with the segmented detection zones (Figure 3.S3B&C).

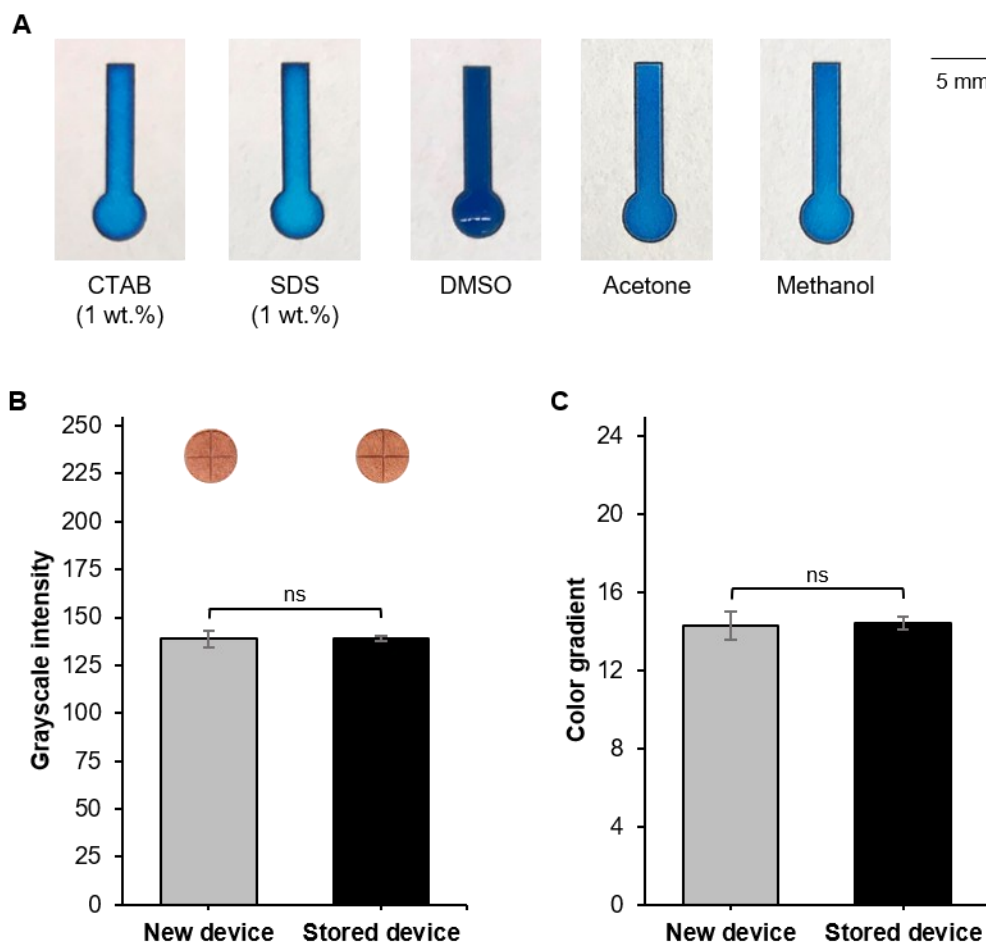
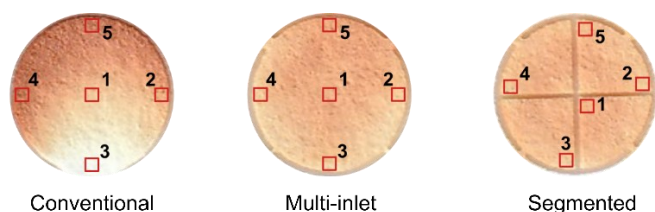


Figure 3.S3. Effect of storage on the performance of the μ PADs fabricated via Parafilm[®]-heating-laser-cutting. The μ PADs were stored in open air for 2 months at ambient conditions ($\sim 22^\circ\text{C}$ and 35-80% relative humidity) and then used for testing. (A) The resistance of the hydrophobic barriers against different solvents and solutions ($n=4$). (B) The grayscale intensity of the glucose assay with 10 mM glucose sample on the devices with segmented detection zones, with obtained values of 138.65 ± 4.48 and 139.03 ± 1.38 for the newly fabricated devices and stored ones, respectively ($n=3$). (C) The color gradient of the glucose assay with the values of $14.29 \pm 0.73\%$ and $14.40 \pm 0.31\%$ for the newly fabricated devices and stored ones, respectively ($n=3$; ns implies that there was no significant difference with a $p\text{-value} > 0.05$).

Color Intensities in Different Selected Areas of Detection Zones

Mean color intensities were measured in different selected areas in the detection zone of conventional, multi-inlet, and segmented devices to assess the uniformity of color distribution. The results suggest that the variation in the color intensities of different areas is significantly decreased from 113 on conventional devices to 13.2 on segmented devices, which means significantly higher color uniformity in the segmented detection zone.



Measured color intensities in different selected areas.

Selected area	Conventional	Multi-inlet	Segmented
1	198.9	187	188.6
2	178.4	203.6	201.1
3	245.3	202.6	201.8
4	132.3	201.4	196.5
5	140.1	177.8	193.7
Max-min	113	25.8	13.2

Figure 3.S4. Color intensities in different selected areas on conventional, multi-inlet, and segmented detection zones.

Analytical Performance of the Conventional Devices and Comparison With Segmented D-Zone

The calibration curve of the glucose detection in artificial urine using conventional devices is demonstrated in Figure 3.S5. Also, to better compare the analytical performance of the segmented D-zone with the conventional devices, the parameters of analytical performance are summarized in Table 3.S2.

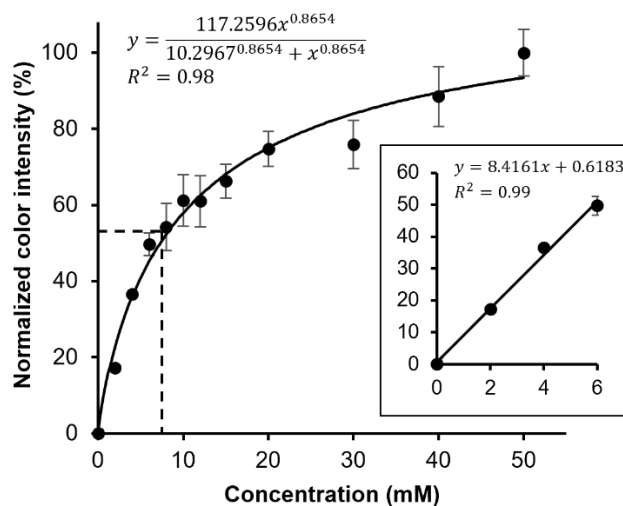


Figure 3.S5. Calibration curve of glucose detection in artificial urine on the devices with conventional detection zone (D-zone). A Hill function was fitted to the data in the range of 0-50 mM with an obtained $R^2 = 0.98$. The inset shows the linear range of the calibration curve. All data are the mean $\pm$ standard deviation of three replicates.

Table 3.S2. Comparison between the analytical performance of conventional devices with the ones with segmented D-zone.

Parameter	Conventional	Segmented D-zone	Description
LOD (mM)	1.26	1.65	Both in the applicable clinical range
Linear range (mM)	0-6	0-10	Improved; higher range for the best performance of the sensor
RSD range of datapoints (%)	1.02-11.51	0.36-6.75	Improved; better precision and reproducibility
Average RSD of data points (%)	7.24	4.20	Improved; better precision and reproducibility
Assay duration (min)	30 $\pm$ 1.7	27.7 $\pm$ 0.6	Improved; faster assay

Laser Settings

In this study, a CO₂ laser cutter (Universal Laser Systems, VLS 2.30DT with a 2.0 lens and 30W laser) was used to create the barriers, with the open-source software Inkscape 1.1.2 to design the patterns. The laser cutter had two ablation modes: 1) raster mode which is mainly used for engraving areas on a surface and 2) vector mode which is used for cutting outlines. In raster mode, the laser engraves designed area in overlapping left-to-right/right-to-left motions line by line, a process similar to regular printers. However, in vector mode, the laser follows the designed vector path, and thus both axes can move simultaneously [362,363]. The VLS 2.30DT laser system automatically treats the lines with the designed thickness above 0.0254 mm as a raster object and the lines below this thickness as a vector object. For the vector lines, the designed line thickness did not have any influence on the thickness of the created barrier on the μ PADs, and laser power and speed were the influential parameters. Also, we found that, in our case, the vector mode created μ PADs with much faster speed than the raster mode. In order to create barriers on μ PADs with different thicknesses, various parameters in both raster and vector mode had to be used that are summarized in Table 3.S3. Also, the z-axis distance and pulses per inch (PPI) was the same for all the devices equal to 0.014 inches and 500, respectively.

Table 3.S3. Laser settings used for the fabrication of different devices in this study.

Figure address	Laser power (%)	Laser speed (%)	z-axis distance (inches)	Designed line thickness (mm)	Mode
Fig. 4.1	50	100	0.014	0.02	Vector
Fig. 4.4A (device 1-4)	37-45-50-60	100	0.014	0.02	Vector
Fig. 4.4A (device 5-7)	28-25-23	30	0.014	0.3-0.5-0.8	Raster
Fig. 4.4B	25	30	0.014	0.35	Raster
Fig. 4.4C	60	100	0.014	0.02	Vector

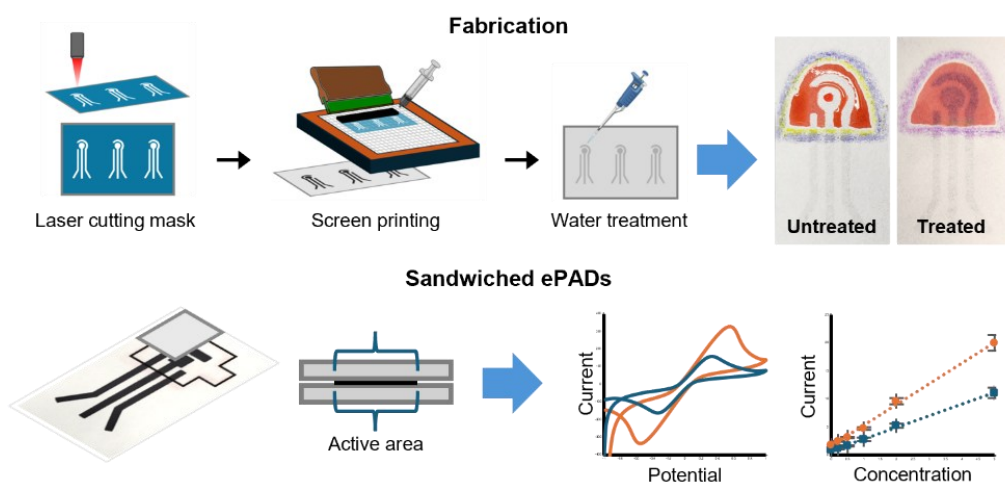
Fig. 4.6 (outline of the whole device and D-zones)	50	100	0.014	0.02	Vector
Fig. 4.6 (outline of the segmentations)	40	100	0.014	0.02	Vector

Chapter 4

Do-it-yourself screen printing of electrochemical paper-based analytical devices with water treatment and sandwiched electrodes for amplified signal

This chapter is adapted from the author's article published in *Electrochimica Acta* (2025), in accordance with the publisher's thesis reuse policy:

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4.1. Abstract

Electrochemical paper-based analytical devices (ePADs) provide a low-cost, portable, and sensitive platform for diverse analytical applications. While screen printing is widely used for electrode fabrication, it often depends on specialized equipment and outsourced mask production, limiting accessibility in laboratories and resource-limited settings. Another key yet under-addressed challenge is the presence of hydrophobic ink components, which impede paper's natural capillary flow and reduce the device's electrochemical performance. In this study, we present a do-it-yourself (DIY) screen-printing method using a portable table with a mesh screen costing under USD 50 and CO₂ laser-cut adhesive vinyl masks. This approach enables rapid, on-demand mask production and achieves electrode resolution of $286 \pm 31 \mu\text{m}$. Additionally, we introduce a simple yet highly effective post-printing water treatment to mitigate ink hydrophobicity. This method has led to a remarkable increase in oxidation peak currents, from $4 \pm 2 \mu\text{A}$ to $305 \pm 18 \mu\text{A}$, when measuring 10 mM ferricyanide/ferrocyanide by cyclic voltammetry (-1 to 1 V, 0.1 V/s), corresponding to more than a 75-fold enhancement. We further developed a novel "sandwiched" electrode configuration, enclosing electrodes between two paper layers, which achieved a 1.96-fold increase in electroactive surface area over conventional single-layer designs. Applied to bienzymatic glucose detection as a proof-of-concept, these sandwiched ePADs showed a ~ 1.8 -fold increase in sensitivity. Finally, we have validated the ePADs for glucose sensing in cell culture media, demonstrating practical applicability. Collectively, these strategies provide a scalable, low-cost fabrication method and design enhancements that improve ePAD performance and usability, particularly in low-resource settings.

4.2. Introduction

Electrochemical paper-based analytical devices (ePADs) have emerged as a promising platform for analytical applications since their introduction in 2009 [19]. These devices combine the advantages of paper, such as low cost, environmental friendliness, and capillary-driven microfluidics, with electrochemical techniques to provide high sensitivity, low limits of detection (LOD), seamless quantification using miniaturized devices, rapid detection times, and easy integration with digital technologies [16–18]. They have been applied across a range of fields, including disease diagnostics, environmental monitoring, and food analysis [17,242]. Notably, ePADs show strong potential to meet the updated REST-ASSURED criteria for point-of-care devices: Real-time connectivity, Ease of specimen collection, Scalable, Transferable, Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and environmentally friendly, and Deliverable to end-users [7].

Fabrication is the first and a crucial step in the production of efficient ePADs, which ideally should be low-cost, rapid, high-resolution, and scalable. To date, various fabrication techniques have been developed, including screen-printing [19,20,260], stencil-printing [261,262], pen- and pencil-drawing [122,263–265], inkjet printing [266,267], and laser pyrolyzing [22]. Among these, screen-printing is an attractive technique due to its ability to produce high-resolution devices at high speeds. It is worth noting that the term “screen printing” is sometimes used interchangeably with “stencil printing,” yet the two methods differ fundamentally in one key aspect: the presence of a mesh screen in screen-printing [364]. This screen allows for more controlled ink transfer onto the substrate, resulting in greater consistency and reproducibility of the printed patterns, as well as faster fabrication compared to stencil-printing [16,17,364]. However, implementing screen-printing in laboratories and low-resource settings presents significant challenges. First, it often requires specialized machinery for printing. Second, the fabrication of custom masks is typically outsourced to third-party manufacturers which is time-consuming and can cost USD 50-100 per mask [16,17,19,20,365]. Consequently, reducing the cost and complexity of screen printing would substantially broaden its accessibility and accelerate ePAD development.

Beyond fabrication, additional challenges arise when depositing ink onto paper substrates. This is mainly because commonly used inks, such as carbon or graphite-based inks, often contain hydrophobic components that hinder capillary flow within the paper structure. This reduced fluid

transport not only limits the applications of ePADs but also can lower sensor sensitivity by decreasing the contact between liquids and the electrode surface [18,21,22]. To enhance sensitivity, several strategies have been explored, including printing electrodes on both sides of the paper [294] or creating three-dimensional (3D) paper wells to utilize the top side of the electrodes [293]. However, these approaches either increase fabrication complexity or disrupt capillary flow and limit applications to spot tests or vertical flow assays. Alternatively, simple origami-based ePAD designs were developed, in which the electrodes were “sandwiched” between two layers of paper, leading to improved sensitivity [295,366]. Yet, the reason behind this enhancement and the key design parameters were not thoroughly investigated [296]. Moreover, these 3D origami designs still restrict ePAD applicability to vertical flow assays. Interestingly, another “sandwich” electrode configuration was later reported to decrease sensitivity compared to conventional 2D designs [297]. Despite these efforts, none fully resolve the persistent issue of ink hydrophobicity, a key yet under-addressed challenge. The contradictory findings also highlight the need for further investigation and design improvement for sandwich-based configurations.

Here, we address these challenges by first introducing a do-it-yourself (DIY) screen-printing technique using a portable screen-printing table that costs less than USD 50 (Figure 4.1). The screen-printing is coupled with laser cutting for mask creation that is fast and allows for on-demand mask creation. Then, we show that simple water-treatment of the ePADs after printing can minimize the hydrophobicity issue of the ink and significantly improve liquid flow and signal intensity in ePADs. Next, we introduce a novel design for “sandwiched” electrodes and thoroughly investigate various influential parameters on the device performance. Finally, as a proof-of-concept, we have detected glucose in cell culture media which is an important marker for various biotechnology applications. We believe that the methods and techniques mentioned in this study can significantly contribute to the fabrication of ePADs in low-resource settings as well as enhancing the applicability of ePADs in real-world situations.

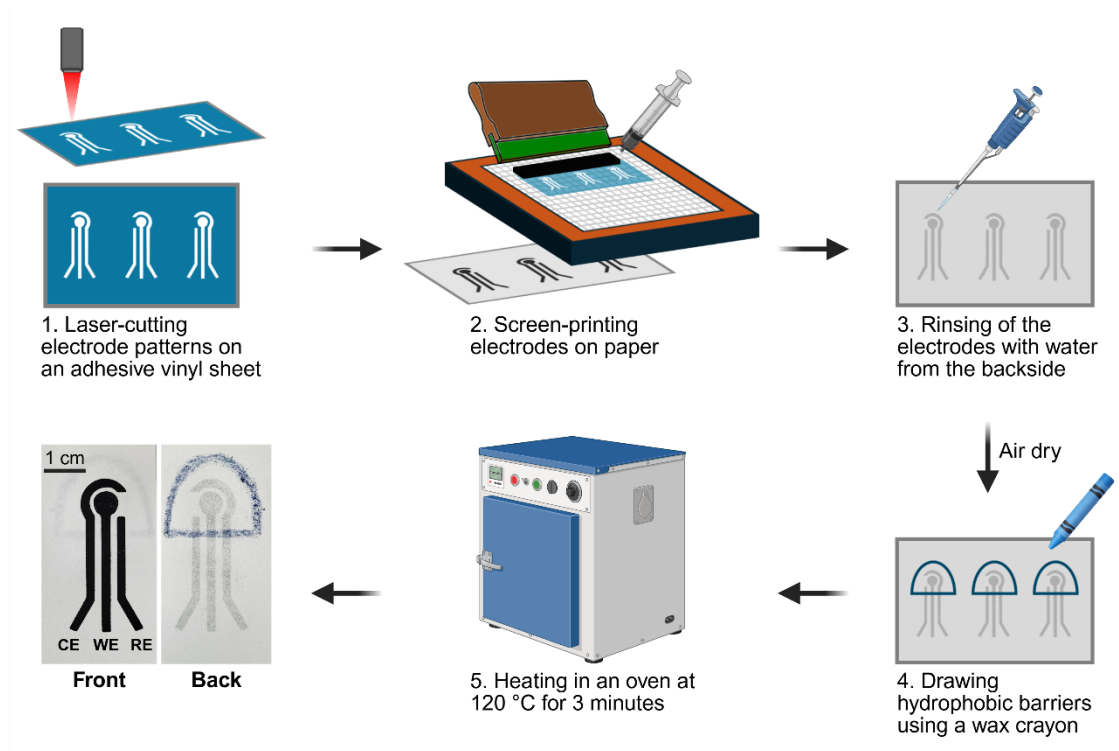


Figure 4.1. Schematic illustration of the do-it-yourself (DIY) screen-printing method for ePAD fabrication. Actual photographs of the fabricated ePADs from the front and back are shown, highlighting the counter (CE), working (WE), and reference (RE) electrodes. Figure created with BioRender.com.

4.3. Material and Methods

4.3.1. Chemicals and Materials

Whatman Grade 1 filter paper (Cat. No. WHA1001917), potassium chloride (KCl, 7447-40-7), tetrahydrofuran (THF, 360589), dibasic ester (DBE, 422053), 2-propanol (190764), methanol (34860), dichloromethane (D65100), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S9763), glucose oxidase from *Aspergillus niger* (GOx, G7141), horseradish peroxidase (HRP, P8250), and glucose (G8270) were obtained from Sigma-Aldrich (Canada). Potassium ferrocyanide trihydrate (P236), potassium ferricyanide (P232), acetone (A18P-4), and carbon/graphite ink (Ercon Inc., E3449) were purchased from Fisher Scientific (Canada). Deionized Milli-Q water and 95% ethanol were supplied by Concordia University. Dulbecco's Modified Eagle Medium (DMEM, glucose-free, 319-061-CL) was purchased from Wisent Inc. Neon food coloring (Clubhouse), wax crayons (Crayola), and transparent tape (Studio) were

sourced locally. Polyethylene terephthalate (PET) sheets (ZHluja, 6 mil; ~0.15 mm), acrylic plastic sheets (Yezinangua, 2 mm), parchment cardstock (Goefun), adhesive-backed vinyl sheets, and rubber squeegees (Exceart Inc.) for screen printing were obtained from Amazon (Canada). The portable screen-printing kit, including a table equipped with screen printing clamps and mesh screen (180/55 white mesh, Screentec, Canada), was acquired from the Concordia University Print Media Facilities (see Figure 4.S1 for more details).

4.3.2. Screen-Printing and Treatment of ePADs

The fabrication procedure for the ePADs is illustrated in Figure 4.1. Electrode patterns were first designed using Inkscape (open-source software) and cut from adhesive vinyl sheets using a CO₂ laser cutter (Universal Laser Systems, VLS 2.30DT, 2.0 lens; see Table 4.S1 for laser settings). The cut vinyl was bonded to a mesh screen, which was then mounted on the screen-printing table (Figure 4.S1). The Ink was thoroughly stirred with a spatula, transferred into a 20 mL syringe, and applied onto the screen. Using a squeegee, the ink was printed onto the paper by applying pressure. This process was repeated to produce the required number of devices. After printing, the screen was cleaned using either THF or a combination of DBE and 2-propanol. In the THF-based approach, the solvent was applied directly to the screen with a paper towel to thoroughly remove any residual ink. In the second approach, the screen was first cleaned thoroughly with DBE and a paper towel to dissolve and remove the ink, after which excess DBE was wiped off. The screen was subsequently cleaned with 2-propanol and a paper towel and then allowed to dry. Because DBE and 2-propanol are less toxic than THF, this second cleaning method is recommended. All printing and cleaning steps were conducted under a fume hood for safety.

For the water treatment of the ePADs, two methods were employed: dipping and pipetting. In both approaches, the treatment was carried out immediately after printing, while the ink was still wet. In the dipping method, each sheet was briefly immersed in a generous volume of water and then removed. In the pipetting method, water was applied to the backside of the paper using a pipette, directly targeting the electrode area. For multiple washes, the water was allowed to dry between applications before repeating the process with the pipetting method. Following treatment, the devices were left at room temperature under a fume hood for the ink to dry (22 °C, 6-8 hours). In experiments involving other chemical treatments, the procedure was similar with the pipetting method, except the treatment was applied only after the ink had completely dried to prevent

damage or dissolution of the printed electrodes. For heat treatment, the devices were placed in a laboratory oven (Thermo Scientific, Cat. No. 11-475-152) at 95-105 °C for one hour immediately after screen-printing, without any prior chemical treatment.

To create hydrophobic barriers, wax crayons were used as previously described [128,367]. Briefly, After the ePADs had dried, a wax crayon was used to define the working zone on the back using a PET or vinyl stencil. The devices were then heated in the oven at 120 °C for 3 minutes to allow the wax to penetrate the paper. Of note, we also tested creating the barriers with a permanent marker as previously reported [11], but the barriers were not strong enough and caused liquid leakage.

4.3.3. Characterization of the Devices

The screen-printing method was evaluated based on the resolution of the fabricated electrodes and the minimum electrode spacing that avoided short-circuiting. Various designs were printed on paper with different electrode thicknesses and inter-electrode distances (see Table 4.S1 for laser settings). Device dimensions were measured by scanning with an office scanner (Brother HLL2390DW) and analyzing the images using ImageJ (open-source software).

For electrochemical testing, all ePADs were fabricated in a three-electrode configuration using carbon/graphite ink, and voltages are reported relative to the pseudo-reference electrode. Cyclic voltammetry (CV) was performed using a solution containing 10 mM potassium ferricyanide and potassium ferrocyanide in 1 M KCl. A 25 μ L sample was applied to the working area from the backside of each device. Then, CV was recorded two minutes after sample introduction over a range of -1 to +1 V versus the reference electrode, at a scan rate of 0.1 V/s, using a portable PalmSens4 potentiostat (PalmSens Inc.). For all calculations, the original (raw) oxidation peak values detected by the device were used.

4.3.4. Optimization and Characterization of Sandwiched ePADs

Here, “sandwiched ePADs” refer to devices where the electrodes are positioned between two layers of paper, while “single-layer” devices have electrodes exposed on only one side. For both types, twice-washed ePADs were used.

Various device configurations were evaluated using CV to determine the optimal setup (see Figure 4.S8 for schematics). In single-layer spot tests (“SL-20 μ L spot test” and “SL-40 μ L spot test”),

20 μL and 40 μL ferricyanide/ferrocyanide sample was pipetted onto the backside of the devices at the center of the working zone, respectively. In the single-layer with clip configuration (“SL-20 μL with clip”), the ePAD was placed between two rigid plastic sheets and secured with a binder clip to hold the system together. Then, 20 μL sample was introduced from the top sample inlet. For sandwiched devices with tape (“SAN-40 μL with tape”), the ePAD was placed on a rigid plastic sheet, covered with a paper layer on top of the electrodes, and secured using transparent tape. For the sandwiched devices with clip (“SAN-40 μL with clip”), a similar paper layer was added over the electrodes, followed by a second plastic sheet, and a binder clip was used to apply pressure and hold the system together. The plastic sheets helped distribute pressure evenly across the working zone. In both sandwiched cases, 40 μL sample was added through a designated sample inlet from the top side of the ePADs.

To compare the electroactive surface areas of single-layer and sandwiched ePADs, CV was performed using 10 mM potassium ferricyanide/ferrocyanide in 1 M KCl over a potential range of -1 to 1 V at scan rates of 0.1, 0.2, 0.3, and 0.4 V/s. The original (raw) detected oxidation peak currents were plotted against the square root of the scan rates in accordance with the Randles-Ševčík equation for diffusion-controlled reversible systems [22,368]:

$$i_p = 0.446nFAC_0^* \sqrt{\frac{nFvD_0}{RT}} \quad (5.1)$$

where n is the number of electrons transferred, F is the Faraday’s constant, A is the electroactive surface area (cm^2), C (mol/cm^3) is the bulk concentration of the redox species, v is the scan rate (V/s), D_0 (cm^2/s) the diffusion coefficient of ferricyanide/ferrocyanide, R is the ideal gas constant and T (K) is the temperature.

4.3.5. Glucose Tests

Glucose detection was performed using a bienzymatic system consisting of glucose oxidase (GOx) and horseradish peroxidase (HRP), with ferrocyanide as the mediator, as previously described [369,370]. To prepare the enzyme solution, 500 U/ml solution of both enzymes was prepared in phosphate buffer 0.1 M (pH 7.2). Another solution containing 100 mM potassium ferrocyanide in 1 M KCl was prepared for the mediator. All measurements were conducted two minutes after sample addition using chronoamperometry (CA) at -0.3 V, with the current recorded at 45 seconds.

To compare and optimize device performance, various device types and reagent addition strategies were evaluated using 2 mM glucose in DI water. In the “untreated SL-20 μ L spot test” (single-layer spot test configuration on non-water-treated devices), 20 μ L of enzyme solution was pipetted onto the backside of the working zone and allowed to dry, followed by 20 μ L of ferrocyanide solution. Then, 20 μ L of sample was added to the working zone. All other configurations were twice-washed ePADs. Similar reagent and sample addition procedure was done for “SL-20 μ L with clip” test (single-layer with clip configuration). For the “SL-40 μ L/2x reagent/spot test” condition, each reagent was applied twice, and the test was performed in a spot-test configuration using 40 μ L of sample. For sandwiched configurations. “SAN-40 μ L/bottom layer/with clip”, “SAN-40 μ L/top layer/with clip” and “SAN-40 μ L/both layers/with clip”, 20 μ L of each reagent was applied to the specified layer(s), and 40 μ L of sample was added via the inlet zone. All sandwich tests were conducted using the clip configuration.

Calibration curves were constructed using twice-washed ePADs with glucose concentrations of 0, 0.2, 0.5, 1, 2, 5, and 10 mM in water, employing both the single-layer (clip, 20 μ L sample) and sandwiched (clip, 40 μ L sample) configurations. For real sample analysis, sandwiched devices were used to test spiked DMEM solutions containing 1.5, 3, and 4.5 mM glucose.

4.4. Results and Discussion

4.4.1. Fabrication and Characterization

The DIY screen-printing procedure for fabricating ePADs involves a few simple steps, as illustrated in Figure 4.1. First, the electrode pattern is cut into an adhesive vinyl sheet using a CO₂ laser cutter. The vinyl mask is then bonded to a mesh screen, and the pattern is printed onto paper using a portable screen-printing table (Figure 4.S1). This method offers two key advantages over conventional screen-printing approaches: (1) masks can be created on-demand, rapidly and at low cost, which significantly accelerates the prototyping process. With entry-level laser cutters available for under USD 1000, and even more affordable alternatives like craft cutters (<USD 300) [262], this approach is highly accessible for both research laboratories and low-resource settings; (2) the portable screen-printing table and mesh screen setup is inexpensive (only USD 49), easy to operate, and requires no specialized equipment, making it suitable for decentralized or DIY fabrication environments.

To evaluate the performance of this fabrication method, we have characterized it in terms of electrode resolution and minimum spacing (Figure 4.2). The method successfully produced electrodes as narrow as $286 \pm 31 \mu\text{m}$ and spaced as close as $339 \pm 21 \mu\text{m}$ without short-circuiting. These resolutions offer high versatility in electrode design and layout, while the fabrication process is compatible with various substrates. For example, Figure 4.S2A shows a device printed on recycled parchment cardstock resembling typical commercial screen-printed electrodes. Additionally, the method enabled at least 30 prints using a single mask without any noticeable change in printing quality (Figure 4.S2B). In terms of printing speed, the pre-printing workflow, including mask fabrication as well as ink and mesh-screen preparation, required approximately 15 minutes. The active printing stage was highly efficient: producing 10 sheets (each containing four devices) took around 8 minutes, corresponding to less than one minute per sheet and roughly 12 seconds per ePAD. Following printing, the cleaning process required an additional ~ 17 minutes. This throughput could be further increased by designing more devices per mask.

It is noteworthy that despite the significant advantages of this technique, one major challenge encountered was cleaning the screen after printing for reuse. This process was labor-intensive and required a substantial volume of solvent. Nonetheless, the use or development of water-based inks that are easier to clean could effectively address this limitation. Overall, the technique demonstrated excellent fabrication speed and resolution. In the following section, the electrochemical responses of the devices are discussed.

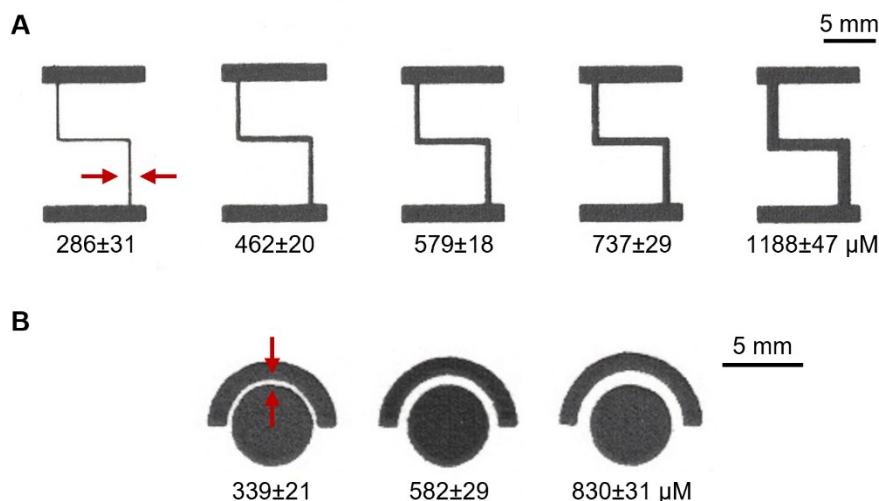


Figure 4.2. Characterization of the screen-printing method. (A) Resolution of the fabricated electrodes (mean $\pm$ SD, $n=6$). (B) Minimum electrode spacing without short circuit (mean $\pm$ SD, $n=6$).

4.4.2. Water Treatment of the ePADs

After characterizing the fabrication method, we have evaluated the electrochemical response of the devices. The ePADs featured three electrodes, all printed with commercial carbon/graphite ink. Electrochemical measurements were performed relative to the same carbon/graphite pseudo-reference electrode, rather than using standard reference electrodes such as silver/silver chloride. This configuration simplifies and accelerates device fabrication while maintaining sufficient accuracy for a range of electrochemical sensing applications [22,122].

Initially, we observed that when the sample was added to the working zone, the liquid did not fully occupy the electrode area, and efficient contact between the electrodes and the sample was not achieved. In some cases, the water droplet did not penetrate into the paper pores in the electrode region at all and instead remained on the surface when added from the backside of the device (Figures 4.3A). The droplet formed a contact angle greater than 90° , confirming that the paper network in this region had become hydrophobic (Figure 4.S3). To assess the impact on electrochemical response, we tested the ePADs using a 10 mM ferricyanide/ferrocyanide solution in 1 M KCl and cyclic voltammetry (CV). As shown in Figure 4.3B, the untreated devices exhibited very low current intensities, with oxidation peaks below $7 \mu\text{A}$, clearly demonstrating inefficient device performance. This behavior was attributed to the hydrophobicity of the ink

formulation, which, once dried on the paper, reduced the local wettability of the paper matrix and impeded capillary flow. As a result, liquid penetration into the paper pores was hindered, preventing proper interaction between the sample and the electrodes, challenges that have also been reported by other groups [18,21,22].

To address the hydrophobicity of the ink, we applied several treatments, including heat treatment at 95-105 °C for 1 hour (similar to a previous study [254]), water treatment immediately after screen printing and before ink drying, and treatment with various solvents after the ink had dried to avoid electrode damage from ink-solvent interaction (Figure 4.3B). Interestingly, water treatment yielded the best results, with an oxidation peak of 269 μ A, followed by acetone treatment (127 μ A), while other strategies showed no or negligible improvement. Electrochemical impedance spectroscopy (EIS) revealed a markedly higher charge transfer resistance in untreated devices (> 40 k Ω) compared to water-treated ones (0.14 k Ω), further confirming that water treatment significantly enhances redox activity (Figure 4.S4). These pronounced differences are mainly due to the fact that applying water to the still-wet ink prevented the paper matrix from becoming hydrophobic (Figure 4.3A), thereby enabling much better liquid-electrode contact and yielding a stronger electrochemical response.

To better understand the effect of water treatment, we performed additional characterization tests. As shown in Figure 4.S3, while water treatment restored the hydrophilicity and capillarity of the paper matrix, it did not significantly change the wettability of the electrode surface on the front side of ePADs. The water contact angles for untreated and water-treated devices were $112 \pm 6^\circ$ and $110 \pm 5^\circ$, respectively. Scanning electron microscopy (SEM) images of the backside of untreated and water-treated devices showed similar cellulose fiber structures, comparable to plain filter paper. SEM analysis of the graphite layer on the top surface of the electrodes revealed stacked graphite flakes covering the printed region, with no visible differences between untreated and water-treated samples (Figure 4.S5). This flake-like morphology is consistent with previous reports of graphite paste printed on paper [371]. Fourier-transform infrared (FT-IR) spectroscopy was used to detect functional groups responsible for possible chemical changes on the backside of the ePADs (Figure 4.S6). A characteristic peak of C=O at 1666 cm^{-1} was present in both untreated and water-treated devices prior to ink drying which can be assigned to isophorone, a major component of the otherwise proprietary commercial ink (50-70% per manufacturer data) [372]. After drying, this peak disappeared due to the evaporation of the solvent, and both device types

exhibited spectra similar to plain filter paper. These results further indicate that the improved performance from the water treatment is likely due to the preservation of the hydrophilicity and wicking behavior of the underlying paper matrix, rather than any changes in structure or chemical composition. Further investigation into this mechanism may be useful but would require inks with well-defined and well-known formulations.

Various water treatment strategies were also tested to evaluate their impact on ePAD performance (Figure 4.3C). Dipping resulted in an oxidation peak of $267 \pm 22 \mu\text{A}$, while one-time pipetting gave a comparable value of $273 \pm 23 \mu\text{A}$, indicating both methods are suitable for the treatment of the devices. Repeated pipetting up to three times enhanced the signal, with two-time and three-time treatments yielding $305 \pm 18 \mu\text{A}$ and $329 \pm 26 \mu\text{A}$, respectively. All strategies up to three repetitions produced acceptable relative standard deviations (RSDs) below 9%, with the two-time treatment showing the lowest variability. However, further repetitions (four to six times) led to diminished signals and greater variability (e.g., six-time: $114 \pm 137 \mu\text{A}$). Therefore, two-time pipetting was selected as the optimal method, providing a balance between signal intensity, reproducibility, and reduced time and labor compared to three-time washing. Using this approach, the simple water treatment led to a remarkable 76.25-fold enhancement in signal intensity compared to the untreated devices.

The performance of two-time washed devices was further assessed by CV analysis. As shown in Figure 4.3D, 11 measurements on different ePADs from the same batch yielded an RSD of 5.9% for the oxidation peak, demonstrating good intra-batch repeatability as compared to other studies [122,262]. Inter-batch variation was also evaluated using devices from six independently fabricated batches, resulting in an RSD of 7.9%, indicating acceptable reproducibility (Figure 4.57). The measured peak-to-peak separation (ΔE_p) was $636 \pm 93 \text{ mV}$, which is higher than the theoretical $\sim 59 \text{ mV}$ for a reversible one-electron redox process. This deviation aligns with previous reports on carbon electrodes and can be attributed to the intrinsic resistance of the cellulose fibers in the paper and carbon electrode material [262,373–375]. Nonetheless, the oxidation-to-reduction peak current ratio was 0.990 ± 0.002 , close to unity, suggesting that the ferricyanide/ferrocyanide species are not adsorbed on the electrode surface and are acceptable mediators for this system.

Altogether, functional and reproducible ePADs were obtained using the water treatment strategy. It should be noted that, based on our observations, several factors can contribute to variability

during the fabrication process and must be carefully controlled to achieve optimal results. These factors include user skills, proper ink storage to prevent solvent evaporation, monitoring ink expiration, and thorough stirring of the ink prior to printing, as well as intrinsic screen-printing parameters such as squeegee pressure and speed [275]. In the next section, we will explore the sandwiching strategy as a simple method to further enhance signal intensity.

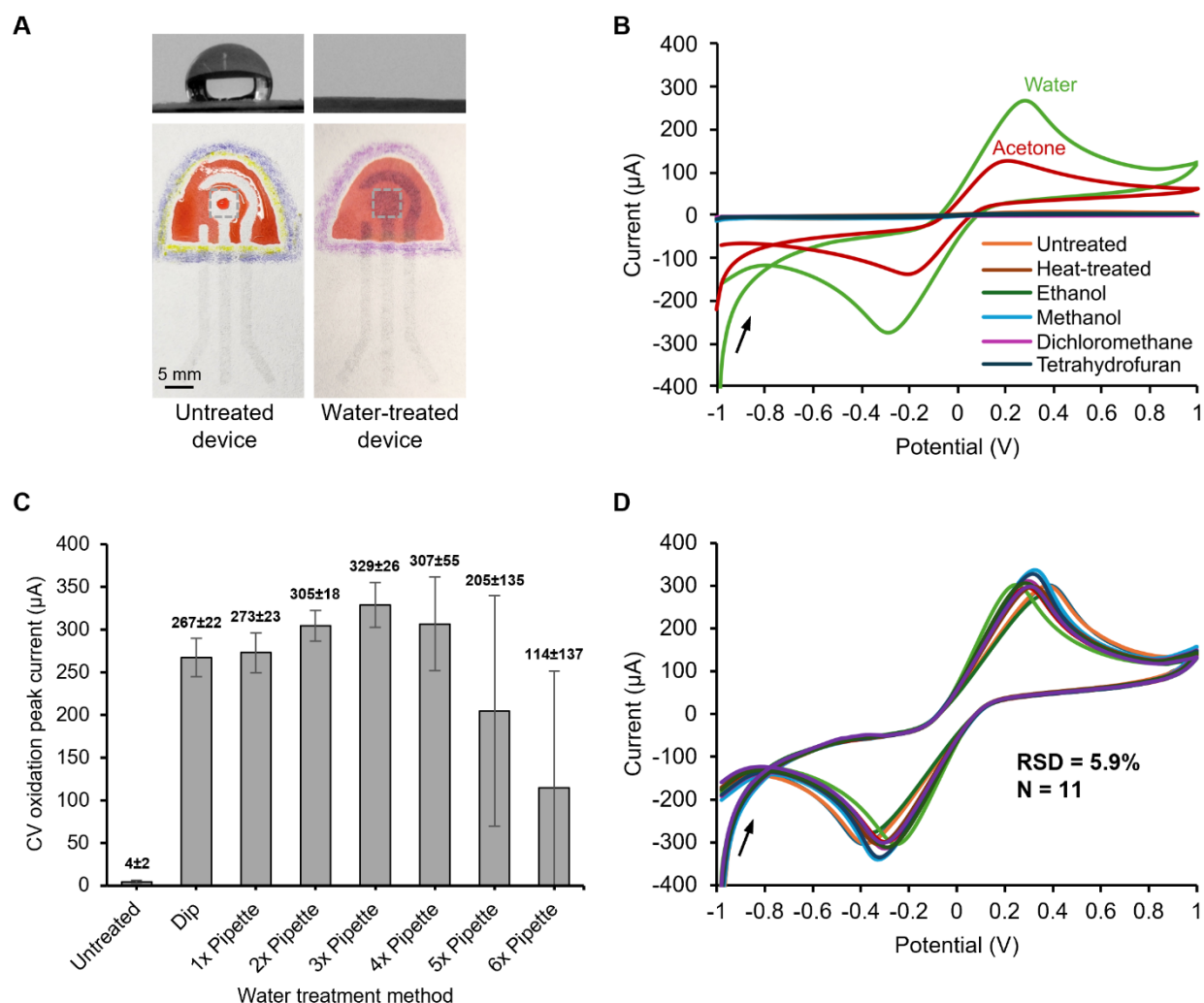


Figure 4.3. Water treatment of the electrodes. (A) Backside view of untreated and water-treated devices; top row shows lateral images of selected regions. Without water treatment, the dye solution did not reach or spread across the electrode area. (B) CV of untreated, heat-treated (95–105 °C, 1 h), water-treated, and solvent-treated ePADs using 10 mM ferricyanide/ferrocyanide in 1 M KCl (scan rate: 0.1 V/s). Only water and acetone treatments produced notable current peaks. (C) Comparison of water treatment methods (dipping vs pipetting) and effect of repeated treatments on oxidation peak currents (mean $\pm$ SD; $n = 11$). (D) CV recordings showing reproducibility of twice-washed ePADs based on oxidation peak currents (RSD: relative standard deviation).

4.4.3. Sandwiched ePADs

After fabricating functional ePADs, we have investigated a novel design in which the electrodes are sandwiched between two layers of paper to increase the electroactive surface area and, consequently, the signal intensity (Figure 4.4A). All other configurations, where electrodes are exposed to only one paper layer, are termed “single-layer” ePADs. We have created ePADs with a new architecture that allowed for adding another paper layer on the top, while having a sample inlet zone for introduction of the sample (Figure 4.4B). Upon sample introduction, the liquid is distributed via capillary action to both paper layers, enabling contact with the electrode from both the top and bottom sides. This novel design makes the ePADs compatible with lateral flow assays unlike prior designs limited to vertical flow or spot test assays [293,295,366]. All tests in this study were performed using twice-washed ePADs.

To optimize the sandwiched ePADs and assess key parameters, we have tested various single-layer and sandwiched configurations (Figure 4.4S8). Each was evaluated using CV with 10 mM ferricyanide/ferrocyanide in 1 M KCl at a scan rate of 0.1 V/s. Initial comparisons revealed that sandwiched devices secured with a binder clip significantly outperformed single-layer spot tests, with notably higher peak currents (328 vs. 158 μ A), indicating a larger effective surface area (Figure 4.4C). We then tested additional variations (Figure 4.4D). A single-layer configuration with a clip and one with double the sample volume (40 μ L) were evaluated and showed no notable increase in the oxidation peak currents. Interestingly, when sandwiched devices were held with transparent adhesive tape, the signal enhancement was limited to only 20% over the single-layer spot test. This was likely due to the hydrophobic nature of the printed ink, which formed a “hydrophobic island” on the hydrophilic paper matrix (Figure 4.4S9). Upon wetting, the adhesive tape lost its strength, leading to partial delamination and restricted liquid access to the top electrode. Therefore, firm contact between the top paper and electrode, achievable using a binder clip, was essential to realize the benefits of the sandwiched architecture. The best-performing configuration yielded a 2.18-fold increase in signal intensity compared to the single-layer device, aligning with theoretical expectations for a doubled electrode-electrolyte interface.

We then assessed the effect of sample volume on the performance of the sandwiched devices with a clip (Figure 4.4E). Increasing the volume from 20 μ L to 40 μ L led to a 2.95-fold enhancement in signal, attributable to more extensive liquid-electrode contact. However, volumes above 40 μ L

caused excess fluid accumulation and did not contribute to signal enhancement. Based on these observations, sandwiched ePADs with a clip configuration and a sample volume of 40 μL were used for all subsequent experiments with sandwiched ePADs.

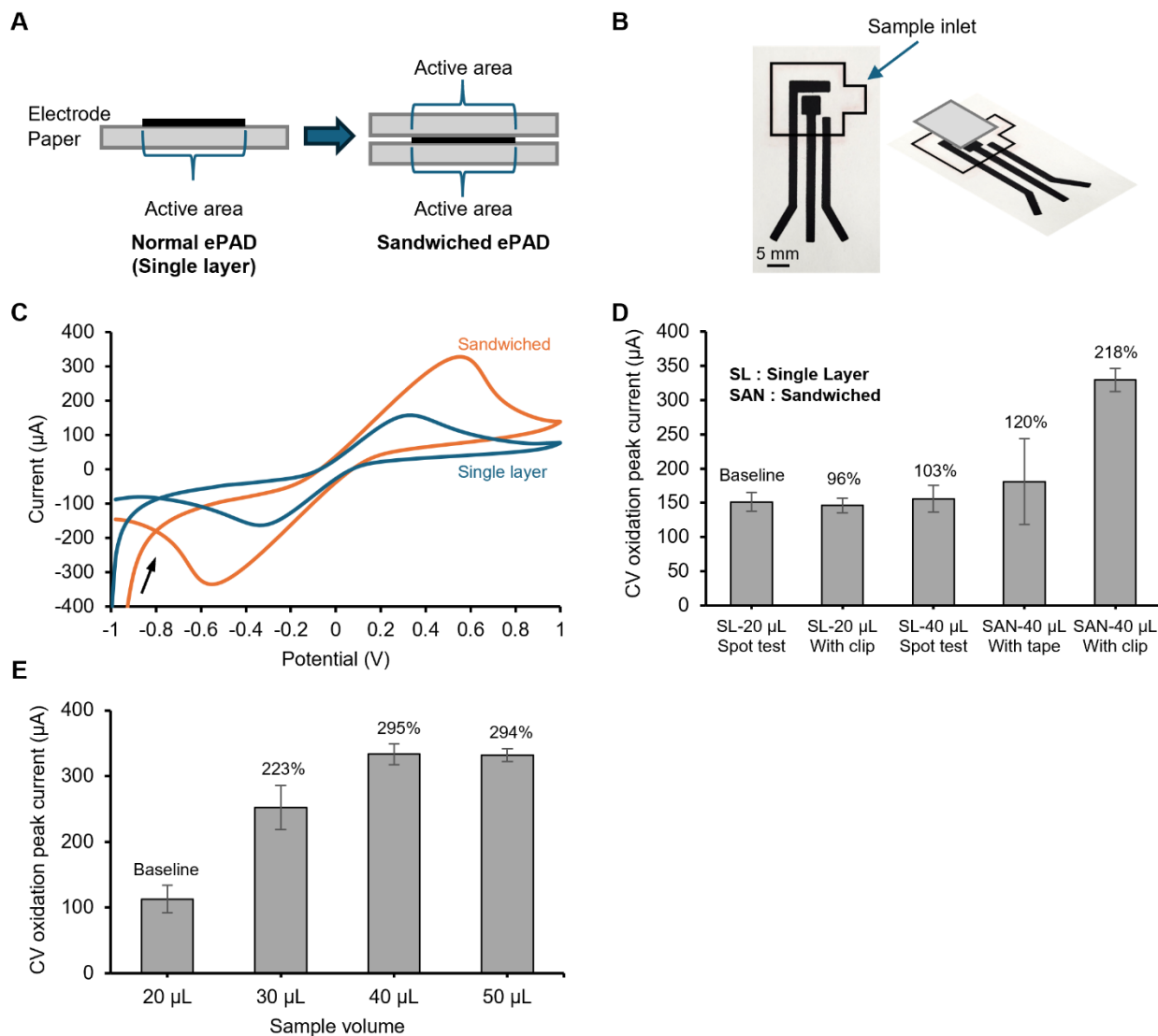


Figure 4.4. Sandwiched ePADs. (A) Schematic illustrating the active surface area of single-layer versus sandwiched devices. (B) Design layout of the sandwiched ePADs. (C) CV comparison between sandwiched with clip and single-layer spot test configurations using 10 mM ferricyanide/ferrocyanide in 1 M KCl (scan rate: 0.1 V/s). (D) Comparison of CV oxidation peak currents across different configurations and test conditions for single-layer and sandwiched ePADs (mean $\pm$ SD; $n = 5$). (E) Effect of sample volume on the CV oxidation peak current of sandwiched ePADs with clip configuration (mean $\pm$ SD; $n = 5$).

To better understand the effect of sandwiching on the electroactive surface area of the ePADs, we have compared single-layer and sandwiched devices using the Randles-Ševčík equation [368]. Although several studies have demonstrated its applicability to ePADs and quasi-reversible systems [22,262,268,293], this equation was originally derived for reversible redox systems at planar, two-dimensional electrodes and is not appropriate for determining exact electroactive surface areas within highly non-planar electrodes [376–378]. However, for the scan rates used here, the data indicates a linear relationship between peak current and the square root of scan rate in both cases, suggesting predominantly diffusion-controlled mass transfer [22,262] and validating that the slope of the curves can be used to compare relative surface areas. To ensure a fair comparison, we have evaluated the single-layer with clip configuration against the sandwiched device with clip, with the only variable being the added top paper layer. CV analysis was performed on both devices in ferricyanide/ferrocyanide at scan rates of 0.1, 0.2, 0.3, and 0.4 V/s (Figures 4.5A and B). The CV oxidation peak currents were plotted against the square root of scan rate, and both devices showed high linearity ($R^2 = 0.99$), indicating good agreement with the Randles-Ševčík equation. Given that all experimental conditions were identical for the two configurations, the slope of the Randles-Ševčík curve was proportional to the electroactive surface area and used to compare surface areas between the single-layer and sandwiched devices. As shown in Figure 4.5D, the slope for the sandwiched devices was 1.96 times greater than that of the single-layer devices, indicating a corresponding 1.96-fold increase in electroactive surface area. This enhancement aligns well with the theoretical expectation of a two-fold increase by adding a paper layer on the top surface of the electrodes.

Overall, these results demonstrate that adding a top paper layer in the sandwiched configuration significantly increases the electroactive surface area and, consequently, the signal intensity of the ePADs. However, ensuring firm bonding between the paper layers and electrodes is critical to maintain robust liquid-electrode contact. In this study, low-cost binder clips effectively bonded the layers, but future work on developing lamination strategies and utilizing hydrophilic inks could eliminate the need for clips and further improve the usability of the device. In the next section, the analytical performance of single-layer and sandwiched devices for detecting glucose will be explored.

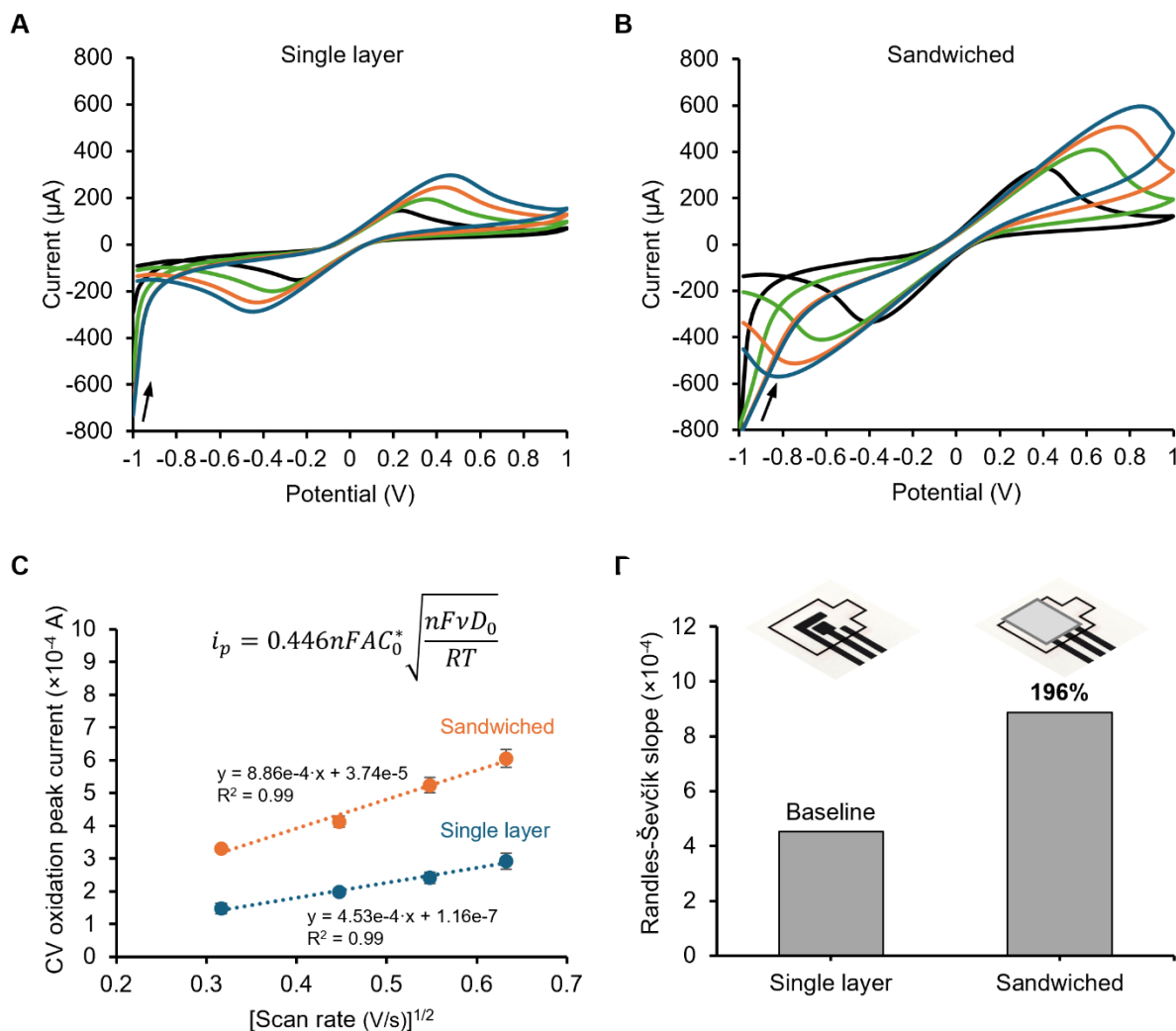


Figure 4.5. Surface area comparison for the single-layer and sandwiched ePADs with clip configuration. CV assessment of (A) single-layer and (B) sandwiched devices using 10 mM ferricyanide/ferrocyanide in 1 M KCl at scan rates of 0.1, 0.2, 0.3, and 0.4 V/s. (C) Plot of CV oxidation peak current versus the square root of scan rate according to the Randles-Ševčík equation (mean $\pm$ SD; $n=5$). (D) Comparison of the Randles-Ševčík slopes between single-layer and sandwiched devices, reflecting differences in electroactive surface area.

4.4.4. Glucose Detection

After optimizing and understanding sandwiched devices, glucose was detected via a bienzymatic reaction involving glucose oxidase (GOx) and horseradish peroxidase (HRP), with potassium ferrocyanide serving as the mediator (Figure 4.6A) [369,370]. Initially, GOx catalyzes the oxidation of glucose, generating gluconic acid and hydrogen peroxide (H_2O_2). Subsequently and simultaneously, H_2O_2 oxidizes ferrocyanide to ferricyanide in the presence of HRP. Finally, at the

electrode surface, an applied potential reduces ferricyanide back to ferrocyanide, producing a measurable current. Chronoamperometry (CA) was used for analysis, in which a fixed potential is applied and the resulting current, governed by the Cottrell equation, is proportional to the analyte concentration at a given time [254]. We performed the CA analysis at an applied potential of -0.3 V for all configurations. Preliminary CV tests confirmed that -0.3 V is a suitable detection potential, showing no notable response in the blank and a clear reduction peak in the presence of glucose, arising from the enzymatic formation of ferricyanide and its subsequent electrochemical reduction to ferrocyanide (Figure 4.S10).

To optimize glucose detection, we compared single-layer and sandwiched devices under various conditions (Figure 4.6B). In all tests, 2 mM glucose in water was used, and the CA current was recorded at 45 seconds. First, a single-layer device without water treatment was evaluated, yielding a current intensity of $0.40 \pm 0.02 \mu\text{A}$, which was significantly lower than that of the water-treated devices. Among the water-treated configurations, the sandwiched devices, in which both paper layers were functionalized with reagents, exhibited the highest current intensity ($9.6 \pm 0.5 \mu\text{A}$), compared to single-layer devices ($5.2 \pm 0.4 \mu\text{A}$), corresponding to a 1.84-fold increase. As a control, a single-layer device with double the amount of reagents and sample (equivalent to the sandwiched configuration) showed no increase in signal intensity, confirming that the enhancement resulted from the addition of the top paper layer. Furthermore, in the sandwiched devices, whether reagents were applied to one or both layers significantly impacted the signal. This observation can be explained by the strong binding of enzymes to the paper matrix, which prevents their migration upon sample addition [203]. Consequently, when reagents are applied to only one layer, ferricyanide production is limited to that layer. Although the sample partially transfers the generated ferricyanide to the opposing layer, this is less efficient than when both layers are functionalized, enabling ferricyanide generation on both sides of the electrodes.

Based on these findings, calibration curves were generated for both single-layer and sandwiched devices. To ensure a fair comparison, both configurations were used with a binder clip, with the only variable being the addition of the top paper layer. Both showed linear responses from 0-5 mM glucose, with deviations observed at 10 mM (Figure 4.S11). As shown in Fig. 4.6C, the sandwiched devices exhibited significantly higher sensitivity, with a slope of 3.69 compared to 2.06 $\mu\text{A}/\text{mM}$ for the single-layer devices, indicating a ~ 1.8 -fold increase. Higher sensitivity not only improves differentiation between analyte concentrations but also enables detection over a

broader range when combined with dilution strategies, as reported previously [379]. Also, the limit of detection (LOD) of the devices was calculated as the concentration on the curve corresponding to three times the standard deviation of the blank above the average signal of the blank [380,381]. The LOD improved from 0.25 mM for the single-layer devices to 0.20 mM for the sandwiched devices. These values for sensitivity and LOD of the sandwiched devices outperform several previously reported ePADs in the literature [19,122,273,370,379].

Finally, as a proof-of-concept application, glucose was detected in spiked cell culture medium. Monitoring glucose levels in culture media is critical for optimal cell growth and biopharmaceutical production [382]. The working range of the ePADs nearly covers the glucose concentrations required for biotechnological applications that use low-glucose media (<5.5 mM) [383,384]. Spiked concentrations of 1.5, 3, and 4.5 mM were successfully detected using the sandwiched devices (Figure 4.6C, green triangles). The measured values showed good agreement with the calibration curve, with percent recovery values of $94.7 \pm 6.7\%$, $99.1 \pm 4.6\%$, and $96.2 \pm 7.4\%$ for the 1.5, 3, and 4.5 mM samples, respectively. Additionally, the RSD values for all spiked tests were below 7%. These results demonstrate the high accuracy and precision of the devices within the scope of this study.

Altogether, we were able to successfully detect glucose using the sandwiched ePADs with a high precision and accuracy. Our findings proved that the sandwiched strategy can be a viable and simple strategy to enhance the sensitivity of ePADs by almost two folds.

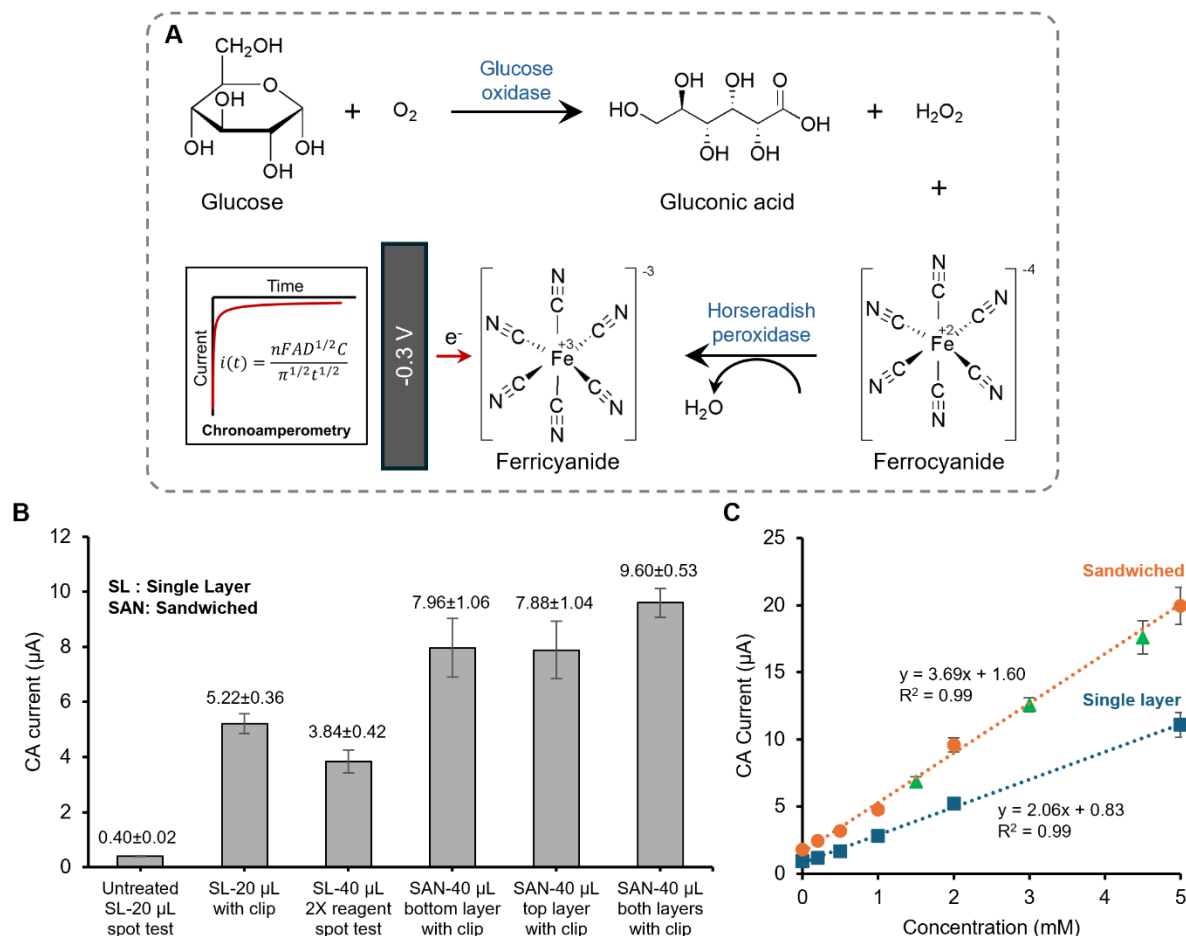


Figure 4.6. Glucose detection. (A) Schematic illustration of the reaction mechanism. (B) Comparison of different testing conditions for the single-layer and sandwiched devices. Bars represent the current measured at 45 seconds from the CA curves for 2 mM glucose in DI water. The “untreated” devices were not rinsed with water after screen-printing, whereas all other devices were washed twice. The maximum current was obtained with the water-treated sandwiched devices in which both paper layers were functionalized with reagents (mean ± SD; n = 5). (C) Calibration curves for glucose detection using CA current after 45 seconds. The sandwiched devices exhibited a 79% increase in sensitivity compared to single-layer devices and successfully detected glucose in spiked cell culture media as shown by green triangles (mean ± SD; n = 5).

4.5. Conclusions

This study presents a low-cost, portable screen-printing setup combined with CO₂ laser-cut adhesive vinyl masks for rapid, on-demand prototyping of electrochemical paper-based analytical devices (ePADs). The approach achieved a high electrode resolution of $286 \pm 31 \mu\text{m}$, offering versatility in fabricating diverse sensor designs. Additionally, a simple post-printing water

treatment effectively mitigated the hydrophobicity effect of carbon-based inks, significantly enhancing signal intensity. Using this fabrication and treatment method, we developed novel sandwiched ePADs compatible with lateral flow assays. These devices exhibited a 1.96-fold increase in electroactive surface area and a 2.18-fold increase in oxidation peak currents for the ferricyanide/ferrocyanide system, demonstrating substantial signal amplification. Furthermore, bienzymatic glucose detection was successfully performed, with the sandwiched devices achieving ~1.8-fold higher sensitivity using chronoamperometry and accurately detecting glucose in spiked cell culture media, validating their practical applicability.

Future research directions include: (1) developing conductive inks formulated with eco-friendly, non-toxic solvents to enable safer and more convenient screen cleaning and reuse; (2) designing hydrophilic inks to improve liquid-electrode interaction on ePADs; and (3) optimizing lamination strategies to streamline the assembly of sandwiched devices. Overall, the proposed methods support rapid, low-cost, and high-resolution fabrication of ePADs with enhanced performance suitable for both laboratory use and resource-limited settings.

4.6. Supporting Information

Laser Settings

A CO₂ laser cutter (Universal Laser Systems, VLS 2.30DT with a 2.0 lens and 30 W laser) was used to fabricate electrode masks on adhesive vinyl sheets. The patterns were designed using the open-source software Inkscape (version 1.1.1). Table 4.S1 summarizes the laser settings used for mask fabrication.

Table 4.S1. Laser settings used to create electrode masks on adhesive vinyl sheets in this study.

Figure address	Laser power (%)	Laser speed (%)	Z-axis distance (inches)	Pulse per inch (PPI)	Mode
Figs 4.1, 4.2A, 4.3-6	85	100	0.014	500	Vector
Fig. 4.2B	100	25	0.014	500	Raster

Screen-Printing Equipment

The screen-printing setup is shown in Figure 4.S1. The table and screen mesh were obtained from the Print Media Facilities at Concordia University. A melamine-faced chipboard (MFC) measuring $61 \times 61 \times 1.6$ cm, equipped with two screen-printing clamps, was used as the printing table (Figure 4.S1A). A $14'' \times 20''$ aluminum-framed screen with a 180/55 white mesh (Screentec, Canada) was used for printing. The mesh was securely pre-glued to the frame, allowing repeated use without detachment. To prevent unintended contact between the screen and the paper during printing, a pencil was attached to the bottom edge of the frame as a spacer (Figure 4.S1B). To create the laser-cut mask, the vinyl sheet was cut to 10×15 cm, and a rigid squeegee was used to transfer the ink onto the paper.

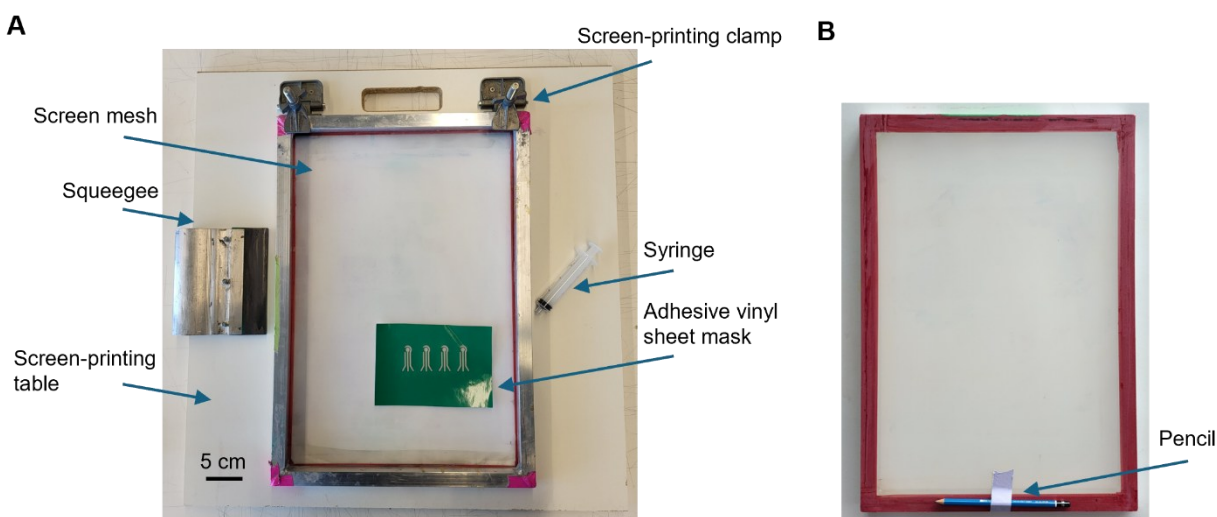


Figure 4.S1. Equipment used for do-it-yourself (DIY) screen printing. (A) Mounted screen on the screen-printing table along with other required tools. (B) Front view of the screen mesh.

Characterizing the Do-It-Yourself Screen Printing

We used the DIY screen-printing technique to fabricate electrochemical devices on parchment cardstock made from recycled paper (Goefun, purchased from Amazon Canada), using a design similar to that of typical commercial screen-printed electrodes (SPEs; CTI Phase Zero, purchased from PalmSens Inc.). As shown in Figure 4.S2A, the DIY approach offers high flexibility for printing on various materials, while achieving geometries comparable to commercial SPEs.

Next, we evaluated how many times a single vinyl sheet screen mask could be used for printing without compromising quality. As shown in Figure 4.S2B, we were able to perform at least 30 prints with no noticeable decline in print quality.

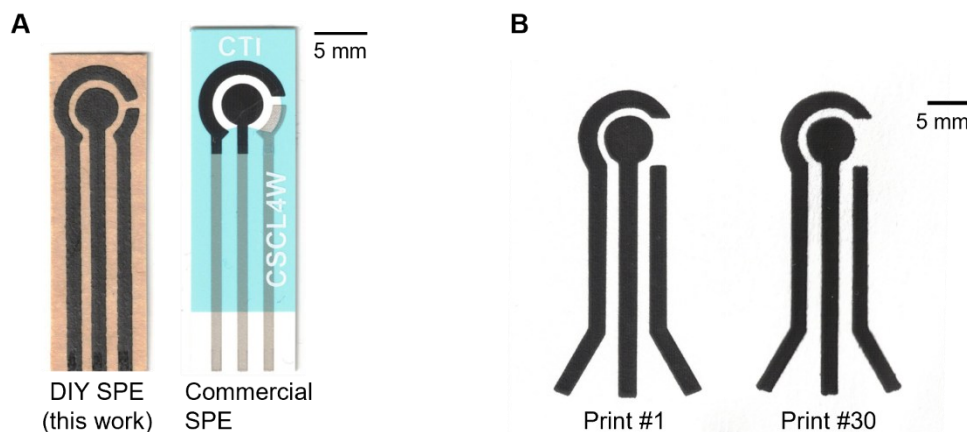


Figure 4.S2. Characterizing DIY screen printing. (A) Comparison between electrodes fabricated on parchment cardstock using DIY screen printing and commercial screen-printed electrodes (SPEs) with similar geometry. (B) Electrode pattern quality after repeated use of the same screen-printing mask.

Water Treatment of the ePADs

Water contact angle measurements of the screen-printed ePADs were performed using a Dropometer instrument (Droplet Lab Inc., Canada) to evaluate the wettability of the electrode region on both the front and backside of the devices. As shown in Figure 4.S3, in some cases, the water droplet did not penetrate the paper matrix on the backside of the printed ePADs, which exhibited hydrophobic behavior with a water contact angle of $108 \pm 2^\circ$. In contrast, devices subjected to water treatment remained hydrophilic and preserved their natural wicking ability. Notably, water treatment did not cause any significant change in the wettability of the electrode surface when measured from the front side of the ePADs, and both untreated and water-treated devices were hydrophobic.

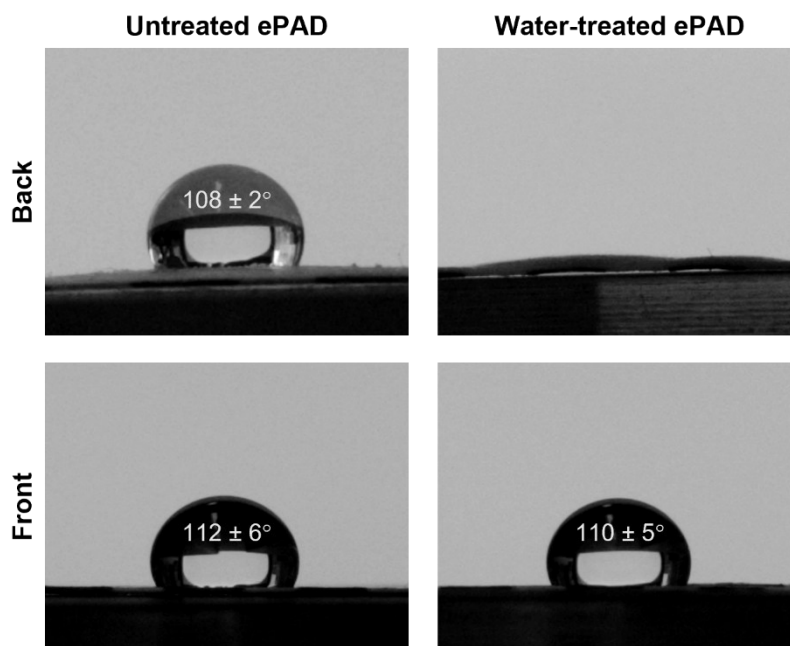


Figure 4.S3. Water contact angle measurements of ePADs before and after water treatment.

Electrochemical impedance spectroscopy (EIS) was performed on untreated and water-treated ePADs to compare their resistance characteristics (Figure 4.S4). The analysis was conducted using 10 mM potassium ferricyanide/ferrocyanide in 1 M KCl, with a DC offset of 0 V, an AC amplitude of 10 mV, and a frequency range of 10 Hz to 310 kHz. The untreated devices exhibited a substantially higher charge transfer resistance than the water-treated ones, indicating hindered electron transfer and less effective redox activity in the absence of water treatment.

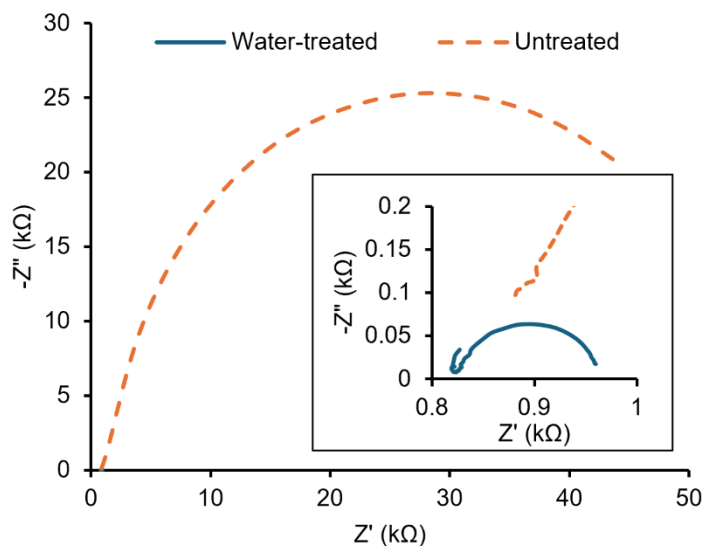


Figure 4.S4. EIS analysis of untreated and water-treated devices. The measurements were carried out in 10 mM potassium ferricyanide/ferrocyanide prepared in 1 M KCl, using a 0 V DC bias, a 10 mV AC amplitude, and a frequency range of 10 Hz to 310 kHz.

Scanning electron microscopy (SEM) was conducted on plain filter paper, untreated ePADs, and water-treated ePADs using a Phenom ProX G5 system (ThermoFisher Scientific, USA). This analysis was performed to assess whether water treatment affected the morphology and physical structure of the electrode regions on both the backside and front side of the devices. For imaging plain paper and the backside of the ePADs, samples were coated with gold nanoparticles using a sputter coater (108 Auto, Cressington Scientific Instruments, UK). All samples were mounted onto conductive carbon tapes (Ted Pella Inc., USA) prior to SEM imaging. The resulting micrographs are presented in Figure 4.S5.

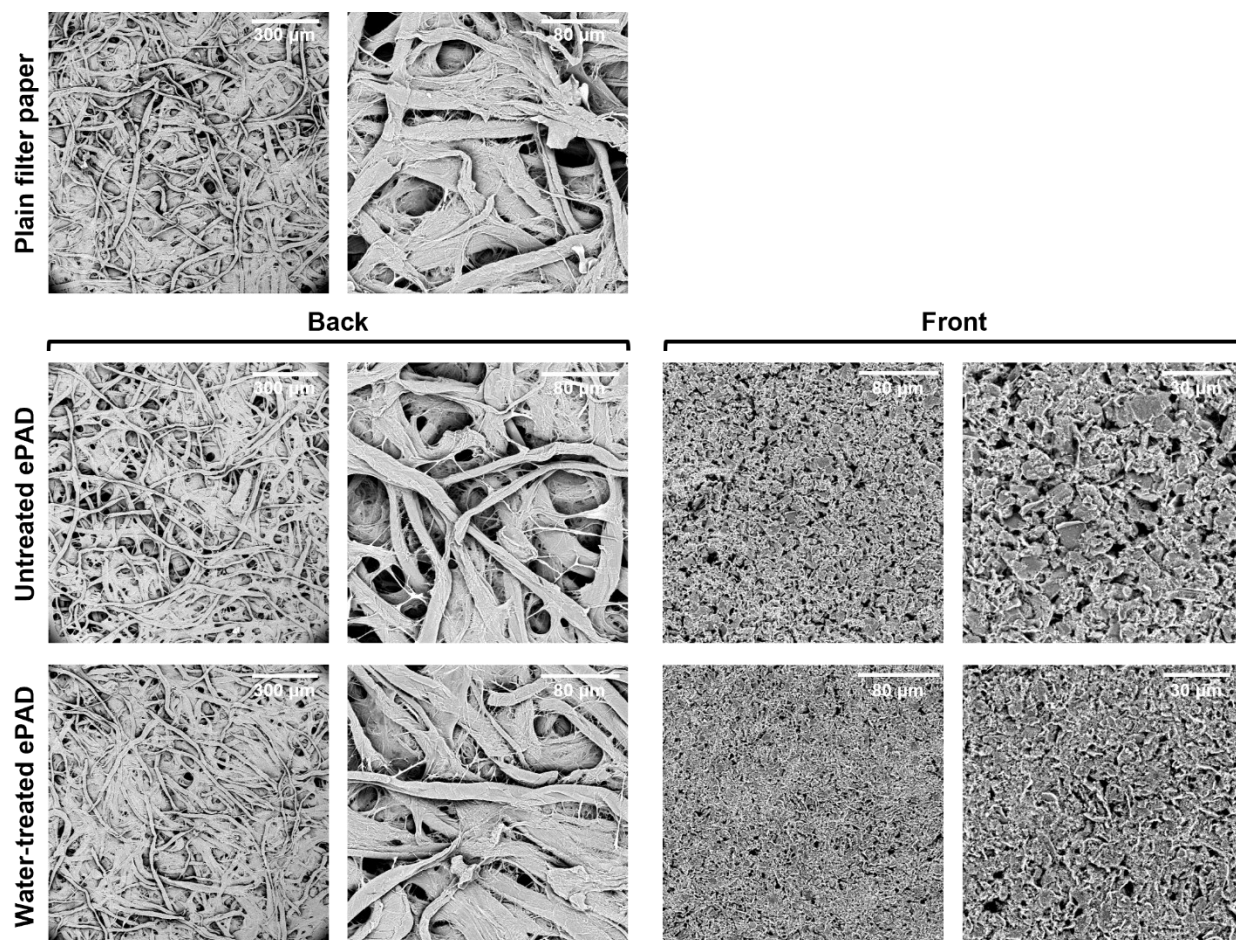


Figure 4.S5. SEM analysis of plain filter paper (Whatman Grade 1), untreated ePADs, and water-treated ePADs.

Fourier transform infrared (FTIR) analysis was performed using a Nicolet™ iS20 FTIR Spectrometer (Thermo Scientific Instrument Co., USA) over the range of 4000-400 cm^{-1} to investigate possible chemical alterations or the creation of new bonds. The analysis compared plain filter paper; freshly printed ePADs before ink drying and without water treatment; freshly printed ePADs subjected to water treatment (after water evaporation but before ink drying); and fully dried printed ePADs with and without water treatment. All measurements on ePADs were conducted from the backside of the device in the electrode region, where changes in paper wettability occur. The results are presented in Figure 4.S6.

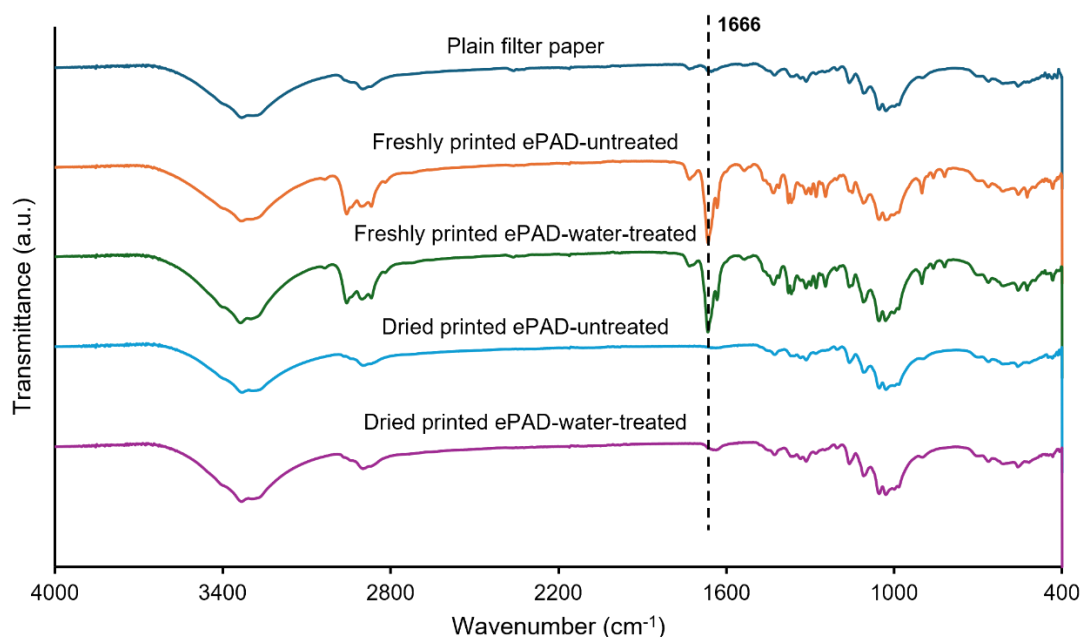


Figure 4.S6. FTIR analysis of plain filter paper (Whatman Grade 1) and various ePAD samples. The ePADs include freshly printed devices without water treatment (before ink drying), freshly printed devices treated with water (after water evaporation and before ink drying), and fully dried devices with and without water treatment. All FTIR measurements on ePADs were performed on the backside of the device in the electrode region.

To assess the inter-batch reproducibility of the water-treated ePADs, twice-washed devices were evaluated using cyclic voltammetry (CV), as shown in Figure 4.S7. The measurements were performed in a solution of 10 mM potassium ferricyanide/ferrocyanide prepared in 1 M KCl, using a scan rate of 0.1 V/s over a potential window of -1 V to 1 V. Ten devices from six independently fabricated batches were tested, and their oxidation peak currents were analyzed. The relative standard deviation (RSD), calculated from the means of each batch, was 7.9%, indicating good inter-batch reproducibility.

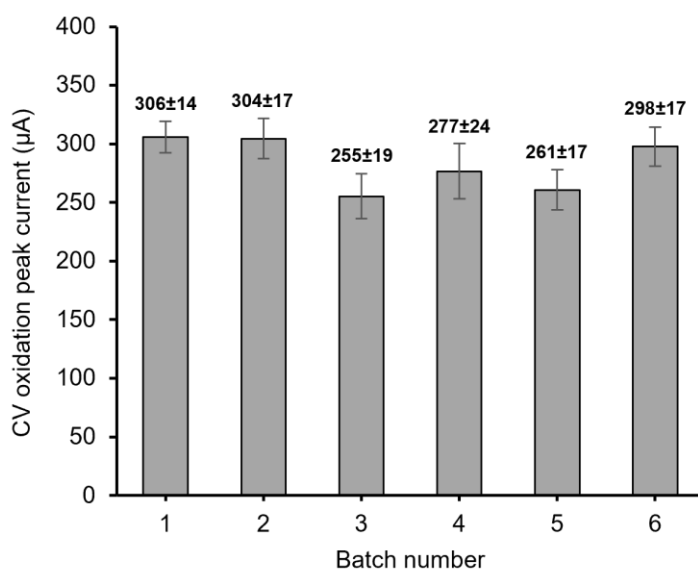


Figure 4.S7. Inter-batch reproducibility of twice-washed ePADs. CV measurements were performed in 10 mM potassium ferricyanide/ferrocyanide prepared in 1 M KCl at a scan rate of 0.1 V/s over a -1 V to 1 V potential range. The average CV oxidation peak current of each batch was used to evaluate device reproducibility (bars represent mean $\pm$ SD; $n = 10$).

Configurations of Single Layer and Sandwiched Devices

Various configurations of single-layer and sandwiched ePADs were compared in this study to identify the optimal setup for sandwiched devices (Figure 4.S8A). For experiments involving binder clips, the ePADs were placed between two sturdy plastic sheets to ensure even pressure distribution over the electrodes. The top sheet was a 2 mm thick acrylic plastic sheet, cut with the laser cutter to a size of 1.8 cm $\times$ 1.8 cm. Toothless copper alligator clips were used to connect the ePADs to the potentiostat (Figure 4.S8B).

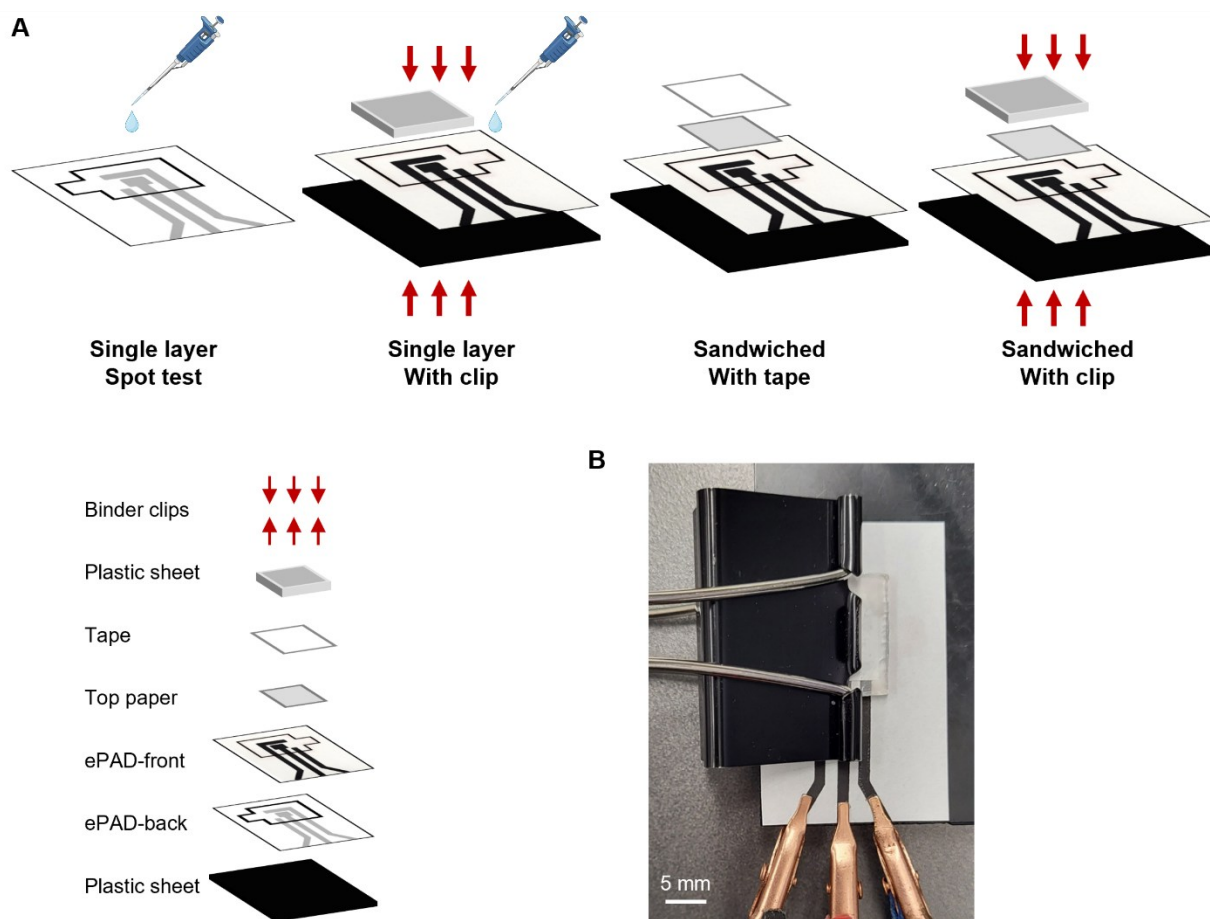


Figure 4.S8. Configurations of the ePADs. (A) Various configurations of single-layer and sandwiched ePADs. For all configurations except the single-layer spot test, the sample was added from the front side at the sample inlet zone. In the spot test, the sample was applied from the backside directly to the center of the working zone. (B) Photograph of the sandwiched ePADs secured with a binder clip.

Impact of electrode hydrophobicity

To evaluate the practical impact of ink hydrophobicity on liquid contact with the top surface of the electrodes, an excess amount of dye solution was added to the working zone of the ePADs to observe its spreading behavior. The liquid accumulated around the electrodes and failed to spread on the top surface of the electrodes, indicating that the hydrophobic nature of the ink limited fluid coverage (Figure 4.S9).

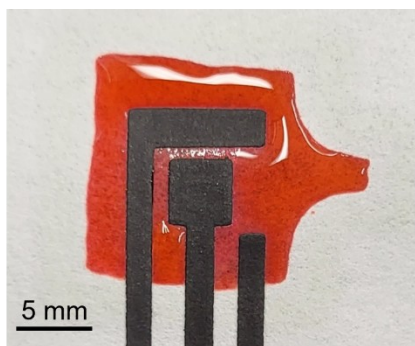


Figure 4.S9. Impact of electrode hydrophobicity. Photograph showing that even after adding an excess amount of liquid to the working zone, the top surface remains mostly dry, with the liquid accumulating around the electrodes.

Glucose Assay

To evaluate potential interference at -0.3 V during chronoamperometry (CA), we first performed cyclic voltammetry (CV) on ePADs using a blank sample (Milli-Q water) and a 5 mM glucose solution in water (Figure 4.S10). The measurements were conducted in the single-layer with clip configuration, scanning from -0.8 to 0.8 V (starting from the open-circuit potential in the backward direction) at a scan rate of 50 mV/s. The results showed no reduction peak up to -0.3 V in the absence of glucose. However, in the presence of glucose, ferrocyanide was oxidized to ferricyanide, and this oxidation product caused a clear reduction peak, confirming that -0.3 V is a suitable detection potential for CA analysis.

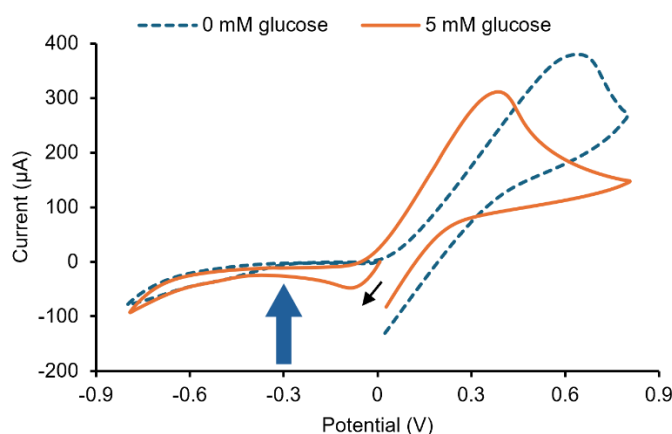


Figure 4.S10. Comparison of CV responses of blank (Milli-Q water) and glucose (5 mM) samples on the ePADs.

To generate calibration curves, glucose tests were performed using both single-layer and sandwiched ePADs with clip configurations, at glucose concentrations of 0, 0.2, 0.5, 1, 2, 5, and 10 mM in water. Figure 4.S11A shows the chronoamperometric responses for each device. The current measured at 45 seconds was used to construct the calibration curves (Figure 4.S11B). Both configurations exhibited linear responses up to 5 mM, while deviations from linearity and increased variability were observed at 10 mM.

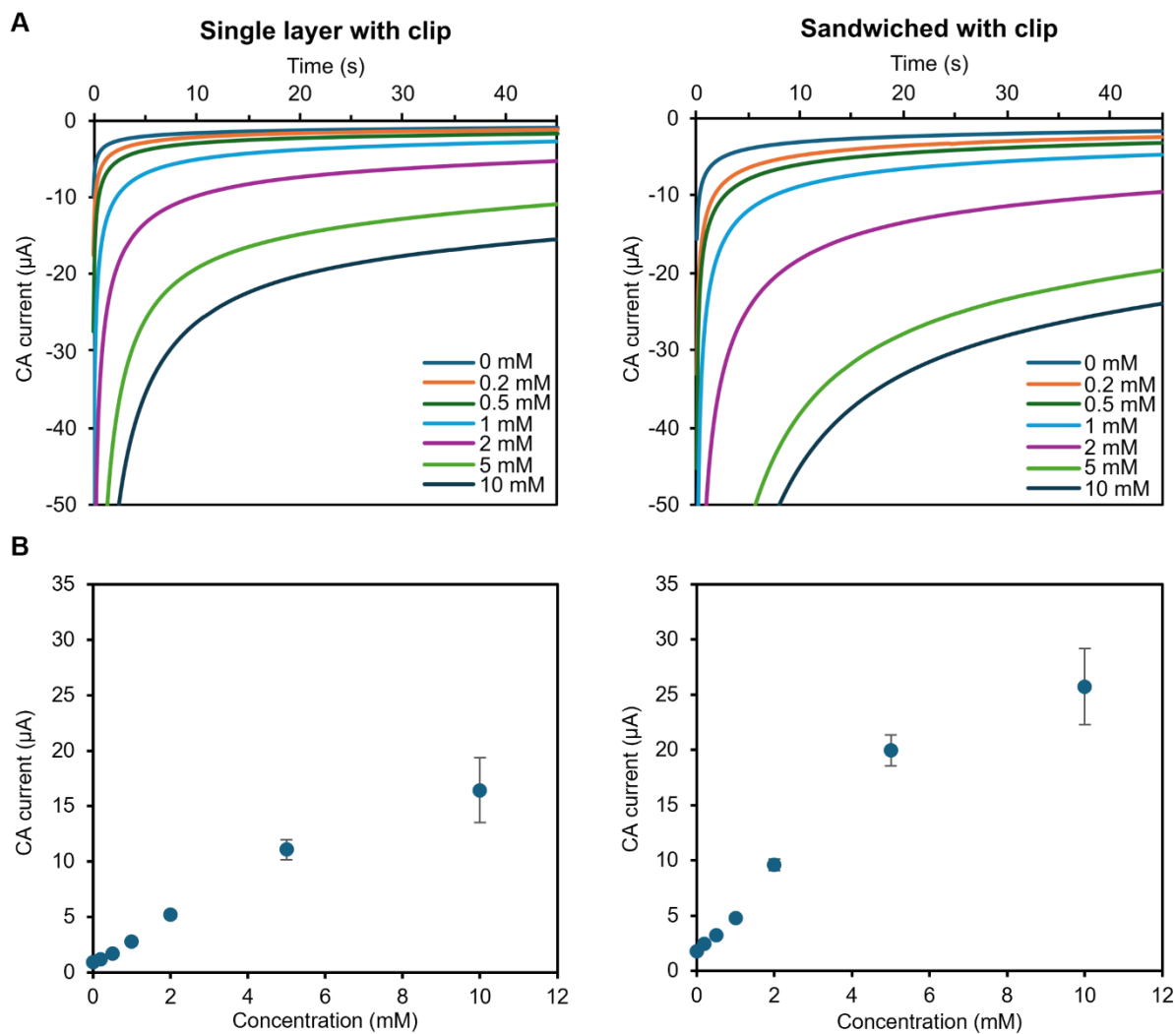


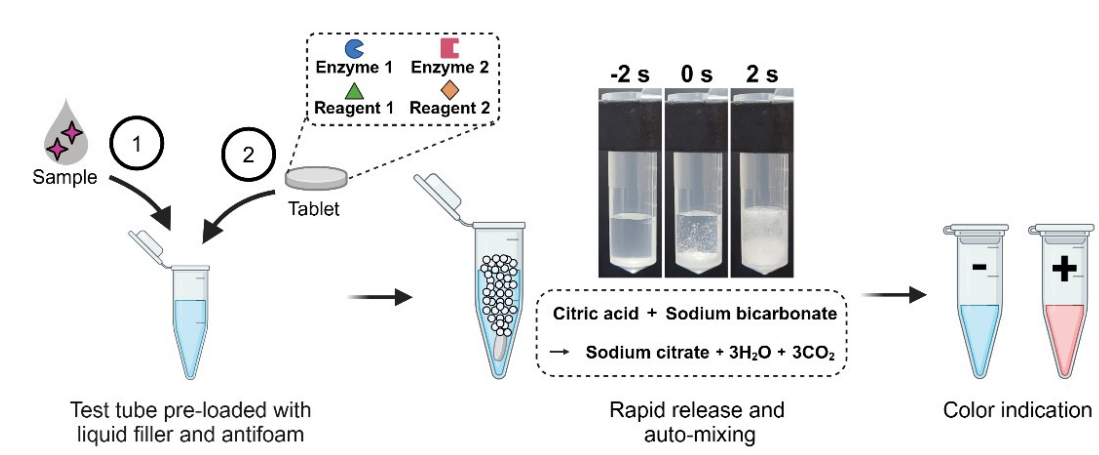
Figure 4.S11. Calibration curves for glucose detection using sandwiched and single-layer ePADs with clip configuration. (A) CA responses at varying glucose concentrations. (B) Calibration curves based on current measured at 45 seconds versus glucose concentration (mean $\pm$ SD; $n = 5$).

Chapter 5

All-Inclusive Sensing Tablet with Integrated Passive Mixer for Ultraviscous Solutions

This chapter is adapted from the author's article published in *ACS Sensors* (2025), in accordance with the publisher's thesis reuse policy:

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5.1. Abstract

Developing low-cost and easy-to-use point-of-care devices is necessary for timely disease diagnosis and health monitoring. Here, we introduce all-inclusive, tablet-based chemo/biosensors with rapid automixing features, capable of mixing in highly viscous solutions with viscosities up to 1700 mPa·s. These tablets are created using a simple powder compression method and contain all necessary reagents to perform assays in a “drop-and-detect” manner, without the need for vigorous shaking or vortex mixing. As proof of concept, we demonstrated the applicability of our Speedy tablets for detecting nitrite in human saliva, a challenging medium due to its viscosity. The strong mixing capability of the proposed tablets ensured consistent and reliable results across range of viscosities, from low to high, while delivering an excellent detection range of 0.03-1.50 mg/dL, covering nitrite levels in human saliva. Additionally, we developed a straightforward method to encapsulate enzymes in trehalose, making them bulkier and more stable using only a mist sprayer, nonstick tray, and spatula, eliminating the need for expensive equipment. This approach allowed us to incorporate small amounts of enzymes into tablet formulations and fabricate the first automixing tablet biosensor. These biosensors were used for the bienzymatic detection of glucose in real human urine within the biologically relevant range of 0.3-2.5 mM, indicating the compatibility of automixing tablets with bioreagents. Each tablet costs less than \$0.30 to produce and remains stable for at least one month at room temperature. The affordability and convenience of our tablets make them a valuable addition to the array of diagnostic tools.

5.2. Introduction

Low-cost and portable diagnostic devices are crucial for timely disease detection and patient management. However, many diagnostic procedures still rely on bulky and expensive equipment, skilled personnel, and time-consuming processes that limit their availability, especially for low-resource settings and developing countries [6,385]. Tablet-based assay platforms offer a promising solution to tackle these issues due to their outstanding characteristics such as portability with pre-measured quantities of reagents, long shelf life, user-friendliness, and low-cost [23–25]. These features enable development of detection tools that can ideally meet the REASSURED criteria for point-of-care (POC) diagnostic devices (Real-time connectivity, Ease of specimen collection, Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free or environmentally friendly, and Deliverable to end-users) [6]. The first use of reagent tablets for POC applications dates back to the early 1940s for detecting total reducing substances in urine [42]. However, recent advancements have re-popularized tablet-based devices, notably following publications by our team members starting in 2014 [23,24].

So far, two major methods have been employed to create tablet-based devices, namely polysaccharide encapsulation of reagents [23,24,330,334] and powder compression [25,42,304,312,324]. Polysaccharide encapsulation is a straightforward method, involving the addition of polysaccharides, such as pullulan and dextran, to a reagent solution and casting the mixture to give highly stable tablet sensors. The powder compression method is a conventional approach that involves weighing and mixing powdered reagents and compressing them into a tablet. These types of tablets are easily mass-producible with the current industrial equipment, and generally, various types of chemicals can be incorporated into their formulation [25]. While each of these methods has its own merits, they suffer from limitations such as requiring intense shaking or vortex mixing to fully dissolve the reagents in the solution, which adds complexity to the assay procedure, and the need for expensive bulky equipment (e.g., freeze dryers) to incorporate bioreagents such as enzymes into tablet formulation. Also, the application of these tablets has been limited to low-viscosity fluids, such as urine and water, restricting their applicability in various POC scenarios.

Here, to address the aforementioned limitations, we suggest incorporating optimized amounts of effervescent chemicals, such as citric acid and sodium bicarbonate, into the formulation to create

“auto-mixing” tablet sensors with strong passive mixing capability. When the tablet is introduced into the solution, the effervescent chemicals react to produce carbon dioxide (CO₂) gas, generating vortices that enhance mixing. Notably, effervescent or rapid-disintegrating tablets have been widely utilized in the pharmaceutical industry for their ease of use and convenience [325,386]. Furthermore, we present a straightforward method to encapsulate enzymes in trehalose, enabling their incorporation into tablet formulations using only a handheld mist sprayer, a tray, and a spatula. Finally, we demonstrate the compatibility of these tablets with bioreagents by creating the first tablet-based “biosensor” with auto-mixing capability.

In the following sections, we first study the characteristics of the tablets in terms of mixing capability and their effect on the pH of the solution. Then, we develop a tablet-based nitrite test to detect nitrite in saliva. Nitrite is one of the commonly detected inorganic biomarkers in saliva, which high levels can be an indicator of oral cancer [387] and periodontal diseases [388], while saliva is a challenging media for POC testing due to its variable viscosity [182]. Next, we create tablet biosensors for glucose detection in urine, containing all reagents including glucose oxidase (GOx), horseradish peroxidase (HRP), and *o*-dianisidine as an indicator in one tablet. Our tablets cost less than \$0.30 to produce, are easy to manufacture using existing tablet production technologies, and allow the end user to perform the test in a simple “drop-and-detect” manner (Figure 5.1). With the global market for POC devices projected to reach approximately \$78 billion by 2028 [389], we believe that our auto-mixing tablet chemo/biosensors are a great addition to the toolbox of POC testing.

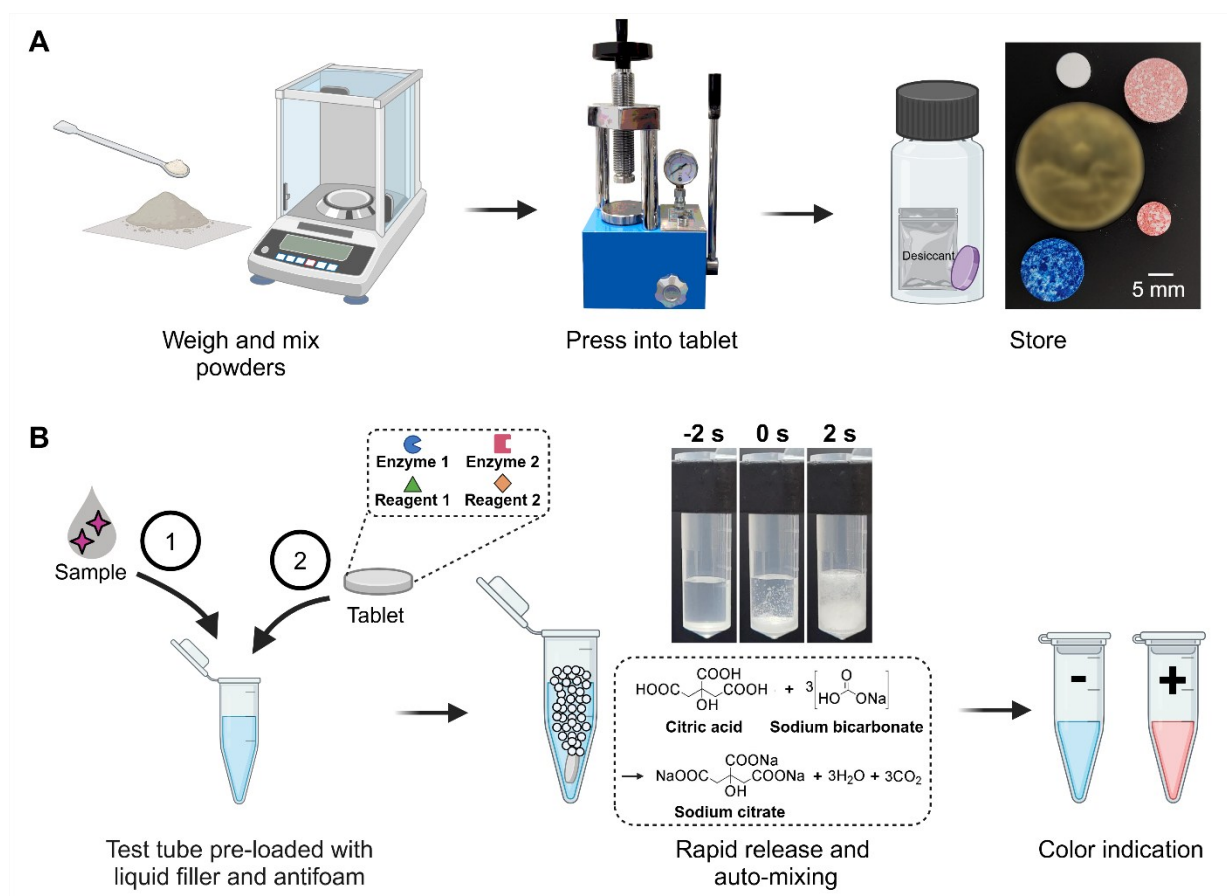


Figure 5.1. Rapid auto-mixing tablet sensors/biosensors. (A) To fabricate the tablets, powders are weighed and mixed, followed by pressing into the tablet format using a tablet press die set and a hydraulic press machine. The tablets are then stored in a glass vial with a desiccant pack (the inset photograph shows sample tablets in different sizes). (B) Drop-and-detect assay workflow for the tablets. A certain amount of sample is added to a test tube pre-loaded with a filler liquid such as water and antifoam agent. Then, a tablet, containing all reagents, is dropped in the solution resulting in the immediate initiation of the effervescence reaction and CO_2 gas release that causes rapid auto-mixing. The result of the assay is then analyzed by the color change in the solution (figure was created in <https://BioRender.com>).

5.3. Material and Methods

5.3.1. Chemicals and Materials

Citric acid monohydrate (Cat. No. C1909), sodium bicarbonate (S6014), polyethylene glycol 6000 (PEG, 8.07491), polyvinylpyrrolidone K30 (PVP, 81420), D-mannitol (M4125), D-(+)-trehalose dihydrate (T0167), sodium phosphate monobasic monohydrate (S9638), sodium phosphate dibasic (S9763), sodium chloride (S9888), sodium carboxymethylcellulose, medium viscosity (SCMC,

21902), N-(1-naphthyl)ethylenediamine dihydrochloride (NED, 33461), sulfanilamide (S9251), glucose (G8270), glucose oxidase from *Aspergillus niger* (GOx, G7141), horseradish peroxidase (HRP, P8250), o-dianisidine dihydrochloride (D3252), and silicone-based antifoam (A8582) were obtained from Sigma-Aldrich, Canada. Potassium phosphate monobasic (P4550) and potassium chloride (P2940) were sourced from ACP Chemicals Inc., Canada, and sodium nitrite (014244) from Fisher Scientific, Canada. PEG was ground into powder using a mortar and pestle; all other chemicals were used as received. A mist sprayer (Tanshine) and 0.5 g silica gel packs (Wisesorb) were purchased from Amazon, Canada; neon food colors (Clubhouse) from a local Walmart; and carbon steel trays (Betty Crocker) from a local store. Real urine and saliva samples were collected from healthy volunteers and used without further processing. This research was approved by Concordia University's Institutional Review Board (Approval No. SC5823; BioPermit No. B-SJA-22-01), with written and verbal informed consent obtained from all participants.

5.3.2. Procedure for Tablet Fabrication

Tablets were fabricated as outlined in Figure 5.1A. First, all powders were weighed on a single weighing paper and mixed directly on it using a spatula. The powder mixture was then transferred to a powder pressing die (13 mm, Specac, UK; or 7 mm and 11 mm, Zhengzhou TCH Instrument Co., China) and compressed using a hydraulic press (Tianjin17, China) under a 2-ton force. The resulting tablets were removed from the die and stored in glass vials containing commercial 0.5 g silica gel packs to protect them from humidity. This simple method ensures uniform tablet composition at the lab scale. For mass production, current industrial scale equipment can be used to easily produce larger numbers of tablets (as high as 1.5 million tablets per hour) with a high consistency between tablets [308,309].

5.3.3. Characterization

Tablets were characterized based on their mixing power, impact on solution pH, and the stability of their CO₂ content and weight over time. Hereafter, for clarity and brevity, “Speedy tablets” will be used in place of “rapid auto-mixing tablets” throughout.

To compare mixing performance, Speedy tablets, traditional tablets, and powder/granule formats were tested using a colored glucose system. Colored glucose was prepared by mixing 10 drops of blue food dye with 1.5 g of glucose in a glass vial, followed by air drying at room temperature.

Each Speedy tablet (500 mg total) contained 300 mg colored glucose, 2 mg PVP (binder), 10 mg PEG (lubricant), 88 mg sodium bicarbonate, and 100 mg citric acid monohydrate, and was pressed using a 13-mm die. Traditional tablets contained 300 mg colored glucose, 2 mg PVP, and 10 mg PEG. The powder/granule group included only 300 mg of colored glucose. For the tests, one tablet or 300 mg powder/granule was added to 10 mL of deionized (DI) water, and the dissolution of the color was recorded over time with a smartphone camera (see Table 5.S1 for a summary of tablet formulations and test procedures).

Next, the power of Speedy tablets was analyzed in the solutions with different viscosities. To create the tablets, first, a powder mixture (86% mannitol, 12% PEG, 2% PVP) was prepared as a 500 mg batch in a glass vial and mixed using a spatula and vortex mixer. From this batch, 30 mg was used per tablet, along with 30 mg citric acid monohydrate and 37 mg sodium bicarbonate. Tablets were compressed using a 7-mm die. Test solutions with viscosities ranging from 8.15 ± 0.02 to 1722 ± 3.46 mPa·s were prepared by dissolving 0.5–3 wt.% sodium carboxymethylcellulose in DI water (see Table 5.S2 for detailed viscosity measurements and viscometer settings). One tablet was dropped into a microtube containing 300 μ L DI water, 10–20 μ L antifoam, and 300 μ L of green dye solution. The mixing process was recorded over time.

The influence of formulation and tablet weight on mixing power was also analyzed. Tablets were made with varying molar ratios of citric acid to sodium bicarbonate, along with 100 mg of base mixture (86% mannitol, 12% PEG, 2% PVP), for a total tablet weight of 300 mg (see Table 5.S1 for detailed formulations). Each tablet was added to 4 mL of DI water in a glass vial, and CO₂ release was monitored by tracking the weight of the solution over time. For the weight effect, tablets with the same 1:3 citric acid-to-bicarbonate ratio were prepared at 150 mg, 300 mg, and 450 mg, and tested under the same conditions.

To assess pH changes, 0.1 M phosphate buffer (PB) solutions with pH values ranging from 5.5 to 8.5 were prepared. Tablets with various citric acid-to-bicarbonate ratios (300 mg total weight) were prepared as discussed. One tablet was added to 2 mL of the solution, and the final pH was measured using a pH meter (Accumet AB200, Fisher Scientific, Canada) after complete tablet dissolution. In addition to these tests, the stability of the CO₂ content, as the main indicator of mixing potential, and tablet weight was monitored over a month (details explained in the Supporting Information).

5.3.4. Nitrite Detection in Saliva

Nitrite detection was performed using the Griess assay method [390]. Speedy tablets (89 mg total, 7-mm die) contained 1.5 mg NED, 1.5 mg sulfanilamide, 24 mg of a powder mixture (88.3% mannitol, 10.4% PEG, 1.3% PVP), 20 mg sodium bicarbonate, and 42 mg citric acid monohydrate. Traditional tablets contained the same reagents (1.5 mg each of sulfanilamide and NED) with 32 mg of a powder mixture (91.2% mannitol, 7.8% PEG, 1% PVP). Two phosphate-buffered saline (PBS) solutions (pH 6.7) were prepared to mimic the viscosity of human saliva, each containing nitrite in the biological range (0–1.5 mg/dL). One contained 0.1 wt.% SCMC (1 mg/mL; 2.73 ± 0.03 mPa·s), and the other 0.4 wt.% SCMC (4 mg/mL; 9.41 ± 0.16 mPa·s), see Table 5.S2 for viscometer settings. For detection, 300 μ L of sample was added to a test tube preloaded with 300 μ L filler liquid and 10 μ L antifoam. The filler liquid was phosphate buffer (pH 5.1), but similar results were also observed with DI water. A single tablet was then dropped in, and images were taken after 20 minutes using a smartphone camera when color development stabilized.

For real saliva testing, samples were left to rest for 20 minutes at room temperature to allow solid components to settle naturally, mimicking point-of-care (POC) conditions without centrifugation. The supernatant (300 μ L) was used as described above. Nitrite concentration was quantified using both the Speedy tablet method and a UV-Vis standard protocol (ThermoFisher Scientific) [391] for comparison (see Supporting Information for full details) .

Tablet stability was assessed over one month by storing Speedy tablets in glass vials with silica gel at room temperature (22 °C), protected from light with aluminum foil. Tablets were tested in 1 mg/dL nitrite PBS solution, and images were captured with a smartphone (Samsung Galaxy S21 FE). Grayscale intensity was analyzed using ImageJ and normalized to day 0 as a measure of reagent activity over time.

5.3.5. Sugar Encapsulation of Enzymes for Glucose Assay

Glucose detection was based on the bienzymatic method, using GOx and HRP, as well as *o*-dianisidine as the indicator [392,393].

To incorporate these bioreagents into the tablet formulation, each was encapsulated in trehalose to enhance stability and facilitate weighing. For encapsulation, GOx (120 U/mL), HRP (65 U/mL), or *o*-dianisidine (1 mg/mL) solutions were prepared in 10 mL DI water and mixed with 5 wt.%

(50 mg/mL) trehalose. The solutions were sprayed onto non-stick trays using a handheld mist sprayer (20-30 cm distance, $\sim 45^\circ$ angle) and air-dried overnight at room temperature ($\sim 22^\circ\text{C}$, relative humidity $<46\%$). The resulting powders were collected and stored in glass vials. Trehalose was chosen over other sugars (i.e., pullulan, dextran) due to better sprayability and solubility.

To assess the activity of the encapsulated reagents, we compared their reaction kinetics with unencapsulated counterparts. Reagent solutions were prepared in DI water using either 25 mg/mL trehalose-GOx, 25 mg/mL trehalose-HRP, and 50 mg/mL trehalose-o-dianisidine, or equivalent concentrations of the original reagents (62.4 U/mL GOx, 33.8 U/mL HRP, 1.04 mg/mL o-dianisidine). For the assay, 50 μL of the sample (real urine spiked with 2 mM glucose) was added to a 96 well plate, followed by the addition of 50 μL phosphate citrate buffer (pH 5.1) and 100 μL of a reagent solution. Reaction progress was monitored at 430 nm using a UV-Vis spectrophotometer (Cytation 5, Agilent Technologies, USA).

5.3.6. Glucose Detection in Urine

The tablet “biosensors” contained 5 mg trehalose-GOx, 5 mg trehalose-HRP, 10 mg trehalose-o-dianisidine, 3.5 mg of a powder mixture (86% PEG, 14% PVP), 30 mg sodium bicarbonate, and 42 mg citric acid monohydrate, forming an all-in-one reagent system. For detection, 200 μL of sample was added to a microtube preloaded with 200 μL filler liquid and 10 μL of $10\times$ diluted antifoam. The filler liquid was citrate-phosphate buffer (pH 5.1), but similar results were also observed with DI water. One tablet was then added to initiate the reaction (see Table 5.S1 for tablet formulations and assay procedures). After color development, the test tube was briefly agitated by hand to homogenize the color, and images were taken with a smartphone camera. All tests were performed using spiked real urine samples containing 0.3–2.5 mM glucose (biologically relevant range). The grayscale color intensity of the solutions was analyzed in comparison to a reference point in the picture by ImageJ to create the calibration curve. For stability testing, tablets were stored at room temperature in a sealed glass vial with a silica gel pack and covered with aluminum foil. Tablet performance was evaluated in 1 mM glucose-spiked urine over a one-month period, and color intensities were normalized to day 0 values as percentage.

5.4. Results and Discussion

5.4.1. Characterization of the Tablets

We assessed the tablets with regard to their mixing power, their effect on the pH of the solution, and stability of their weight and CO₂ content in order to better understand their behavior and to develop proper functional tablet assays. The tablets were created by the main three steps of: 1) weighing and mixing the powders on a weighing paper, 2) pressing the mixture by a hydraulic press machine in 7, 11, or 13-mm die sets, and 3) storing the tablets in a glass vial containing silica gel packs to prevent humidity damaging the tablets (Figure 5.1A). The Speedy tablets contained citric acid monohydrate and sodium bicarbonate as effervescence materials that produce CO₂ gas upon contact with water, which causes vortex creation in the solution and, in turn, provides the auto-mixing capability (Figure 5.1B) [325]. Other than that, the tablets also contained mannitol as the bulking agent, PEG as the lubricating agent to facilitate removing the tablets from the die set after pressing, and PVP as the binding agent.

First, we compared the mixing behavior of the Speedy tablets with that of the traditional tablets and powder/granules (Figure 5.2A, [Movie 5.S1](#)). All formats contained the same 300 mg of colored glucose to be dissolved in 10 mL of DI water. We observed that the Speedy tablets were able to completely dissolve the colored glucose in the solution within ~ 3 min as evidenced by the uniform distribution of the color in the solution. However, for the traditional tablet and powder/granule format, the colored glucose was not completely dissolved even after 5 hours. These results indicate that the Speedy tablets are significantly effective in reducing the mixing time, without the need for any extra steps such as shaking or equipment such as a vortex mixer. Also, compared to the powder format, the tablet format facilitates portability of pre-measured amounts of reagents and enhances the ease-of-use, packaging, and dosage accuracy in final application.

Next, we evaluated the mixing power of the Speedy tablets in the solutions with different viscosities (Figure 5.2B and Table 5.S2). The solutions were prepared by dissolving 0.5 to 3 Wt.% SCMC in DI water to give different viscosities from 8.16 ± 0.02 to 1722 ± 4 mPa·s, respectively. One Speedy tablet was added to a microtube pre-loaded with 300 μ L of the viscous solution, 10-20 μ L antifoam, and 300 μ L of green dye solution, and the mixing behavior was recorded over

time. The results indicated that the tablets successfully mixed the green dye solution with the viscous solution up to a high viscosity of 1722 ± 4 mPa·s as confirmed by the uniform color distribution in the solution ([Movie 5.S2](#)). We also observed that mixing time increased with solution viscosity, rising from 1.7 ± 0.1 minutes in DI water to 4.5 ± 0.1 minutes in green dye solution containing 3 wt.% SCMC. Notably, in the absence of a tablet, the same viscous solution showed incomplete mixing even after 6 hours. These results clearly indicated that the Speedy tablets were able to mix the solutions with a high viscosity in an acceptable time range (less than 5 min), and they can be an effective platform to be used in challenging media with a high viscosity.

After that, we assessed the effect of tablet formulation and weight on their mixing power (Figure 5.2C). Tablets with the same weight (300 mg) but different molar ratios of citric acid to sodium bicarbonate were tested in 4 mL of DI water, and the release of CO₂ gas was recorded over time as an indicator of mixing power. Tablets with a 1:3 citric acid to sodium bicarbonate ratio released the most CO₂ in 3 min (34.2 ± 0.6 mg), compared to 1:2 (32.5 ± 3.3 mg) and 1:6 (23.6 ± 0.2 mg), indicating the highest mixing power. These results align with theoretical expectations, as the 1:3 ratio reflects the stoichiometric balance between citric acid and sodium bicarbonate in the effervescence reaction, ensuring maximum reactant availability in a given formulation. Any deviation from this ratio will cause reduced CO₂ generation, and in turn, mixing power. Additionally, tablets with the same 1:3 formulation but different weights were tested. The 450 mg tablet showed the highest mixing power, releasing 56.1 ± 2.7 mg of CO₂, followed by the 300 mg and 150 mg tablets with 34.2 ± 0.6 mg and 15.1 ± 1.5 mg, respectively. These results demonstrate that mixing power can be tuned by adjusting both formulation and tablet weight, allowing customization for specific applications.

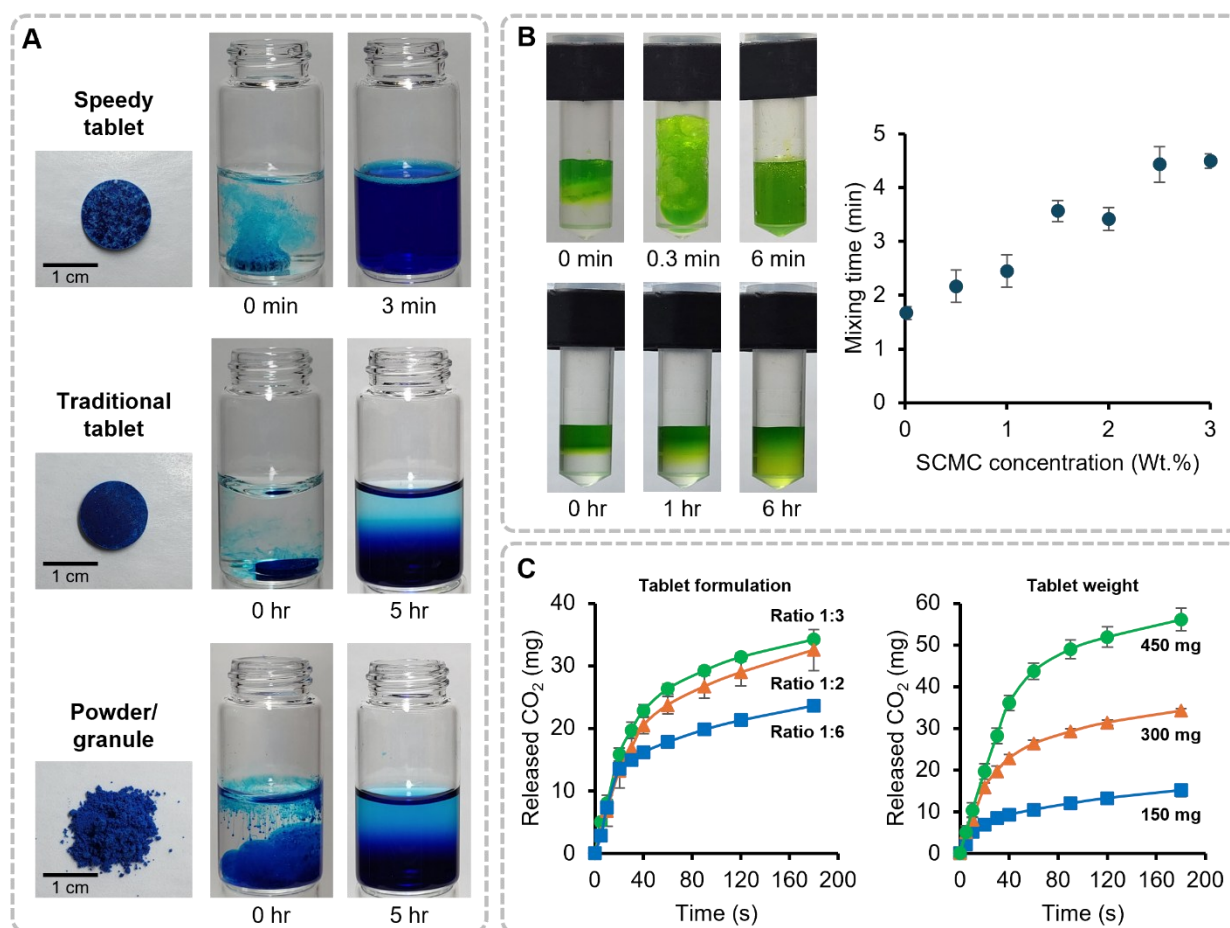


Figure 5.2. Characterization of the Speedy tablets. (A) Comparison of mixing performance between Speedy tablets, traditional tablets, and powder/granule format for dissolving 300 mg colored glucose in 10 mL water (see [Movie 5.S1](#)). (B) Mixing behavior of Speedy tablets in green dye solutions with viscosities ranging from 8.15 ± 0.02 to 1722 ± 3.46 mPa·s, prepared using 0.5–3 wt.% SCMC. Tablets were added to test tubes containing equal parts water and dye solution. Mixing completed within 4.5 ± 0.1 min for viscosities up to 1722 mPa·s; image taken at 6 min (top row), while without tablets, solutions remained unmixed after 6 h (bottom row, see [Movie 5.S2](#)). Graph shows mixing time vs. viscosity (mean $\pm$ SD, $n = 3$). (C) Effect of tablet formulation (left) and weight (right) on mixing power. Tablets (300 mg) with different citric acid:sodium bicarbonate molar ratios of 1:2, 1:3 (stoichiometric), and 1:6 were tested in 4 mL DI water, and CO₂ release was monitored as an indicator for mixing power. For weight tests, tablets of 150, 300, and 450 mg (1:3 ratio) were compared (mean $\pm$ SD, $n = 4$).

We also evaluated the effect of Speedy tablets on solution pH, an important parameter in many assay protocols (Figure 5.3). Tablets (300 mg) with varying citric acid:sodium bicarbonate molar ratios were tested in 2 mL of DI water and phosphate buffer (PB) solutions at pH 5.5, 7.0, and 8.5. The tablets exhibited strong buffering capacity, effectively minimizing pH changes across a wide pH range. This buffering behavior was tunable, and increasing the citric acid content shifted the

final pH from neutral toward acidic. Tablets with a 1:6 ratio maintained pH between 6.9–7.1, although some sodium bicarbonate remained undissolved due to the reduced mixing power at this ratio. Ratios of 1:3 (stoichiometric) and 1:2 resulted in pH ranges of 5.9–6.3 and 5.0–5.3, respectively. The reason for this strong buffering behavior is the presence of two buffer systems in the effervescence reaction mechanism: 1) citric acid-sodium citrate buffer and 2) bicarbonate buffer. When citric acid reacts with sodium bicarbonate, it produces sodium citrate, which is the conjugate base of the citric acid, creating a buffer system that mainly acts in the acidic range [394]. On the other hand, sodium bicarbonate creates a buffer system with weak carbonic acid that can easily turn into carbon dioxide and water. This system is one of the main buffer systems in the body (blood) as well [395]. Other effervescent formulations without acidic components can also be used to further adjust the pH if needed [396].

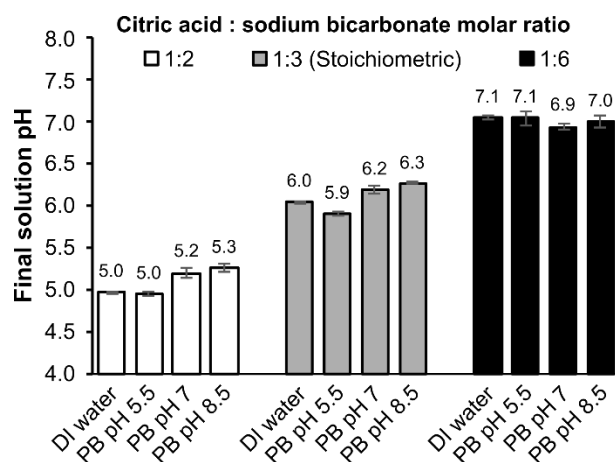


Figure 5.3. Effect of Speedy tablets on solution pH. Tablets with varying citric acid:sodium bicarbonate molar ratios were dissolved in DI water and 0.1 M phosphate buffer (PB) solutions with initial pH values ranging from 5.5 to 8.5. Final pH was measured after the dissolution of the tablet (mean $\pm$ SD; $n = 3$). The tablets exhibited strong buffering capacity, effectively minimizing pH changes.

Finally, we investigated the stability of the tablets' weight and CO₂ content up to 1 month as the CO₂ content is the indicator of the mixing potential of the tablets (Figure 5.S1). The weight of the tablets reduced by ~2.5%, which is mainly due to the loss of the water content in the tablets [396]. Also, the tablets retained ~92% of their CO₂ content over a month, while no statistically significant difference was observed between the CO₂ content of day 0 and week 4, indicating the stability of the mixing potential. Overall, the tablets showed a significant mixing power to reduce the mixing time and enhance ease-of-use both in low and high viscosities. Also, the buffering effect of the

tablets enables the creation of all-in-one tablet assays without the need to add extra buffer reagents to either the tablet composition or the solution.

5.4.2. Analytical Performance in Viscous Fluids - Nitrite Detection in Saliva

After characterizing the tablets' mixing power and pH effects, we developed Speedy tablet sensors for semi-quantitative detection of nitrite in saliva as a proof-of-concept application. Saliva is an attractive diagnostic fluid due to its non-invasive collection and rich composition of biomarkers. Compared to blood, it allows easy, stress-free sampling without specialized equipment, making it suitable for frequent monitoring [397]. However, its high and variable viscosity presents challenges for POC testing by affecting molecular transport and assay consistency [182,398,399]. On the other hand, nitrite is a key inorganic biomarker in saliva, with elevated levels linked to periodontal disease [182,388,400], oral cancer [387,401], and end-stage renal diseases [402,403]. Some studies also have found correlations between salivary nitrite and nitrite in blood, indicating that salivary nitrite may be used to monitor the blood nitrite levels non-invasively [404,405]. This is particularly important as abnormal body nitrite has been reported with various diseases including gastric cancer and blue baby syndrome [406].

We developed Speedy tablet sensors for nitrite detection based on the Griess assay, in which sulfanilamide reacts with nitrite under acidic conditions to form a diazonium cation that couples with NED, producing a purple-pink azo dye (Figure 5.4A) [390]. The Tablets were fabricated using a 7-mm die and contained sulfanilamide and NED (reagents), mannitol (bulking agent), PEG (lubricant), PVP (binder), and citric acid with sodium bicarbonate (effervescence agents). Mannitol is a sugar-alcohol that is typically used in tablet production [407,408]. The non-hygroscopicity and inertness of mannitol makes it an optimal choice over other sugar bulking agents (e.g., sorbitol) to incorporate in tablet sensors to help in long-term stability [25]. The post-fabrication weight of the tablets was 87.3 ± 0.7 mg, with a thickness of 1.53 ± 0.02 mm. For the detection, one sensor tablet was added to a test tube pre-loaded with 300 μ L of filler liquid, 10 μ L of antifoam agent, and 300 μ L of the sample. The filler liquid was phosphate buffer (pH 5.1), although we observed similar results with DI water due to the tablets' strong buffering effect. The pre-loaded filler liquid ensured sufficient solution volume, allowing the Speedy tablets to efficiently mix the reagents, minimize the required sample volume, and adjust sensor performance by diluting the sample to the desired concentration range.

In order to assess the nitrite tablets' performance in different viscosities, we prepared two PBS solutions pH 6.7 with viscosities of 2.73 ± 0.03 and 9.41 ± 0.16 mPa·s by dissolving 0.1 Wt.% and 0.4 Wt% SCMC in the solutions, respectively, to mimic the low and high saliva viscosity. Different studies have reported various viscosity ranges for human saliva depending on their sample collection techniques and the shear rates used to determine viscosity [409]. In one study, the viscosity of saliva was reported to be from 3.8 to 8.8 mPa·s, which is covered by our low and high viscosity PBS solutions [409,410]. Also, we chose the pH of 6.7 for the samples to match the pH of actual human saliva, which ranges from 6.2-7.6, with 6.7 being the average [411].

As shown in Figure 5.4B, we tested the Speedy tablets across a nitrite concentration range of 0–1.5 mg/dL in two PBS solutions with low and high viscosity. The tablets produced similar color responses in both viscosities at all concentrations, without requiring shaking or other user intervention. Unlike traditional tablets, which failed to dissolve even after 1 hour (Figure 5.4C, [Movie 5.S3](#)) and required ~1.5 minutes of vigorous hand shaking to mix, the Speedy tablets dissolved completely within ~3 minutes, yielding uniform color development. Calibration curves for both viscosities demonstrated high linearity ($R^2 > 0.99$) with similar trendlines (Figure 5.4D).

To rigorously evaluate reproducibility and any minor differences between viscosities, we performed six replicates at 1.5 mg/dL (Figure 5.S2A). The relative standard deviations (RSDs) were 10.17% for the low-viscosity solution and 5.52% for the high-viscosity solution, with no statistically significant difference between them. The observed variation was primarily due to weighing and handling errors, as each tablet contained only 1.5 mg of Griess reagents. This reason became evident when we tripled the tablet formulation to reduce these errors, and the RSDs improved to 3.53% (low viscosity) and 2.59% (high viscosity), confirming improved performance in both viscosities (Figure 5.S2B). Overall, the tablets provided consistent results across varying viscosities. Using well-established powder mixing technologies or encapsulation methods to make the powders bulkier will further improve the reproducibility of the tablets for testing in low volumes. Also, due to the tablets' buffering effect, the solution pH automatically adjusted to 3.73 ± 0.02 when DI water was used as the filler liquid. Their working range was 0.03–1.5 mg/dL, covering nitrite levels typically found in healthy human saliva (0.6–1 mg/dL) [412]. Moreover, the experimental limit of detection (LOD) of the tablet sensors was 0.03 mg/dL, which is lower than the LOD of several reported POC devices for nitrite detection (Table 5.S3).

Based on the above-mentioned results, we concluded that semi-quantitative analysis is well-suited for the real-world application of the tablets. To validate their performance, we tested the nitrite content in saliva samples from 4 individuals and classified their concentrations into different ranges according to the colors produced in PBS tests (Figure 5.S3). The tablets successfully detected the nitrite concentration ranges in all samples, which were consistent with the results obtained using the standard UV-Vis method. These findings demonstrate the tablets' applicability in real scenarios and complex biological matrices. Finally, we evaluated the tablets' stability over one month (Figure 5.4E). According to Griess reagent manufacturers, the liquid mixture must be used within 8 hours due to NED's susceptibility to oxidation by air and light, and it requires refrigerated storage [391]. We stored our tablets at room temperature (22 °C) in glass vials, protected from light with aluminum foil and from moisture with a silica gel pack. When tested in 1 mg/dL nitrite PBS solutions, the tablets retained $97.0 \pm 5.9\%$ of their initial activity after 29 days, demonstrating high stability without refrigeration.

It should be noted that other tablet-based assays have been reported for either detection of analytes in saliva or nitrite detection. In one study, pullulan encapsulated gold nanoparticle tablets were employed to detect glucose in saliva [26]. However, the detection method required preparation of separate solutions of GOx and dissolved gold nanoparticle tablets as well as utilization of a vortex mixer to give the final assay results. These steps would complicate the assay procedure, especially for POC testing. In another study, multi-layered tablets with sequential release of reagents were developed to detect nitrite by the Griess reaction method [324]. While these tablets showed good analytical performance, their applicability in viscous fluids such as saliva was not studied. Also, the fabrication of multi-layered tablets required several steps, making it difficult for mass production. Overall, our nitrite tablets are low cost (~\$0.03) and are easy to fabricate and scale up with current industrial technologies, while the auto-mixing capability allows for POC testing in viscous fluids such as saliva without the need for any equipment or extra steps.

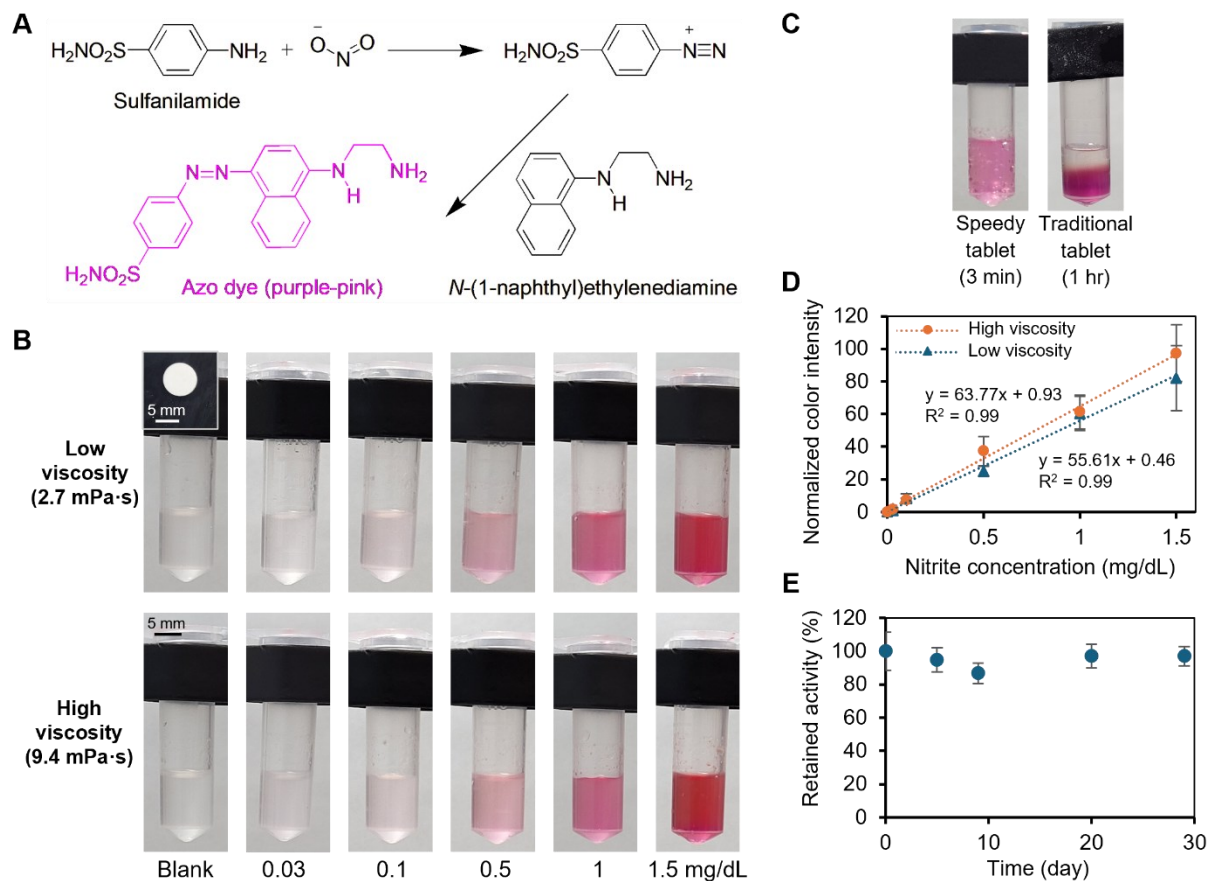


Figure 5.4. Nitrite detection by Speedy tablet sensors. (A) Schematic of the Griess assay used for nitrite detection. (B) Sensor performance in PBS solutions with low and high viscosities, mimicking variability in saliva. The tablets yielded consistent color responses across 0-1.5 mg/dL nitrite, regardless of viscosity. (C) Comparison with traditional tablets containing the same Griess reagents. Speedy tablets fully dissolved within ~3 min, while traditional tablets did not completely mix even after 1 hour (see [Movie 5.S3](#)). (D) Calibration curves for low and high viscosity solutions with linear trendlines (mean $\pm$ SD; $n = 3$). (E) Stability of Speedy tablets stored at room temperature for 1 month, retaining $97.0 \pm 5.9\%$ of activity (mean $\pm$ SD; $n = 3$).

5.4.3. Tablet-Based “Biosensors” - Glucose Detection in Urine

So far, we have shown that Speedy tablets have a significant mixing power and pH buffering effect with applications in challenging viscous fluids such as saliva. To further expand the applicability of the proposed tablets, we developed Speedy tablet “biosensors” for the first time, with a proof-of-concept application for glucose detection in urine. Bioreagents such as enzymes play a pivotal role in many detection strategies owing to their high specificity and sensitivity, and having functional tablet biosensors with rapid auto-mixing is a great step forward in tablet assays. Also,

glucose detection in urine can play a pivotal role in the diagnosis and management of diabetes. International Diabetes Federation reported ~540 million adults living with diabetes worldwide in 2021, while 240 million people remaining undiagnosed [413]. If diabetes is not managed properly, it can result in various serious health complications such as increased risk of cardiovascular diseases [414], kidney failure [415], and vision loss [416], emphasizing the importance of timely diagnosis of diabetes. While, currently, electrochemical test strips are widely used for POC detection of glucose in blood for diabetes monitoring, this method is invasive and painful [417,418]. Thus, glucose detection in urine provides a convenient non-invasive approach to complement current methods.

Here, we detect glucose by the bienzymatic method shown in Figure 5.5A. In this method, first, GOx catalyzes glucose into gluconic acid and hydrogen peroxide. Then, hydrogen peroxide oxidizes *o*-dianisidine in the presence of HRP to give the brown-colored oxidized *o*-dianisidine as the indicator of the reaction [392,393]. However, incorporating bioreagents such as enzymes into tablet assays via powder compression presents challenges. First, the small quantities required for an assay make it difficult to weigh, mix, and distribute enzymes uniformly within the formulation. Second, enzymes are sensitive to environmental factors like humidity and temperature and typically lack functional stability without modification [4,5]. To address these issues, encapsulation is necessary to both bulk up the enzyme material for easier handling and enhance its stability over time.

We developed a convenient method to encapsulate enzymes and *o*-dianisidine in trehalose (Figure 5.5B). First, a solution of the target substance was prepared in DI water, followed by the addition of 5 wt.% (50 mg/mL) trehalose. The solution was sprayed onto a non-stick tray using a handheld mist sprayer and air-dried at room temperature, yielding solid trehalose-encapsulated particles. The particles were then collected with a spatula. Trehalose was selected for its well-established stabilizing effect on biomolecules [196,336,419]. The experimental conversion rate from solution to powder was 48 mg solid/1 mL solution, enabling accurate calculation of active reagent content in the trehalose-encapsulated powder. For example, we used 5 mg of trehalose-HRP powder per tablet, corresponding to 104 μ L of the initial 65 U/mL enzyme solution. This amount contained only ~0.03 mg of HRP, an amount too small to weigh accurately prior to encapsulation. Of note, the common methods of encapsulation are freeze drying (lyophilization) and spray drying that require bulky and expensive equipment [420,421]. Previous studies have used freeze drying to

encapsulate enzymes and oligonucleotides for tablet assays [25,324], but our method offers a low-cost alternative using only a mist sprayer, tray, and spatula.

Next, we compared the reaction kinetics of the encapsulated enzymes and indicator with that of the unencapsulated substances to investigate the impact of encapsulation on the reaction. Two reagent solutions were prepared with the same concentrations of enzyme and indicator and were used to detect 2 mM glucose. The development of the brown product of o-dianisidine oxidation was monitored over time at 430 nm by a UV-Vis machine. As shown in Figure 5.5C, the trehalose-encapsulated reagents gave a similar kinetic trend compared to that of the original reagents. The trehalose-encapsulated reagents exhibited slightly higher absorbance, likely due to background light absorption caused by the presence of trehalose. These results confirmed that our encapsulation method could be reliably used to prepare reagents for use in tablets without loss of performance.

The tablet biosensors contained 5 mg trehalose-GOx (equivalent to ~0.09 mg original GOx powder), 5 mg trehalose-HRP (~0.03 mg HRP), 10 mg trehalose-o-dianisidine (~0.21 mg o-dianisidine), along with PEG, PVP, citric acid, and sodium bicarbonate, for a total weight of 92.35 ± 0.58 mg. Tablet dimensions were 7 mm in diameter and 1.57 ± 0.02 mm in thickness. For testing, one tablet biosensor was dropped in the test tubes preloaded with 200 μ L filler liquid, 10 μ L diluted antifoam, and 200 μ L sample. The filler liquid was citrate-phosphate buffer (pH 5.1), but similar results were observed for DI water due to the tablet's buffering capability. The filler liquid in the assay protocol provided enough liquid for the tablets to generate bubbles and mix the solutions, adjusted the sensor performance for the desired concentration range, and minimized the color background of the urine sample by diluting it. As shown in Figure 5.5D, the tablets successfully detected glucose in spiked real urine samples with concentrations of 0.3 to 2.5 mM, giving a gradual color change as expected. The RSD of all data was below 6%, indicating high reproducibility of the results. The tablets also auto adjusted the pH of the solution to 4.54 ± 0.03 when used with DI water, making them an all-in-one tablet without any additional steps.

We further assessed the performance of the tablet biosensors by quantitative analysis of the color intensity in the assay results (Figure 5.5E). The data followed a typical saturation curve consistent with enzymatic reactions, similar to what is reported in the literature [13,196]. We fitted a Michaelis-Menten equation to the data, resulting in an $R^2 > 0.99$ that indicates a high correlation

between the data and the Michaelis-Menten equation as a saturation curve. We also performed a parallel study with the same reagents in a 96 well plate and analyzed the results with UV-Vis (Figure 5.S4). The UV-Vis data showed a similar Michaelis-Menten behavior within the same glucose range, providing strong validation of the results obtained using the Speedy tablets and smartphone. The experimental LOD for the tablets was 0.3 mM, lower than that of several reported POC devices (Table 5.S3). The working range of 0.3–2.5 mM covers the biological range of urinary glucose, which is typically 0–0.8 mM. Elevated glucose levels in urine may indicate hyperglycemia and can serve as a non-invasive marker for conditions such as diabetes mellitus [422].

Other tablet-based assays have also been reported for glucose detection in urine. Some articles employed copper sulphate in the tablet for the detection of total reducing substances in urine for glycosuria using auto-mixing tablets [304,311]. In this type of assay, copper is reduced to cuprous oxide in the presence of reducing substances such as glucose to produce different colors. However, this method is not specific to glucose and measures the total reducing substances in urine that can lead to over estimation of glucose concentration [311]. Previously, our group developed dextran-encapsulated GOx and HRP tablets for glucose detection in urine. However, that method required separate addition of an indicator solution and manual mixing (vortexing or shaking) to dissolve the tablet contents [334]. The auto-mixing tablet biosensors are highly specific due to the incorporation of enzymes in the formulation and are all-in-one, requiring no additional steps to perform the assay.

Finally, we evaluated the stability of the tablet biosensors over 1 month (Figure 5.5F). Tablets were stored at room temperature (22 °C) in a glass vial with a silica gel pack and wrapped in aluminum foil. Stability was assessed using 1 mM glucose, with color intensity normalized to day 0 as a measure of performance. After 30 days, tablets retained $94.2 \pm 6.6\%$ of their functionality, demonstrating excellent stability for storage and transport without refrigeration. With a production cost of just \$0.27 per tablet, this platform offers a low-cost, robust solution for POC applications.

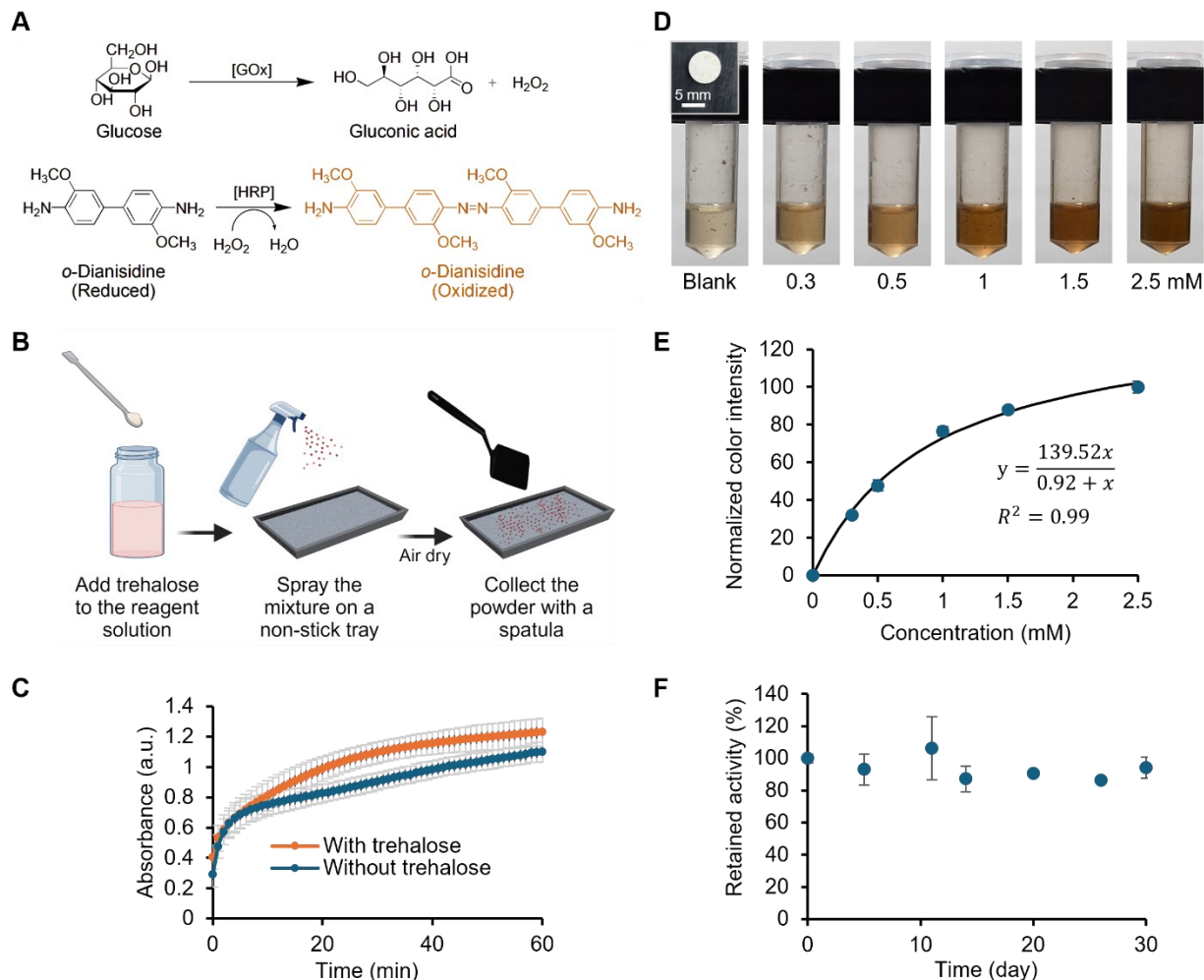


Figure 5.5. Glucose detection by Speedy tablet biosensors. (A) Schematic of the bienzymatic glucose detection reaction. (B) Schematic of the spray-tray method for encapsulating GOx, HRP, and o-dianisidine in trehalose without any bulky equipment. (C) Comparison of encapsulated vs. unencapsulated reagents in a 2 mM glucose assay; no difference in the performance was observed (mean $\pm$ SD; $n = 3$). (D) Detection of glucose in spiked real urine samples using Speedy tablets. (E) Calibration curve from color intensity analysis fitted to a Michaelis–Menten model ($R^2 > 0.99$; mean $\pm$ SD; $n = 3$). (F) Tablet stability after 1 month of storage at room temperature, retaining $94.2 \pm 6.6\%$ activity (mean $\pm$ SD; $n = 3$).

5.5. Conclusions

To broaden the applicability of tablet-based platforms, we developed rapid auto-mixing tablet sensors and biosensors for point-of-care applications. These tablets contain appropriate amounts of effervescent chemicals, such as citric acid and sodium bicarbonate, enabling rapid release and mixing of the reagents. The tablets demonstrated over a 100-fold improvement in mixing

efficiency compared to traditional methods and effectively auto-mixed in highly viscous solutions up to 1700 mPa·s. As proof of concept, we created an all-inclusive tablet, containing all necessary reagents, and successfully detected nitrite in real human saliva, a challenging medium due to its variable viscosity. The tablet sensor demonstrated a working range of 0.03-1.5 mg/dL, covering the range of nitrite in healthy humans. Next, we demonstrated a simple method to encapsulate enzymes in trehalose to make them bulkier and more stable under ambient conditions, using only a handheld mist sprayer, a non-stick tray, and a spatula. Using the encapsulated enzymes, we created the first auto-mixing tablet “biosensors” and used them in detecting glucose in real human urine within the biologically relevant range of 0.3-2.5 mM. The nitrite and glucose tablets cost only ~\$0.03 and \$0.27 to produce, respectively, while both maintained their activity for at least one month at room temperature, demonstrating their potential for low-resource settings.

One limitation of the auto-mixing tablets is that the effervescent chemicals may interfere with the assay chemistry; however, optimizing the amount of effervescent chemicals and selecting proper assay reagents may overcome this issue. Additionally, the effervescent chemicals have a strong buffering effect that limits their application in sensing strategies involving pH alterations. Nevertheless, this buffering effect can be advantageous for other applications, such as creating tablets with automatic pH adjustments. We believe that our proposed auto-mixing tablets can provide an efficient, low-cost, and portable platform for a wide range of applications, including nucleic acid amplification tests, challenging assays for intracellular molecules, and monitoring in agriculture, water, soil, and food. Also, integrating tablet-based assays with smartphones and telecommunication will further extend the use of these devices beyond the laboratory, enhancing their real-world applicability.

5.6. Supporting Information

Summary of the Tablet Formulations and Test Procedures

Table 5.S1. Summary of the tablet formulation components and test procedures for each of our case studies.

Reference to section	Tablet formulation	Die size	Test procedure	Study goal
Characterization of the tablets	300 mg colored glucose, 2 mg PVP, 10 mg PEG, 88 mg sodium bicarbonate, and 100 mg citric acid monohydrate	13 mm	Drop one tablet in 10 mL of DI water in a glass vial	Format comparison-Speedy tablet
Characterization of the tablets	300 mg colored glucose, 2 mg PVP, 10 mg PEG	13 mm	Drop one tablet in 10 mL of DI water in a glass vial	Format comparison-traditional tablet
Characterization of the tablets	30 mg of powder mixture (86% mannitol, 12% PEG, 2% PVP), 37 mg sodium bicarbonate, and 30 mg citric acid monohydrate	7 mm	Drop one tablet into a microtube pre-loaded with 300 μ L of DI water, 10-20 μ L of antifoam, and 300 μ L of the viscous fluid	Mixing power in viscous fluids
Characterization of the tablets	100 mg powder mixture (86% mannitol, 12% PEG, and 2% PVP), 88.9 mg sodium bicarbonate, and 111.1 mg citric acid monohydrate	11 mm	For mixing power: drop one tablet in 4 mL of DI water in a glass vial For pH: drop one tablet in 2 mL of DI water in a centrifuge tube	Effect of tablet formulation on mixing power and pH-Ratio 1:2

Characterization of the tablets	100 mg powder mixture (86% mannitol, 12% PEG, and 2% PVP), 109 mg sodium bicarbonate, and 91 mg citric acid monohydrate	11 mm	For mixing power: drop one tablet in 4 mL of DI water in a glass vial For pH: drop one tablet in 2 mL of DI water in a centrifuge tube	Effect of tablet formulation on mixing power and pH-Ratio 1:3
Characterization of the tablets	100 mg powder mixture (86% mannitol, 12% PEG, and 2% PVP), 141.2 mg sodium bicarbonate, and 58.8 mg citric acid monohydrate	11 mm	For mixing power: drop one tablet in 4 mL of DI water in a glass vial For pH: drop one tablet in 2 mL of DI water in a centrifuge tube	Effect of tablet formulation on mixing power and pH-Ratio 1:6
Characterization of the tablets	50 mg powder mixture (86% mannitol, 12% PEG, and 2% PVP), 54.5 mg sodium bicarbonate, and 45.5 mg citric acid monohydrate	11 mm	Drop one tablet in 4 mL of DI water in a glass vial	Effect of tablet weight on mixing power-150 mg
Characterization of the tablets	150 mg powder mixture (86% mannitol, 12% PEG, and 2% PVP), 163.5 mg sodium bicarbonate, and 136.5 mg citric acid monohydrate	11 mm	Drop one tablet in 4 mL of DI water in a glass vial	Effect of tablet weight on mixing power-450 mg
Characterization of the tablets	124 mg powder mixture (86% mannitol, 12% PEG, and 2% PVP), 152 mg sodium bicarbonate, and 124 mg citric acid monohydrate	13 mm	Drop one tablet in 100 mL of sulfuric acid (0.5 M)	Stability of the CO ₂ content and weight of the tablets

Analytical performance in viscous fluids- nitrite detection in saliva	1.5 mg NED, 1.5 mg sulfanilamide, 24 mg powder mixture (88.3% mannitol, 10.4% PEG, and 1.3% PVP), 20 mg sodium bicarbonate, and 42 mg citric acid monohydrate	7 mm	Drop one tablet into a microtube pre-loaded with 300 μ L of filler liquid, 10 μ L of antifoam, and 300 μ L of the sample. Filler liquid was either phosphate buffer (pH 5.1) or DI water	Nitrite detection-Speedy tablet
Analytical performance in viscous fluids- nitrite detection in saliva	1.5 mg NED, 1.5 mg sulfanilamide, and 32 mg powder mixture (91.2% mannitol, 7.8% PEG, and 1% PVP)	7 mm	Drop one tablet into a microtube pre-loaded with 300 μ L of phosphate buffer (pH 5.1), 10 μ L of antifoam, and 300 μ L of the sample	Nitrite detection-traditional tablet
Analytical performance in viscous fluids- nitrite detection in saliva	4.5 mg NED, 4.5 mg sulfanilamide, 72 mg powder mixture (88.3% mannitol, 10.4% PEG, and 1.3% PVP), 60 mg sodium bicarbonate, and 126 mg citric acid monohydrate	11 mm	Drop one tablet into a glass vial pre-loaded with 900 μ L of phosphate buffer (pH 5.1), 30 μ L of antifoam, and 900 μ L of the sample	Nitrite detection-Speedy tablet-big
Tablet-based “biosensors”- glucose detection in urine	5 mg trehalose-GOx, 5 mg trehalose-HRP, 10 mg trehalose-o-dianisidine dihydrochloride, 3.5 mg powder mixture (86% PEG and 14% PVP), 30 mg sodium bicarbonate, and 42 mg citric acid monohydrate	7 mm	Drop one tablet into a microtube pre-loaded with 200 μ L of filler liquid, 10 μ L of 10x diluted antifoam, and 200 μ L of the sample. Filler liquid was either citrate-phosphate buffer (pH 5.1) or DI water	Glucose detection-Speedy tablet

PVP: polyvinylpyrrolidone K30; PEG: polyethylene glycol 6000; DI water: deionized water; NED: *N*-(1-naphthyl)ethylenediamine dihydrochloride

Viscosity of the solutions

In this study, we prepared solutions with different viscosities by adding different Wt.% of sodium carboxymethylcellulose (SCMC) to the solutions. The viscosity of the solutions was measured by a rotational viscometer (SNB-2 model) using different settings needed with respect to the viscosity of the solution. The results of the measurements and the settings of the viscometer for each measurement are summarized in Table 5.S2.

Table 5.S2. Viscosity of the solutions and the settings of the viscometer used in this study.

Solution	Viscosity (mPa·s) ^a	Rotor number	Speed (rpm)
0.5% SCMC in water	8.15 ± 0.02	0	60
1.0% SCMC in water	28.21 ± 0.45	0	12
1.5% SCMC in water	66.06 ± 0.21	0	6
2.0% SCMC in water	395 ± 1	1	12
2.5% SCMC in water	751 ± 1	1	6
3.0% SCMC in water	1722 ± 3	1	3
0.1% SCMC in PBS	2.73 ± 0.03	0	60
0.4% SCMC in PBS	9.41 ± 0.16	0	30

^aThe values for the measured viscosities are mean ± standard deviation for 3 replications.

SCMC: sodium carboxymethylcellulose; PBS: phosphate buffer saline.

Stability of the CO₂ Content and Weight of the Tablets

The stability of the CO₂ content and weight of the tablets was monitored over 1 month. These parameters are important to evaluate the stability of the mixing potential of the tablets. The tablets were created with the 13-mm die set and contained 124 mg citric acid, 152 mg sodium bicarbonate, and 124 mg mixture of other powders (86% mannitol, 12% polyethylene glycol (PEG), and 2% polyvinylpyrrolidone (PVP)). The tablets were stored in a glass vial containing 1 silica gel pack at room temperature (22 °C). The CO₂ content was calculated by measuring the weight difference of solution before and after dissolving a single tablet in 100 ml of 0.5 M sulfuric acid, as described by other groups.[386,396]

The initial weight of the tablets was 398.5 ± 0.4 mg on day 0, and it decreased by 2.5% over 4 weeks to 388.5 ± 0.2 mg. This weight loss is mainly attributed to the loss of water content in the tablets [396]. The CO₂ content of the tablets was 80 ± 10 mg on day 0, and the tablets maintained ~92% of the CO₂ content after 4 weeks, while statistical analysis revealed no significant difference between the two data (t-student test, p-value = 0.37).

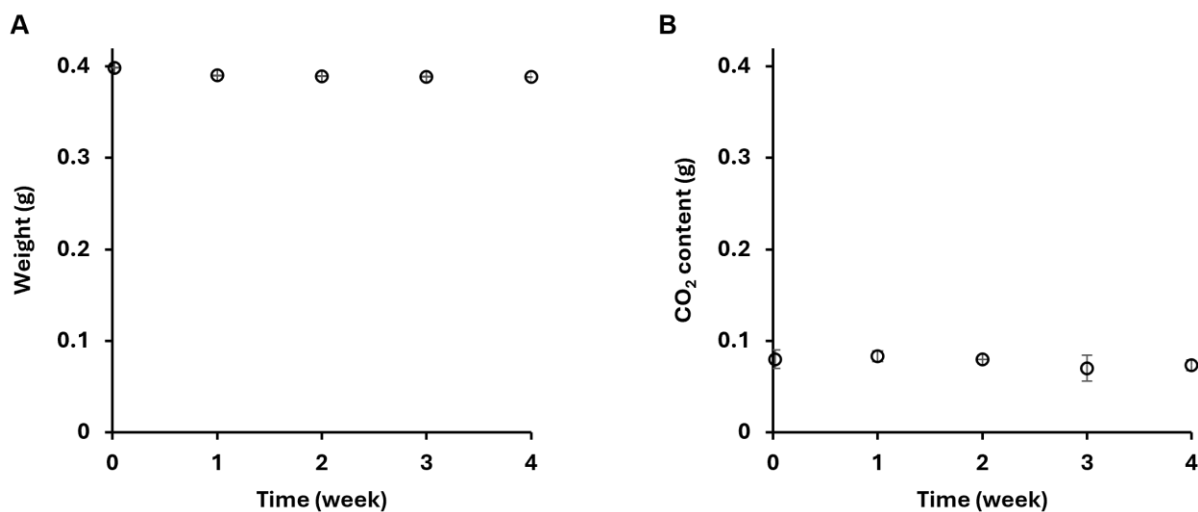


Figure 5.S1. The stability of the weight and CO₂ content of the tablets. (A) Weight of the tablets at different time points (n=5), (B) CO₂ content of the tablets calculated by measuring the CO₂ release after dissolving one tablet in 100 ml of 0.5 M sulfuric acid (n=3). Each data point is mean $\pm$ standard deviation of the replications.

Reproducibility of the Nitrite Assay

To assess the reproducibility of the nitrite Speedy tablets, we performed six replicates of the nitrite assay in 1.5 mg/dL nitrite solutions (Figure 5.S2). First, 7-mm tablets were tested in phosphate buffer saline (PBS) solutions of both low and high viscosity (Figure 5.S2A). The greyscale color intensities were compared to a reference point in the images. The results showed a relative standard deviation (RSD) of 10.17% for low-viscosity PBS and 5.6% for high-viscosity PBS. A two-tailed Student's t-test (equal variance) revealed no significant difference between the color intensities for the two viscosities ($p = 0.33$).

Next, to assess the effect of tablet size on assay reproducibility, we prepared nitrite Speedy tablets using three times the original formulation and assay solution volume (Figure 5.S2B; see Table 5.S1 for formulations). These larger tablets were fabricated using an 11-mm die set. The RSD was 3.5% for the low-viscosity solution and 2.6% for the high-viscosity solution. Again, no statistically significant difference was observed between the color intensities ($p = 0.53$). These results demonstrate that both small and large tablets perform reliably in solutions of varying viscosity. However, larger tablets reduced variability associated with powder weighing and handling, thereby improving assay reproducibility.

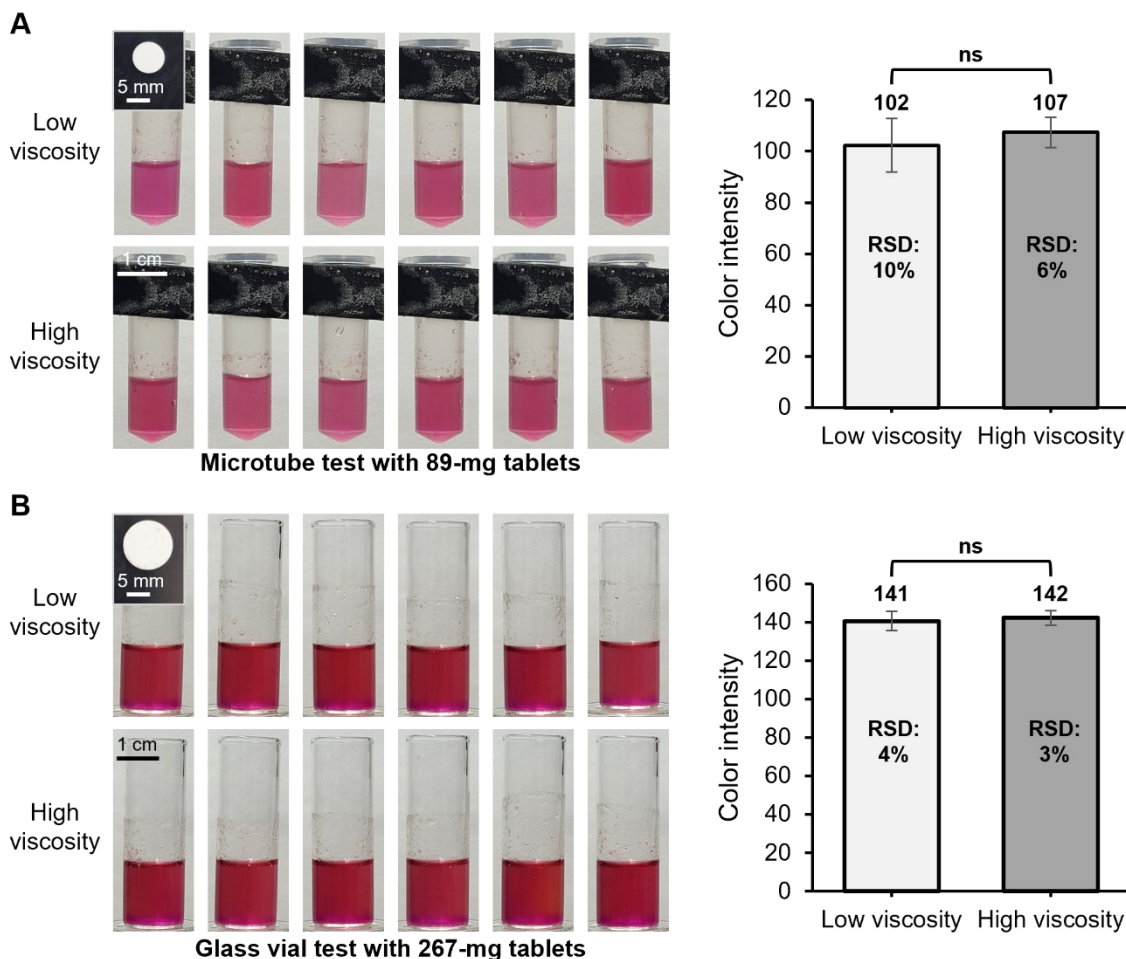


Figure 5.S2. Reproducibility tests for nitrite Speedy tablets. (A) Tablets with 7-mm diameter and 89 mg were prepared and tested in 1.5 mg/dL nitrite solutions in low and high viscosity PBS. The color intensities were analyzed for relative standard deviation (RSD, mean $\pm$ SD, n=6). No significant difference was observed between the color intensities (p-value > 0.05). (B) Tablets with 11-mm diameter and tripled formulation (267 mg) were prepared and tested in both viscosities and RSDs were obtained (mean $\pm$ SD, n=6). No statistically significant difference was observed between the results of low and high viscosity solutions (p-value > 0.05).

Standard UV-Vis Method for Nitrite Detection and Real Saliva Tests

Standard UV-Vis measurements for nitrite detection in real saliva were performed following the ThermoFisher Scientific protocol [391] with modifications. Reagent solutions were prepared by dissolving 1 mg/mL of N-(1-naphthyl)ethylenediamine dihydrochloride (NED) in deionized water and 10 mg/mL of sulfanilamide in 5% phosphoric acid. Prior to measurements, equal volumes of the NED and sulfanilamide solutions were mixed (1:1) to prepare the Griess reagent. For the assay, 20 μ L of the Griess reagent mixture was added to a well of a 96-well plate, followed by 150 μ L of the sample and 130 μ L of deionized water. The plate was shaken for 30 minutes on a microplate vortex mixer (Fisherbrand, USA) at room temperature. Absorbance was then measured at 540 nm using a UV-Vis spectrophotometer (Cytation 5, Agilent Technologies, USA).

To generate the calibration curve, nitrite standards ranging from 0.01 to 0.3 mg/dL were prepared in deionized water and tested using the UV-Vis method (Figure 5.S3A). For the analysis of real saliva samples, the samples were allowed to rest at room temperature for 20 minutes to allow solid particles to settle. The supernatant was collected and diluted 10-fold to bring the concentration within the assay's detection range. The diluted sample was then tested using the UV-Vis method, and nitrite concentration was calculated using the calibration curve equation. As shown in Figure 5.S3B, the UV-Vis results were consistent with those obtained using the Speedy tablets.

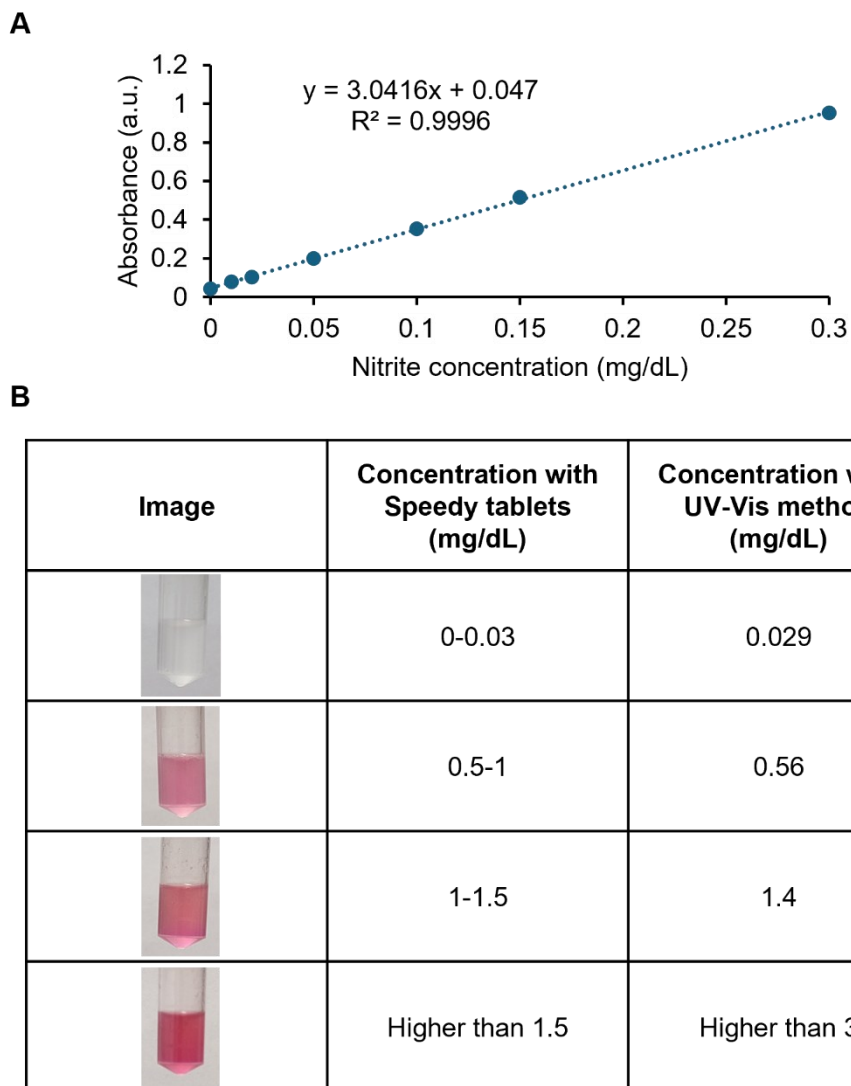


Figure 5.S3. Standard UV-Vis method for nitrite detection. (A) Calibration curve obtained using the UV-Vis method by testing standard nitrite concentrations ranging from 0.01 to 0.3 mg/dL in deionized water at 540 nm (mean $\pm$ SD, n=6). (B) Comparison of nitrite detection results in real saliva samples using the Speedy tablets and the UV-Vis method.

UV-Vis Test for the Glucose Assay

UV-Vis measurements for the glucose assay were performed in 96-well plates to compare with the performance of the tablet-based assay using smartphone imaging (Figure 5.S4). The UV-Vis assay employed the same reagents as the tablet-based method. A reagent solution was prepared in deionized water containing 62.4 U/mL glucose oxidase, 33.8 U/mL horseradish peroxidase, and 1.04 mg/mL o-dianisidine dihydrochloride.

For the assay, sample concentrations of 0, 0.3, 0.5, 1, 1.5, and 2.5 mM were prepared by mixing 50 μ L of glucose-spiked real urine samples with 50 μ L of phosphate-citrate buffer (pH 5.1) in a 96-well plate. Next, 100 μ L of the reagent solution was added to initiate the reaction. After color development, the solution was gently agitated with a pipette tip, and absorbance was measured at 430 nm using a UV-Vis plate reader (Cytation 5, Agilent Technologies, USA).

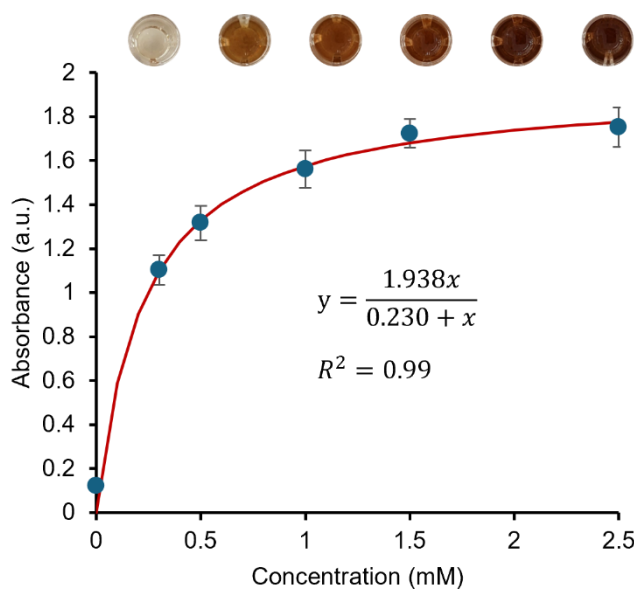


Figure 5.S4. Glucose assay using the UV-Vis method. A reagent solution containing glucose oxidase, horseradish peroxidase, and o-dianisidine dihydrochloride was prepared in DI water and used for testing in spiked real urine samples. The absorbance was measured at 430 nm. A Michaelis-Menten calibration curve was fitted to the data with an $R^2 > 0.99$ (mean $\pm$ SD, $n=3$).

Comparison with the Literature

Table 5.S3. Comparison between the performance of the tablet chemo/biosensors in this study with other reported colorimetric POC assays.

Glucose	Method/Ref.	Platform	LOD (mM)	Nitrite	Method/Ref.	Platform	LOD (mg/dL)
	Bienzymatic/ [13]	Paper	1.65		Griess assay/ [182]	Paper	0.1
	Bienzymatic/ [196]	Paper	0.5		Griess assay/ [423]	PDMS microfluidics	1.15
	Bienzymatic/ [424]	Paper	0.4		An azo coupling reaction/ [425]	Film-shaped polymer	0.08
	Enzyme-free/ [426]	Paper	0.34		Griess assay/ [427]	Ionogel-based polymer-paper	0.04
	Bienzymatic/ [428]	Hydrogel disk	0.32		Griess assay/ [429]	Hydrogel on glass fiber	0.04
	Bienzymatic/ [430]	Transparent nanopaper	1.4		Polymer-carbon nanodots/ [425]	Solution	0.04
	Bienzymatic/ this work	Tablet	0.3		Griess assay/ this work	Tablet	0.03

Chapter 6

Conclusions, Future Directions, and Knowledge

Dissemination

6.1. Conclusions

This work makes several significant contributions to the advancement of low-cost, easy-to-use, and portable analytical devices for point-of-care (POC) and point-of-use (POU) applications. The thesis begins with a comprehensive review of detection methods used for COVID-19 diagnosis, then shifts its focus to microfluidic paper-based analytical devices (μ PADs) and tablet-based assays as two promising platforms for developing POC/POU analytical tools. For both platforms, a state-of-the-art review is presented, highlighting recent advancements, their advantages and limitations, and future directions. Subsequent chapters address key challenges in the fabrication, color formation, and signal intensity of colorimetric and electrochemical μ PADs. Finally, the thesis introduces novel tablet-based devices with rapid auto-mixing capabilities for challenging sample media and biosensing applications. The key findings and contributions of each chapter are summarized below:

Comprehensive review of μ PADs and tablet-based assays (Chapter 2):

This chapter surveys the state-of-the-art in μ PADs and tablet-based assays. For μ PADs, it examines their historical development, fundamental principles of operation, channel fabrication techniques, and detection methods (colorimetric and electrochemical). Tablet-based assays are reviewed in terms of their history, powder-compressed tablets, and polysaccharide-encapsulated tablets. Notably, to the best of the author's knowledge, this thesis presents the first comprehensive literature review dedicated to tablet-based assays, consolidating decades of progress previously scattered across the literature. Both platforms are critically assessed with regard to their advantages, limitations, and potential directions for future research.

Simplified fabrication techniques and engineered detection zones for μ PADs (Chapter 3):

This chapter introduces a simplified method to create channels in μ PADs using widely available Parafilm[®], a laboratory oven, and a CO₂ laser cutter. The technique achieves high resolutions of $172 \pm 15 \mu\text{m}$ (barriers) and $269 \pm 32 \mu\text{m}$ (channels). In addition, two novel engineered detection

zone designs (multi-inlet and segmented) are presented, which reduced the color gradient in glucose assays from 28.77 ± 1.49 to 12.35 ± 1.18 and $8.95 \pm 0.98\%$, respectively, improving the precision of the devices. These zones can be fabricated in a single step alongside channel formation, are compatible with multiplex detection formats, and broaden the applicability of μ PADs.

Do-it-yourself fabrication of electrochemical paper-based analytical devices (ePADs) and signal enhancement strategies (Chapter 4):

This chapter presents a simple do-it-yourself screen-printing approach for fabricating electrodes on paper, achieving high resolutions of $286 \pm 31 \mu\text{m}$ for electrodes and $339 \pm 21 \mu\text{m}$ for electrode spacing, with a fabrication speed of 30 seconds per device. The entire screen-printing setup costs less than USD 50 and is compatible with common CO₂ laser cutters or craft cutters for mask fabrication, thereby enhancing accessibility and accelerating prototyping. A critical challenge in ink-deposited ePADs, ink hydrophobicity, is addressed via a straightforward water-treatment strategy, which significantly improved signal intensity by over 75-fold. Furthermore, a sandwiching strategy, where electrodes are enclosed between two paper layers, nearly doubled signal intensity and sensitivity for glucose detection in cell culture media.

Novel tablet-based assays with rapid auto-mixing capability (Chapter 5):

This chapter introduces tablet-based assays with rapid auto-mixing, achieving more than a 100-fold increase in mixing speed compared to conventional tablets. They also demonstrate effective auto-mixing in highly viscous media of up to 1700 mPa·s and were successfully applied to nitrite detection in saliva, a challenging medium with variable viscosity. Furthermore, the first “biosensor tablet” with auto-mixing capability is presented, incorporating all necessary reagents (enzymes, indicators, etc.) for glucose detection in urine. Reagents were encapsulated in trehalose to enhance stability and bulkiness, enabling robust tablet formation. To achieve this, a simplified method was developed requiring only a handheld mist sprayer, a non-stick tray, and a spatula, thereby eliminating the need for costly equipment such as freeze dryers.

Literature review on COVID-19 detection techniques (Appendix):

This chapter presents a thorough review of COVID-19 detection technologies, including molecular methods for viral RNA detection (e.g., RT-PCR, RT-LAMP, CRISPR-Cas), antigen detection techniques (e.g., LFAs), and serological immunoassays (e.g., ELISA, magnetic bead-based CLIA,

and LFAs). The principles of each method is explained with schematic figures, followed by critical discussion of the method in terms of performance, advantages, and limitations. Written during the COVID-19 pandemic, this chapter provides a valuable foundation for future pandemic preparedness and highlights gaps in current diagnostic technologies that need to be addressed.

Overall, the knowledge and methods presented in this thesis pave the way for the development of more efficient, accessible, and affordable analytical devices. Also, by simplifying fabrication and improving device performance, these advances can make μ PADs and tablet-based assays more widely available to research laboratories worldwide and accelerate progress toward their commercialization for real-world applications.

6.2. Future Directions

Building upon the research presented in this thesis, several avenues for future work can be explored to further enhance the developed techniques and expand their potential applications, as outlined below.

Equipment-free lamination with eco-friendly materials for μ PAD fabrication. Laminated papers are widely used in μ PADs, particularly in conjunction with CO₂ laser etching. However, manual lamination typically requires extra equipment such as ovens, hot presses, or laminators. Using adhesive-coated sheets that can easily and uniformly bond to paper would greatly simplify both lamination and fabrication. For this approach to work, the backing sheet must be sufficiently thick or resistant to the heat of CO₂ laser cutting to allow etching of the paper layer without damage to the backing. In addition, most current backing materials are plastic-based and therefore not environmentally friendly, which undermines one of the key merits of μ PADs, their sustainability. Identifying materials that are both eco-friendly and compatible with equipment-free lamination would thus be highly advantageous for improving both the sustainability and the ease of μ PAD fabrication.

Temperature-actuated valves based on gelatin for μ PADs. Gelatin is a natural biopolymer that can form hydrogels, dissolving at higher temperatures and solidifying upon cooling. This property could be harnessed to create initially closed valves in μ PADs, enabling controlled fluid flow for complex or multi-step assays.

Initially open valves with simple integration and operation in μ PADs. Initially open valves are useful for allowing a defined liquid volume to pass through channels, particularly in multi-step and complex assays. However, most existing designs are either challenging to integrate in μ PADs or cumbersome to operate. Swelling hydrogels with tunable swelling rates could offer a straightforward solution to this limitation.

Enhancement of bioreagent stability on ePADs. One of the major limitations of ePADs is the poor long-term stability of bioreagents (e.g., enzymes) for electrochemical detection [16]. Improving their stability would significantly expand the applicability of ePADs and accelerate their translation toward market use. Encapsulation of reagents with stabilizing sugars such as pullulan or trehalose could be a promising approach to achieve this.

Using hydrophilic and eco-friendly carbon inks for ePADs. Hydrophilic carbon inks [431] could improve electrode-liquid interactions and enhance the sensitivity of ePADs, while being compatible with the capillary action of paper substrate. In addition, many commercial inks contain components that require harsh solvents for cleaning mesh screens for reuse. Employing or developing eco-friendly or water-based inks that adhere effectively to paper substrates and can be removed easily from screens would offer clear advantages for sustainable and practical fabrication. Water-based inks are also attractive because they can be tailored to incorporate biosensing reagents directly into the ink formulation, thereby enabling single-step fabrication of ePADs.

Developing effective lamination strategies for sandwiched ePADs. Sandwiching electrodes between two layers of paper is a simple approach that can nearly double the sensitivity of ePADs. However, as shown in Chapter 4, this strategy may require binder clips to apply pressure for proper binding of the layers, which adds operational complexity. Developing effective lamination methods to ensure strong interlayer adhesion could eliminate the need for clips. In addition, the use of hydrophilic inks for sandwiched electrodes may further improve electrode-liquid interaction and reduce reliance on external pressure.

Automatic and adjustable frequent monitoring of analytes with ePADs. The wicking ability of paper, combined with adjustable flow delays, can be harnessed to design devices for frequent analyte monitoring with tunable timing. Multiple channels branching from a single sample zone could be created, each allowing the sample to pass after a defined delay. These channels would pass over a common three-electrode sensor to record the electrochemical response from each

channel. Adjustable delay strategies, such as incorporating dissolvable sugars and varying channel length, could be employed to regulate timing. Such a system would provide a simple solution for frequent analyte monitoring over a desired time range with adjustable intervals.

Single-step fabrication of electrodes and hydrophobic barriers for ePADs using CO₂ laser cutters. CO₂ laser cutters can be employed to directly create carbon electrodes on paper through laser scribing/pyrolysis. However, the working zones of these devices often need to be fabricated separately, complicating the process. To address this, at the intersection of the electrodes and the boundaries of the working zone, the paper could be patterned in specific geometries that maintain electrical conductivity while blocking fluid flow, enabling single-step fabrication of the entire ePAD and thereby improving both speed and simplicity. Laminated papers (e.g., with Parafilm[®]) may also be used to enhance mechanical strength and further streamline fabrication.

Effervescent tablets for cell lysis and intracellular molecule detection. The bubbling effect of effervescent tablets could be combined with chemical agents (e.g., detergents) to facilitate the lysis of pathogenic microorganisms and viruses, thereby releasing intracellular molecules such as DNA and RNA. The same tablet or another tablet could also incorporate sensing reagents, enabling detection of the released targets. Coupling this cell-lysis strategy with nucleic acid amplification tests could provide an effective tool for pathogen detection.

Tablet-based assays with electrochemical readout. To date, tablet-based assays have primarily relied on optical readouts such as colorimetric, fluorometric, or chemiluminescent methods. However, these approaches can be time-consuming, prone to interference from sample color, or dependent on specialized and expensive equipment such as spectrophotometers. On the other hand, electrochemical sensors face challenges with reagent immobilization and stability on electrodes. Developing tablets that incorporate reagents for electrochemical detection could therefore address these limitations and open new directions in both tablet-based assays and electrochemical sensing. In this system, tablets would be introduced into the sample to initiate an electrochemical reaction, which could then be measured using commercial or custom-built screen-printed electrodes. When combined with low-cost, smartphone-compatible potentiostats [245], such tablets could provide a robust platform for portable and sensitive analyte detection. Moreover, their integration with electrochemical 96-well plate arrays [432] could enable high-throughput and multiplexed testing in laboratory settings.

Multiplexed tablet-based assays with colorimetric and electrochemical readouts. A key limitation of tablet-based assays has been the difficulty of achieving multiplexed detection. One potential solution is to combine colorimetric reagents for one analyte with electrochemical reagents for another, provided the reagents do not interfere with each other. Similarly, dual-modal assays could be designed, where equipment-free colorimetric readouts enable qualitative or semi-quantitative analysis, while electrochemical detection provides more sensitive and quantitative measurements.

Sample-volume independent tablet-based assays. Current tablet-based assays require the application of a defined sample volume, often using liquid-handling equipment such as pipettes. This adds complexity and may reduce reproducibility in field applications. To overcome this limitation, tablet-shaped hydrogels could be developed that do not dissolve in solution but instead absorb a defined volume of sample into their structure. Within the hydrogel, the sample would react with pre-immobilized reagents, producing a visible color change. In this way, the tablet could simply be placed in any amount of sample and indicate the presence of the analyte through colorimetric change. However, this approach would be more suitable for qualitative or semi-quantitative detection rather than fully quantitative analysis.

Development of white magnetic particles coupled with gold nanoparticles for colorimetric assays. Magnetic particles are widely used in detection strategies such as magnetic bead-based CLIA for analyte separation and enhancement of detection limit. While they hold promise for improving colorimetric assays, conventional iron-based beads are dark in color and interfere with optical readouts. This limitation could be addressed by developing white magnetic particles, such as holmium-based ones [433], provided they are engineered to remain well-dispersed in aqueous solutions. Conceptually, such particles could be combined with gold nanoparticle-based color formation strategies, similar to those used in LFAs, to enable sensitive colorimetric detection. In this envisioned approach, magnetic carriers functionalized with recognition elements could capture the analyte, followed by signal formation through gold nanoparticle accumulation, producing a visible color readout. Such strategies may be adaptable to existing LFA platforms or could inspire new types of colorimetric sensing devices.

Highly sensitive nucleic-acid-amplification-free detection of DNA/RNAs. Current gold-standard methods for RNA and DNA detection, such as RT-PCR, rely on nucleic acid amplification, which is time-consuming and requires temperature control. An alternative approach is to achieve comparable detection limits by amplifying the signal through cascade reactions rather than amplifying the nucleic acids themselves. In this strategy, recognition elements (e.g., CRISPR-Cas enzymes) bind to the analyte of interest and trigger a cascade of reactions that generate strong outputs (e.g., colorimetric or electrochemical). For instance, for DNA detection, DNA-based hydrogels [434,435] could potentially be explored as carriers for programmable CRISPR-Cas enzymes, enabling signal release upon activation by a Cas12 probe. Conceptually, complementary hydrogel systems might be designed to interact with one another in a cascade fashion, resulting in accelerated reagent release in response to initial target recognition by the probe. A similar strategy could be envisioned for RNA detection.

6.3. Knowledge Dissemination

The outcomes of the research presented in this thesis, along with related academic activities conducted during the PhD program, were actively disseminated to the broader scientific and professional community. Dissemination activities included publications in peer-reviewed journals, presentations at national and international conferences, media interviews, and delivering workshops. A detailed summary of these activities is provided in the following section.

Journal publications

First-authored publications

1. Safiabadi Tali SH, Saebi R, Mehrjou A, Mirzaie F, Sadiq Z, Goulet MA, Jahanshahi-Anbuhi S. Do-it-yourself screen printing of electrochemical paper-based analytical devices with water treatment and sandwiched electrodes for amplified signal. *Electrochimica Acta*. 2025 Dec 1:147931.
2. Safiabadi Tali SH, Al-Kassawneh M, Mansouri M, Sadiq Z, Jahanshahi-Anbuhi S. All-Inclusive Sensing Tablet with Integrated Passive Mixer for Ultraviscous Solutions. *ACS sensors*. 2025 May 6;10(5):3493-503.
3. Safiabadi Tali SH, Hajimiri H, Sadiq Z, Jahanshahi-Anbuhi S. Engineered detection zone to enhance color uniformity on paper microfluidics fabricated via Parafilm®-heating-laser-cutting. *Sensors and Actuators B: Chemical*. 2023 Apr 1;380:133324.
4. Safiabadi Tali SH[†], LeBlanc JJ[†], Sadiq Z, Oyewunmi OD, Camargo C, Nikpour B, Armanfard N, Sagan SM, Jahanshahi-Anbuhi S. Tools and techniques for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)/COVID-19 detection. *Clinical microbiology reviews*. 2021 Jun 16;34(3):10-128. ^[†] Equal contribution. ([Featured in media](#)).

Co-authored publications

5. Saebi R, Safiabadi Tali SH, Jahanshahi-Anbuhi S, Goulet MA. Electrochemical Tablet-based Assay for Stable and Reproducible Biosensing. 2025 (submitted).

[#]My contributions to this research include Conceptualization, Methodology, Validation, Formal Analysis, Visualization, and Writing-Review&Editing.

6. Ezeoke CH, Sadiq Z, Safiabadi Tali SH, Jahanshahi-Anbuhi S. Reverse Titration Using Tablets for Accurate Water Hardness Measurement with Improved Resistance to Interference. *Chemosensors*. 2025;13(10):365.
#My contributions to this research include Methodology, Validation, and Writing-Review&Editing.
7. Mansouri M, Safiabadi Tali SH, Sadiq Z, Jahanshahi-Anbuhi S. User-friendly detection of nitrite in soil samples with tablet-based sensor. *Microchemical Journal*. 2025 Jul 16:114584.
#My contributions to this research include Conceptualization, Methodology, Formal analysis, Visualization, and Writing-Review&Editing.
8. Sadiq Z, Safiabadi Tali SH, Mansouri M, Jahanshahi-Anbuhi S. A dual-functional nanogold tablet as a plasmonic and nanozyme sensor for point-of-care applications. *Nanoscale Advances*. 2025;7(10):2967-78.
#My contributions to this research include Visualization, and Writing-Review&Editing.
9. Sadiq Z, Al-Kassawneh M, Safiabadi Tali SH, Jahanshahi-Anbuhi S. Dextran-Gold Nanoparticle-Based Tablets and Swabs for Colorimetric Detection of Urinary H₂O₂. *ACS Applied Nano Materials*. 2025 Jan 8;8(2):1008-20.
#My contributions to this research include Visualization, and Writing-Review&Editing.
10. Sadiq Z, Safiabadi Tali SH, Hajimiri H, Al-Kassawneh M, Jahanshahi-Anbuhi S. Gold nanoparticles-based colorimetric assays for environmental monitoring and food safety evaluation. *Critical Reviews in Analytical Chemistry*. 2024 Oct 2;54(7):2209-44.
#My contributions to this research include Methodology, Data Curation, Investigation, Visualization, Writing-Original Draft, Writing-Review&Editing.
11. Sadiq Z, Al-Kassawneh M, Safiabadi Tali SH, Jahanshahi-Anbuhi S. Tailoring plasmonic sensing strategies for the rapid and sensitive detection of hypochlorite in swimming water samples. *Microchimica Acta*. 2024 Apr;191(4):183.
#My contributions to this research include Visualization, and Writing-Review&Editing.
12. Hajimiri H, Safiabadi Tali SH, Al-Kassawneh M, Sadiq Z, Jahanshahi-Anbuhi S. Tablet-based sensor: a stable and user-friendly tool for point-of-care detection of glucose in urine. *Biosensors*. 2023 Sep 19;13(9):893.

#My contributions to this research include Conceptualization, Methodology, Validation, Writing-Review&Editing, and Supervision.

13. Sadiq Z, Safiabadi Tali SH, Jahanshahi-Anbuhi S. Gold tablets: gold nanoparticles encapsulated into dextran tablets and their pH-responsive behavior as an easy-to-use platform for multipurpose applications. ACS omega. 2022 Mar 22;7(13):11177-89.

#My contributions to this research include Visualization, and Writing-Review&Editing.

14. Esfahani AR, Sadiq Z, Oyewunmi OD, Safiabadi Tali SH, Usen N, Boffito DC, Jahanshahi-Anbuhi S. Portable, stable, and sensitive assay to detect phosphate in water with gold nanoparticles (AuNPs) and dextran tablet. Analyst. 2021;146(11):3697-708.

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15. Oyewunmi OD, Safiabadi-Tali SH, Jahanshahi-Anbuhi S. Dual-modal assay kit for the qualitative and quantitative determination of the total water hardness using a permanent marker fabricated microfluidic paper-based analytical device. Chemosensors. 2020 Oct 9;8(4):97.

#My contributions to this research include Investigation, Formal analysis, Visualization, and Writing-Review&Editing.

Conference presentations

1. Safiabadi Tali SH, Saebi R, Mirzaie F, Mehrjou A, Goulet M-A, Jahanshahi-Anbuhi S. Simplified screen-printing fabrication of electrochemical paper-based analytical devices coupled with sandwiched electrodes for enhanced sensitivity. Chemical Engineering Research Day Conference, Montreal, Canada, Mar 2025 (oral; [awarded Best Presenter among Concordia University students](#)).
2. Safiabadi Tali SH, Saebi R, Mirzaie F, Jahanshahi-Anbuhi S, Goulet M-A. Enhancing stability of bioreagents on electrochemical paper-based analytical devices (ePADs). 75th Annual Meeting of the International Society of Electrochemistry, Montreal, Canada, Aug 2024 (poster).

3. Safiabadi Tali SH, Al-Kassawneh M, Mansouri M, Jahanshahi-Anbuhi S. Easy-to-use tablet-based sensor for point-of-care detection of nitrite in human saliva. Chemical Engineering Research Day Conference, Montreal, Canada, Mar 2024 (oral; [awarded Best Presenter among Concordia University students](#)).
4. Safiabadi Tali SH, Hajimiri H, Sadiq Z, Jahanshahi-Anbuhi S. Enhanced color uniformity on paper microfluidics with engineered detection zones fabricated via Parafilm[®]-heating-laser-cutting. Canadian Chemical Engineering Conference (CSCChE 2023), Calgary, Canada, Oct-Nov 2023 (oral).
5. Safiabadi Tali SH, Al-Kassawneh M, Jahanshahi-Anbuhi S. Low-cost tablet-based sensor for on-site detection of nitrite in water. PEOPLE 2023 International Conference, Montreal, Canada, Aug 2023 (poster).
6. Safiabadi Tali SH, Hajimiri H, Jahanshahi-Anbuhi S. Parafilm-heating-laser-cutting fabrication of paper microfluidics enabling micro-engineered detection zones with enhanced color uniformity. Chemical Engineering Research Day Conference, Montreal, Canada, Mar 2022 (oral; [awarded 2nd Best Presenter among Concordia University students](#), [awarded 3rd Place in TGWT Clean Technologies Inc. Sustainability Competition](#)).
7. Safiabadi Tali SH, Jahanshahi-Anbuhi S. Point-of-care diagnostic tools for detection of SARS-CoV-2/COVID-19: where we are and what to do next? Chemical Engineering Research Day Conference, Montreal, Canada, Mar 2021 (oral).
8. Safiabadi Tali SH, Jahanshahi-Anbuhi S. Paper-based analytical devices (PADs): the technology tree and future directions. BIOFOR International Conference, Montreal, Canada, Feb 2020 (poster).
9. Safiabadi Tali SH, Jahanshahi-Anbuhi S. Microfluidic paper-based analytical devices: fabrication methods and emerging applications. CQMF-QCAM Student Symposium, Montreal, Canada, Nov 2019 (poster).

Media interviews

1. Le Devoir. “L’évolution des tests diagnostiques de la COVID-19.” February 7, 2022.
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2. Concordia University News: “Concordia researchers break down the COVID-19 diagnostic arsenal.” June 16, 2021. Available at:
<https://www.concordia.ca/news/stories/2021/06/16/concordia-researchers-break-down-the-covid-19-diagnostic-arsenal.html>.

Workshops organized and delivered

1. “Chemo/biosensors from lab to everyday life”. Girls Summer Engineering and Technology Program (GirlSET), Concordia University, July 2024. Hands-on workshop delivered as a science instructor for high school girls on the concepts and applications of sensors and biosensors.
2. “Chemo and biosensors”. Girls Summer Engineering and Technology Program (GirlSET), Concordia University, July 2021. Workshop delivered as a science instructor for high school girls on the concepts and applications of sensors and biosensors.

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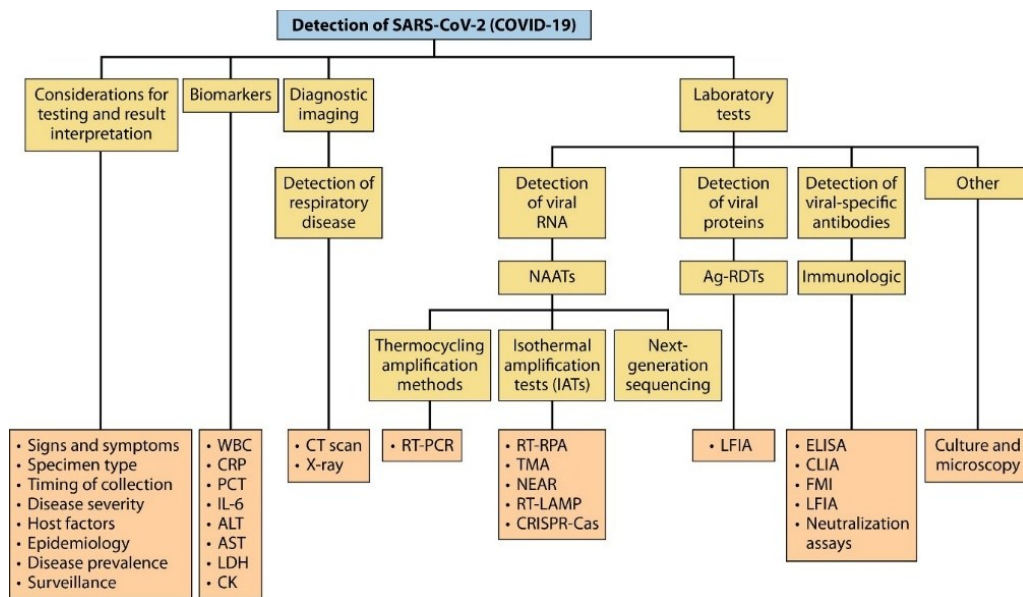
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Appendix

Published Review Paper: COVID-19 Detection

This section is adapted from the author's article published in *Clinical microbiology reviews* (2021), in accordance with the publisher's thesis reuse policy:

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A.1. Abstract

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory disease coronavirus 2 (SARS-CoV-2), has led to millions of confirmed cases and deaths worldwide. Efficient diagnostic tools are in high demand, as rapid and large-scale testing plays a pivotal role in patient management and decelerating disease spread. This paper reviews current technologies used to detect SARS-CoV-2 in clinical laboratories as well as advances made for molecular, antigen-based, and immunological point-of-care testing, including recent developments in sensor and biosensor devices. The importance of the timing and type of specimen collection is discussed, along with factors such as disease prevalence, setting, and methods. Details of the mechanisms of action of the various methodologies are presented, along with their application span and known performance characteristics. Diagnostic imaging techniques and biomarkers are also covered, with an emphasis on their use for assessing COVID-19 or monitoring disease severity or complications. While the SARS-CoV-2 literature is rapidly evolving, this review highlights topics of interest that have occurred during the pandemic and the lessons learned throughout. Exploring a broad armamentarium of techniques for detecting SARS-CoV-2 will ensure continued diagnostic support for clinicians, public health, and infection prevention and control for this pandemic and provide advice for future pandemic preparedness.

A.2. Introduction

While coronavirus disease 2019 (COVID-19) is not the first pandemic of the 21st century [436], it has generated unprecedented global concern and responses. COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is thought to have emerged from a zoonotic source [437] and spread rapidly in humans through respiratory droplets and contact. There is some concern for airborne transmission, but it is unclear of the role of this transmission route outside of the potential aerosolizing procedures in health care settings [438–440]. With an estimated reproductive number, R_0 between 1.4 and 5.6, SARS-CoV-2 rapidly spread worldwide [441–444]. Since the first cases reported in December 2019 [445–447], there have been over 106 million confirmed cases and 2.3 million deaths reported worldwide (as of February 9, 2021) [448].

From a disease manifestation perspective, SARS-CoV-2 infection can be asymptomatic [449], and COVID-19 disease spans from a mild influenza-like illness (ILI) to life-threatening complications [450,451]. SARS-CoV-2 does not only affect the respiratory tract, resulting in pneumonia, but can also affect the gastrointestinal, neurological, or cardiovascular systems. Atypical presentations of COVID-19 include cutaneous manifestations such as a Kawasaki-like disease in children and ophthalmic/gustatory dysfunction (i.e., anosmia and ageusia, which is the loss of smell and taste, respectively), which may have been underestimated in initial reports [452–455].

Despite numerous therapeutic options being explored (e.g., convalescent plasma), no large-scale treatments are available. Public health interventions have evolved over time to limit viral spread (Figure A.1) and have included the use of personal protective equipment (PPE) like masks, hand washing, and containment measures such as city lockdowns, travel restrictions, and physical distancing [456–465]. Although these strategies have been essential to reduce the virus's spread, they have had significant adverse socio-economic impacts, and adherence to these prevention strategies is challenging to sustain [457]. Currently, cases of COVID-19 have declined following a first pandemic wave in some areas, whereas other areas are experiencing subsequent waves of activity. Fortunately, many vaccine candidates are under development and undergoing regulatory approval processes [466–470]. Recently, COVID-19 mRNA vaccines have been the first licensed for use and are rapidly being administered as supplies are provided [463,471]. However, given the time required for adequate immunization coverage in the population at large, subsequent pandemic waves are anticipated [466,472–474]. Therefore, detection methods for SARS-CoV-2 remain a

crucial part of containment and mitigation strategies, and lessons learned from this pandemic may help prepare against future pandemics.

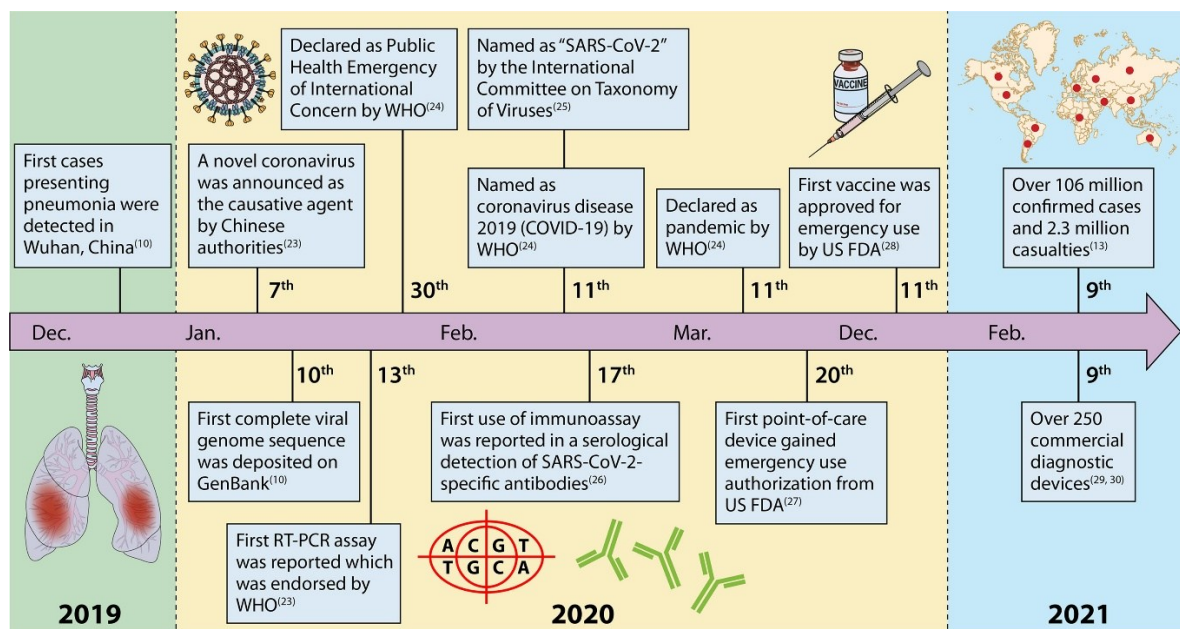


Figure A.1. Timeline of COVID-19 spread and global response to it. Of note, while SARS-CoV-2 was initially thought to have emerged from China in December 2020, there are data to suggest it may have circulated more broadly earlier than initially recorded in other countries, and further studies are underway to investigate this possibility in other areas [475–478]

In terms of testing, real-time reverse-transcription polymerase chain reaction (RT-PCR) remains the most common method used to identify SARS-CoV-2 [479]. While common in diagnostic laboratories worldwide, many laboratories remain faced with supply chain shortages for real-time RT-PCR reagents and consumables, all while being asked to increase testing capacity. As such, delays were common for test results, prompting the exploration of alternative testing options such as specimen pooling or laboratory testing using methods other than RT-PCR. Methods that could enhance testing capacity, streamline testing (i.e., automation), or those which provide more rapid results in easy to use formats that are amenable to point-of-care applications without complex instrumentation (e.g., isothermal technologies) were all desired [23,24,480–484]. Rigorous research escalated quickly from the academic to industry partners, and this research is ongoing to develop testing alternatives or complements to existing technologies.

While recent reviews have been published on the management of SARS-CoV-2 [480,484–492], recent advancements in novel diagnostic methods justify the need for a more comprehensive

synthesis of the current literature. In this review, first, the biological characteristics of SARS-CoV-2 are described in order to fully understand molecular and immunologic methods for its detection. Following a brief discussion on the COVID-19 disease manifestations, compatible signs and symptoms, and disease biomarkers, diagnostic imaging techniques are described in relation to COVID-19 lower respiratory tract involvement, including applications such as monitoring disease severity, the progression of the illness, or complications. Then, a comprehensive review of current and recent advances in molecular, antigen, and serological immunodiagnostic methods will be covered, including rapid diagnostic tests (RDTs) used in the laboratory setting and point-of-care (POC) applications. Overall, this review expands our knowledge of current and exploratory avenues for detecting SARS-CoV-2 virus and COVID-19 disease.

It should be noted that some of the references used in this review were pre-prints that have not been peer-reviewed, and recognizing that data on detection of SARS-CoV-2 or COVID-19 disease is rapidly evolving, some details on testing options and guidelines may no longer be recent and should thus be reviewed in the context of recent findings and recommendations. Nonetheless, the review provides a comprehensive synthesis of the most current data available to date, along with current recommendations for detection of SARS-CoV-2 or diagnosis of COVID-19.

A.3. Sars-Cov-2 Genome and Structure

Understanding the genetic and structural properties of SARS-CoV-2 is a prerequisite to developing effective diagnostic tools. SARS-CoV-2 was first isolated and sequenced in China in January 2020 [445–447]. Transmission electron microscopy revealed that SARS-CoV-2 has a diameter in the range of 60 to 140 nm, and its morphology was consistent with other members of the *Coronaviridae* family (Figure A.2A) [447,460]. SARS-CoV-2 is an enveloped, positive-strand RNA virus and, on the genetic level, it shares 96%, 80%, and 50% sequence identity to bat coronavirus (RaTG13), SARS-CoV-1, and middle eastern respiratory syndrome coronavirus (MERS-CoV), respectively [446,493]. Based on these analyses, the International Committee on Taxonomy of Viruses named the virus SARS-CoV-2, which was formerly referred to as the 2019 novel coronavirus (2019-nCoV) or human coronavirus 2019 (hCoV-19) [460].

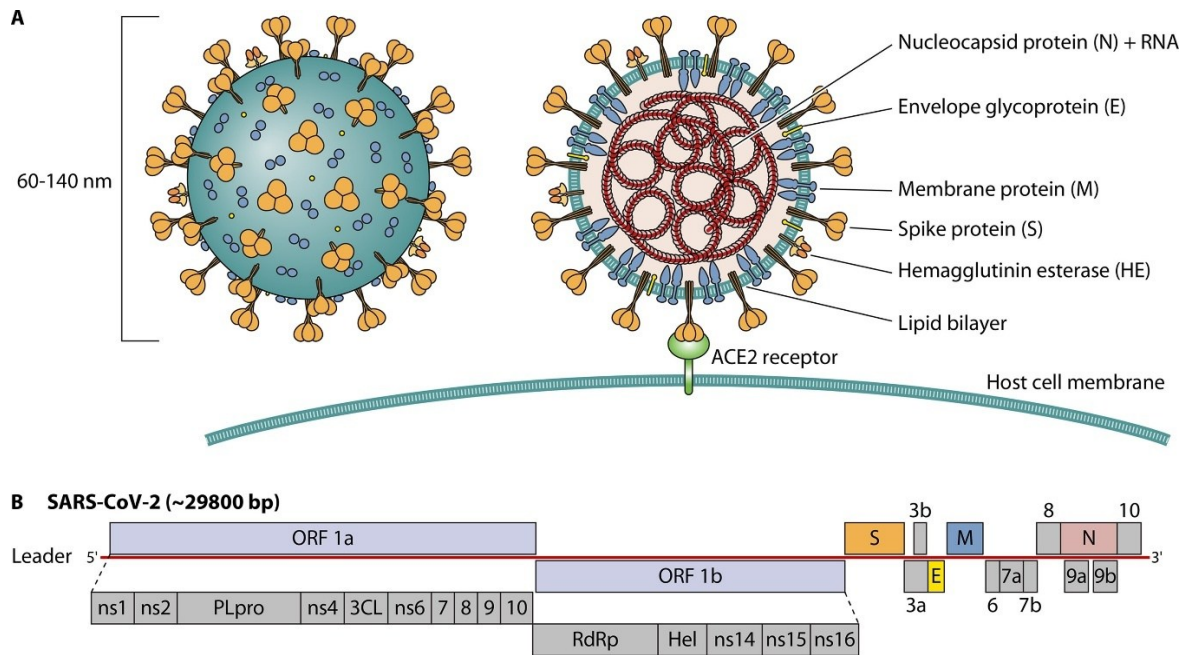


Figure A.2. Physical and genome structure of SARS-CoV-2. (A) Diagram of the SARS-CoV-2 virion. (B) Genome organization and proteins with known or unknown functions. This figure was inspired by the work in Refs. [456,494].

Our understanding of SARS-CoV-2 structure and function has been largely derived from research on SARS-CoV-1, MERS-CoV, and seasonal coronaviruses. SARS-CoV-2 has a single-stranded positive-sense RNA genome between 26 and 35 kilobases, encoding approximately 27 proteins with the similarity of proteins of known functions, while others are unclear/unknown or putative (Figure A.2B) [456,472,490,494,495]. The first open reading frame (Orf1a/b) on the 5'-end of the viral genome occupies ~71% of the entire genome and produces two polyproteins (pp), pp1a and pp1ab. These two polyproteins are processed by the viral proteases into 15 non-structural proteins (nsp), and these proteins are collectively involved in polyprotein processing, viral RNA replication, and mRNA synthesis [490,494]. The remaining proteins, including the structural and accessory proteins, are expressed from several nested subgenomic mRNAs produced through a process known as the discontinuous transcription by the viral RNA-dependent RNA polymerase (RdRp).

The structural proteins include the small envelope (E) protein, membrane (M) protein (also known as the matrix protein), nucleocapsid (N) protein, hemagglutinin-esterase (HE) protein, and spike (S) glycoprotein (Figure A.2A) [494,496]. The E and M proteins are primarily involved in viral assembly, budding, and virion morphogenesis [497–499], while the N protein complexes with the

viral genomic RNA to generate the nucleocapsid [500]. The S protein is the major surface glycoprotein on SARS-CoV-2, forming approximately 40 trimers that play an important role in both receptor binding and membrane fusion through the two functional subunits, S1 and S2 [472,501]. The S protein trimers contain a stable stalk separated from the globular heads by three flexible hinges, allowing for orientation freedom to interact with host cell receptors [502]. The S1 subunit contains the receptor-binding domain (RBD) that directly interacts with the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell surface, whereas the S2 subunit contains a structural loop responsible for fusion events between the viral and host cell membranes, resulting in the release of the viral genomic RNA into the cytoplasm [503,504]. Of note, along with engaging the ACE2 host cell receptor, the cellular serine protease TMPRSS2 is engaged for S protein priming, and this cofactor has been investigated as a possible antiviral target using viral entry inhibitor [505,506].

Overall, having knowledge of SARS-CoV-2 pathogenesis can help understand disease manifestations and help guide the development of molecular and immunologic tools for the identification of this virus.

A.4. Clinical Manifestations of Covid-19 Disease

The spectrum of SARS-CoV-2 infection can vary from asymptomatic infection to life-threatening complications of COVID-19 disease [472]. Using modeling, it was estimated that over 59% of transmissions arise from asymptomatic individuals, with 35% from pre-symptomatic stages of infection and 24% from individuals who never develop symptoms [507]. These estimates are concerning but emphasize the need for the wide use of vaccines and maintaining key public health interventions like wearing masks, hand hygiene, and social distancing.

In most symptomatic cases, COVID-19 presents as a mild to moderate upper respiratory illness, with signs and symptoms compatible with other respiratory viruses [508]. As such, the diagnostic accuracy of any individual sign or symptom is very poor, and neither the presence nor absence of any sign or symptom can be used to rule in or out COVID-19 [508]. With the possibility of other pathogens that could present like SARS-CoV-2 infection, case definitions based on clinical presentation are not sufficiently specific but can help support the investigation of suspect COVID-19 cases. On the other hand, given the list of possible presentations and atypical manifestations of

COVID-19 could mirror those of other diseases, identifying the etiology of illness as SARS-CoV-2 requires laboratory testing.

In a recent Cochrane review, a summary of 16 studies (7706 patients) was presented [508]. Only six of the possible signs and symptoms of COVID-19 had sensitivities greater than 50%, and results were highly variable between studies and settings. The performance of the most common signs and symptoms is summarized as follows: cough (with sensitivity and specificity from 43 to 71% and 14 to 54%, respectively); sore throat (5 to 71% and 55 to 80%); fever (7 to 91% and 16% to 94%); musculoskeletal symptoms (e.g., arthralgias or myalgias) (19 to 86% and 45 to 91%); fatigue (10 to 57% and 60 to 94%); and headache (3 to 71% and 78% to 98%) [508]. It was noted that possible confounders were present, and the high heterogeneity between data suggested that signs and symptoms are variable between individuals [508]. Other less common clinical presentations have been documented, including alterations in smell or taste (i.e., anosmia or dysgeusia), as well as neurologic or cutaneous manifestations [452–454,509–513]. It is noteworthy that in the early stages of the pandemic, some of these symptoms may have been missed or underreported, but knowledge on possible clinical presentations of COVID-19 have evolved over time.

In some cases of COVID-19, progression to lower respiratory tract illness (e.g., pneumonia) can occur and may require hospitalization, intensive care unit (ICU) support, and mechanical ventilation, and complications can arise that include acute respiratory distress, multi-organ dysfunction, and death [508,514–522]. In general, adverse outcomes and deaths are more common with increasing age or in individuals with underlying medical comorbidities such as respiratory system disease, cardiovascular disease, and diabetes [515–517]. Fatality rates vary between studies and countries but are generally higher in the hospital setting (e.g., 4 to 11%) compared to the overall case fatality rates (e.g., 2 to 3%) in the general population [517,519,522,523]. In terms of recovery, the median duration of hospital stay is 10 to 14 days, and resolution generally occurs within 2 to 3 weeks [522]. There is a lack of evidence whether some symptoms can persist after recovery. In one study, patients were followed up to 60 days post-recovery, with 87.4% reporting at least one symptom [523]. The most common symptoms were fatigue (53.1%), dyspnea (43.4%), joint pain (27.3%), and chest pain (21.7%).

Overall, while some signs or symptoms may be compatible with COVID-19, none are specific, and laboratory testing is required to confirm the diagnosis. Further studies are required to help identify the frequency of atypical clinical presentations, and additional data looking at known clinical presentations of COVID-19 should consider possible confounders such as the possibility of other etiologies, host factors (e.g., comorbidities), disease severity, and the time from infection and symptom onset.

A.5. Biomarkers for Covid-19 and Routine Laboratory Investigations

Apart from laboratory tests specific for detecting SARS-CoV-2 discussed throughout this review, routine laboratory testing spanning hematology, biochemical, and chemical markers is used to assess a patient's health or identify possible clues to a disease state [524–527]. Such routine laboratory work-up of individuals is used to refine a medical differential diagnosis, thereby supporting or refuting potential causes of the clinical presentation based on typical outcomes of these investigations for a defined disease. Many of these investigations can evolve through the clinical course of illness, and additional testing can be ordered by physicians based on the clinical presentation. These can include tests such as a white blood cell (WBC) counts, markers for inflammatory conditions (C-reactive protein [CRP], procalcitonin [PCT], or interleukin 6 [IL-6]), tests for anticoagulation, and indicators of tissue damage (alanine aminotransferase [ALT], aspartate aminotransferase [AST], lactate dehydrogenase [LDH], and creatine kinase [CK]). While biomarkers for COVID-19 have been the subject of much investigation during the current pandemic, none of these tests are sensitive or specific for COVID-19 disease. In a Cochrane review analyzing 67 lab tests from 21 studies encompassing 14,126 COVID-19 cases and 56,585 non-COVID-19 cases, only three markers showed sensitivity and specificity values greater than 50%: decrease in lymphocyte count and increase in inflammatory markers CRP and IL-6 [527]. Overall, no individual biomarker can be used reliably to rule COVID-19 disease in or out, and laboratory testing should be performed. However, it should be noted that some laboratory markers have values for patient management as they can help assess the severity of the disease, progression of the illness, or even act as risk factors for death. In the most recent Centers for Disease Control and Prevention (CDC) guidance documents for clinicians caring for patients with COVID-19, a summary of important laboratory tests is described, with lymphopenia being the most common laboratory finding in patients hospitalized with COVID-19 [524]. Laboratory markers associated

with increased illness severity include lymphopenia and neutropenia, elevated serum ALT, AST, LDH, CRP, and ferritin [524,525]. Patients with the critical illness have high plasma levels of inflammatory makers, and elevated d-dimer and lymphopenia have been associated with an increased risk of death.

Of note, this section is not intended to be a comprehensive review of all biomarkers used in routine or exploratory investigations for COVID-19. The authors recognize the availability of guidelines for clinicians caring for patients with suspect or confirmed infection with SARS-CoV-2 [524,525], as well as the expertise of medical staff in ordering laboratory tests to help guide evolving differential diagnoses throughout the clinical course of illness. However, this section also recognizes the ongoing efforts of researchers who are dedicated to understanding the role of existing or novel biomarkers. Overall, no laboratory marker to date is diagnostic for COVID-19, but they do have value in patient management over time, regardless of SARS-CoV-2 infection status. Biomarkers for COVID-19 disease severity or prognosis remains an active area of research that may not only lead to new diagnostic approaches but also helps us understand disease progression and host responses to COVID-19 [528–531].

A.6. Diagnostic Imaging for Covid-19 Disease

While testing of specimens collected from the upper respiratory tract is common for diagnosing SARS-CoV-2 infection, the progression of the disease may involve the lower respiratory tract (e.g., pneumonia), with or without detectable SARS-CoV-2 virus in the upper respiratory tract [492,532–540]. Testing of specimens from the lower respiratory tract (e.g., bronchoalveolar lavages [BALs]) is possible using nucleic acid amplification tests (NAATs) like RT-PCR, but obtaining lower respiratory tract specimens is not always possible [541–544]. Along with laboratory testing, diagnostic imaging can complement investigations of COVID-19 to assess the involvement of disease in the lower respiratory tract or other anatomic sites. Diagnostic imaging techniques include chest radiography (or chest X-ray; CXR), computed tomography (CT) scan, ultrasound, magnetic resonance imaging (MRI), and positron emission tomography-CT (PET/CT) [545–553]. Among these, CT scans are the most frequently used methods for diagnosing lower tract involvement or follow-up of COVID-19 cases [547–549]. CT scans produce cross-sectional images at different angles, thereby providing a three-dimensional look at the targeted anatomy. Chest CT scan images can be assembled and assessed by radiologists to check for possible

abnormalities suggestive of lower tract disease such as viral pneumonia [490,549,554]. Typical features of a chest CT in COVID-19 are ground-glass or reticular opacities (GGO) with or without consolidations that present bilaterally, peripherally, or in posterior distributions [550].

The utility of diagnostic imaging for routine screening for COVID-19 has been a subject of debate and has not been recommended by most radiology societies [550,551,555–558]. On the other hand, due to the shortage of RT-PCR supplies during the early days of the pandemic and the possibility of false-negative RT-PCR results from sampling the upper respiratory tract, some hospitals in the Hubei province of China included CT scans in the diagnosis of SARS-CoV-2 infection [490,554,559,560]. While diagnostic imaging techniques like CT have merits to help assess lower respiratory tract disease involvement, to monitor disease progression, or to investigate other complications of COVID-19, it should be noted that diagnostic imaging methods are less sensitive than sampling the lower respiratory tract and testing using molecular methods, and specificity is low, given that typical features of COVID-19 disease are common to other respiratory viruses or illnesses [550–553,561–565]. Initial reports of the utility of CT scans in the diagnosis of COVID-19 suggested increased sensitivity of CT over real-time RT-PCR, but others have suggested explanations for the disparities between RT-PCR test results and diagnostic imaging assessments, including poor sampling techniques, differences in the performance of testing methods, the anatomic site of RT-PCR testing (upper vs. lower tract), and disease prevalence [548,561,563,566–571]. High sensitivities (i.e., >90%) have been reported for CT scans in high prevalence populations, while low sensitivities (<60%) were reported in studies with low prevalence populations [549–551,555–558,560]. In a Cochrane review for confirmed cases of COVID-19, the pooled sensitivity was 93.1% (95% CI: 90.2% to 95.0%) for chest CT and 82.1% (95% CI: 62.5% to 92.7%) for CXR, but heterogeneity between studies was considerable [558]. Specificity for diagnostic imaging is low, at 18.1% (95% CI: 3.7 to 55.8%) [558]. In other words, approximately 80% of individuals would have received a diagnosis of COVID-19 in the absence of disease. As such, the use of diagnostic imaging techniques should be accompanied with careful consideration for factors such as disease prevalence in the study population, the severity of the illness, performance and context of the methods used, differences in radiologist opinions, and possible confounding diagnoses [549,550,570–572,551,555–558,560,563,569]. On the other hand, it is also important to recognize that diagnostic imaging is a useful tool for patient management with or

without a confirmed etiology through laboratory testing, as it can be used to monitor the severity of illness, disease progression, and assess possible complications [573–578].

Understanding the benefits and limitations of diagnostic imaging for COVID-19 is an active area of research, along with applications of artificial intelligence (AI) (a.k.a. machine learning) [579–581]. AI-based methods can be used in diagnostic imaging to help recognize abnormal features in images and classify them in defined categories, thus increasing accuracy, standardization, and speed of analyses by radiologists [487,546,579–583]. AI-approaches can be categorized into three main groups: approaches that analyze CT-scan images, methods based on X-ray, and those that realize diagnosis through jointly analyzing of CT-scan and X-ray images [584–588]. While AI-based applications have shown benefits to diagnostic imaging methodologies [487,546,582,583], more clinical investigations are needed to evaluate their possible incorporation in routine procedures for investigations of suspect cases of COVID-19, and laboratory testing is required to confirm the disease etiology. Further, acquiring a reliable AI-based system requires access to a comprehensive training dataset that includes all variations of COVID-19 as well as other lung diseases; providing such all-inclusive dataset is difficult and labor intensive.

A.7. Laboratory Methods for the Detection of Sars-CoV-2

Diagnosis of COVID-19 can be performed using molecular detection of SARS-CoV-2 RNA, which is now widely available. Immunodiagnostic methods for identifying viral antigens and serology to recognize an immune response to the virus are also available. The following sections will describe the commonly used and potential methods for the laboratory detection of SARS-CoV-2, with important consideration for factors like specimen type and timing of specimen collection.

A.7.1. Specimen Types

Prior to describing methods for SARS-CoV-2 detection, it should be recognized that accurate detection of any infectious disease requires adequate specimen collection at the anatomical site of infection, during a time when the pathogen of interest should be present [589–591]. While the virus has been detected in a variety of specimen types using molecular methods [461,533,592–598], few have been widely adopted due to unreliable detection or lack of sensitivity. The use of

blood, serum, or plasma for SARS-CoV-2-specific serology and other immunodiagnostics tests will be discussed in later sections of the manuscript.

For respiratory viruses like SARS-CoV-2, specimens collected from the upper respiratory tract using a flocked nasopharyngeal (NP) swab that is placed in universal or viral transport media (UTM or VTM, respectively) are the gold standards [599–601]. In contrast to other swabs (e.g., cotton swabs on wooden sticks), specimen collection using flocked NP swabs that are coated with multi-length fibers allows for enhanced recovery of respiratory viruses and bacteria, and the UTM or VTM allows a stable media for transport to the laboratory [570]. Other than NP swab specimens, alternative specimens and collection methods have been validated and gained interest, including the use of nasal mid-turbinate swabs, anterior nares (Na), oropharyngeal (OP) swabs, or washes/aspirates from the nasopharynx, nose, or throat [533,534,609,591,602–608]. Specimen combinations can also be used. For example, a paired collection using an OP swab along with a sampling of the anterior nares was shown to be equivalent to NP swab collection for the detection of SARS-CoV-2, and different options are available for OP/Na collections [570,591,603,604,610]. It is worth mentioning that during the COVID-19 pandemic, procurement of NP swabs and UTM (or VTM) was challenged by global supply chain shortages. Several groups have developed and validated the possibility of using 3D-printed swabs as alternatives to commercial NP or nasal swabs, but while some have been clinically validated for detecting SARS-CoV-2 RNA, further investigations are required for their applicability in SARS-CoV-2 antigen detection [611–615]. For media used for swab transport to laboratories, other than the typical UTM or VTM, alternatives have been investigated for use for SARS-CoV-2 testing, including Amies transport medium, or sterile normal saline, phosphate-buffered saline (PBS), M4, and minimal essential media (MEM), and stability analyses have assessed ideal transport and storage conditions [604,605,616].

While NP swabs are considered the gold standard for respiratory virus sampling of the upper respiratory tract, hospitalized adults with the progression of COVID-19 to lower tract disease may require additional specimen types [606]. When lower tract infection is suspected through clinical presentation or with the aid of diagnostic imaging, specimens such as bronchoalveolar-lavage (BAL), endotracheal secretions, or sputum should be considered [492,532–539,617].

Recently, the use of non-invasive collection methods like saliva and throat gargles have gained much interest, as these samples are amenable to self-collection and have the potential for large

scale population-based surveillance [618–623]. While some studies have demonstrated that the performance of saliva for detection of SARS-CoV-2 was comparable to NP or nasal swab collections [620,621,624–627], others challenged the performance of saliva for SARS-CoV-2 detection [628]. The variability in saliva collection or differences in the patient populations tested might explain these inconsistencies, but further analyses are required [628,629]. Also, although not used routinely in many laboratories, detecting SARS-CoV-2 RNA from stool is possible in the presence or absence of gastrointestinal symptoms [630]. The possibility of culturable SARS-CoV-2 from stool opens discussions regarding the possibility of fecal-oral transmission and human health or ecological risks [631,632] and also opens the opportunity for research into community-based surveillance in low prevalence settings using wastewater [630,633].

In post-mortem examinations, the extent of investigations will be dependent on several factors, but NP swabs, swabs from the lungs, and tissue samples can be used for diagnostic testing for SARS-CoV-2 [634–637]. Specimens in 10% buffered neutral saline or formalin-fixed paraffin-embedded (FFPE) commonly used for histopathological examinations can also be used, but these pose challenges for NAATs like real-time RT-PCR as RNA can be degraded by formalin, and sensitivity for detection of SARS-CoV-2 RNA by real-time RT-PCR could be compromised [638]. The CDC recommends these media be used in limited settings [634]. Immunohistochemical (IHC) and *in situ* hybridization (ISH) assays for the detection of SARS-CoV-2 have now been developed, but limited data is available on their performance [639].

A.7.2. Timing of Specimen Collection

SARS-CoV-2 has been identified in various clinical specimen types [461,533,591–598], but the timing of detection differs between methods and the specimen types collected for testing. SARS-CoV-2 RNA can be detected early in the pre-symptomatic stage of the disease and later on, even after recovery. However, the timing for specimen collection is critical, as testing too early or too late following exposure can potentially lead to false-negative results [640]. It was shown that real-time RT-PCR false-negative rates could be minimized by testing 2 to 3 days after symptom onset, with an average time of symptom onset of 5 days post-exposure [641–647]. Repeat testing can be considered for individuals with an initial negative test result, but there is a high level of clinical suspicion [571]. Of note, viral shedding studies are often performed using RNA detection alone and less often in combination with virus culture; however, the absence of cultivable virus does not

preclude the potential for SARS-CoV-2 transmission, and laboratory detection of SARS-CoV-2 using molecular methods does not imply infectious virus [645,648–654]. For the purpose of this section, viral shedding is described in the context of RNA detection without implying the potential for viral transmission. A discussion regarding the association of SARS-CoV-2 RNA detection and potential infectivity will be covered later in this section, as well as in the real-time RT-PCR section.

The magnitude of the viral load and duration of shedding depends on specimen type, the anatomic site of illness, the severity of illness, and likely host immune response to infection [606,645,655–657]. The average duration of SARS-CoV-2 RNA detection in the upper respiratory tract of patients with mild disease ranged from 7.9 to 20 days post-symptom onset and from 6 to 30.8 days in cases with moderate to severe illness. Detection of SARS-CoV-2 in the lower respiratory tract ranged from 8 to 38.4 days for mild cases of COVID-19 and spanned between 6 and 26.9 days for moderate to severe illness [658]. In a systematic review and meta-analysis, the pooled estimate of the mean duration of SARS-CoV-2 RNA detection from symptom onset in mild adult cases was 12.1 days (95% confidence intervals [95% CI]: 10.1 to 14.1) in the upper respiratory tract and 24.1 days (95% CI: 10.0 to 38.2) in the lower respiratory tract. For moderate to severe cases, the pooled estimated for duration of SARS-CoV-2 RNA positivity in the upper respiratory tract was 15.8 days (95% CI: 11.1 to 20.6) and 23.2 days (95% CI: 21.5 to 25.0) in the lower respiratory tract [658]. In a systematic review and meta-analysis, the temporal dynamics of SARS-CoV-2 viral loads were stratified by COVID-19 disease severity and sampling site. In cases of mild adult disease, SARS-CoV-2 RNA in the upper respiratory tract was maximal on day four at approximately 6.6×10^8 copies/ml, whereas lower tract viral loads peaked at approximately 2.7×10^8 copies/ml on day six post-symptom onset [658]. In cases of moderate to severe adult disease, maximal SARS-CoV-2 RNA detected in the upper respiratory tract occurred on day eight at 4.6×10^9 copies/ml and on day 11 at approximately 3.5×10^8 copies/ml in the lower respiratory tract [658]. Regarding the difference in viral loads and duration of shedding between symptomatic and asymptomatic patients, the literature is inconsistent. Some publications observed little to no difference in viral loads between the two groups [659–663], while others suggested significantly higher viral loads in symptomatic patients [661]. Whether these differences are attributed to differences in disease severity, variations in the performance of methods used, or host factors, remains to be determined.

As highlighted above, the median duration of viral shedding is variable between individuals and likely dependent on disease severity and several host factors such as age, immunocompromising,

or medical comorbidities [645,649–654,664–666]. While most individuals with mild disease clear the virus within 10 to 20 days, in some cases with severe COVID-19, the duration of shedding can be prolonged [654,667]. The longest duration of viral RNA shedding reported to date were 83 and 111 days after symptom onset [668,669]; however, the persistence of RNA suggestive of low viral loads may be of little clinical significance, as the detection of SARS-CoV-2 RNA does not necessarily imply infectivity [606,645,657,664–666,649–656]. Moreover, many factors can affect the detection of SARS-CoV-2 RNA, such as the quality of sample collection, transport, and variables in laboratory processing; RNA positivity can be intermittent and inaccurate at the later stages of illness [670]. Therefore, the CDC recommends that the discontinuation of transmission-based precautions for patients with confirmed SARS-CoV-2 infection should be based on the resolution of symptoms, not based on testing [667]. While some countries have similar recommendations for discharge from quarantine, there is some heterogeneity in approaches, and these often vary based on the severity of illness and presence or absence of symptoms [671].

While not used routinely in many laboratories, detecting SARS-CoV-2 RNA from stool is possible in the presence or absence of gastrointestinal symptoms [630,672]. However, only 1% of patients had detectable RNA in their stool in the absence of positive respiratory specimens [630,672]. For some patients, viral shedding in stool can occur for a longer period than in the respiratory samples and could help diagnose if upper and lower respiratory tract specimens are negative, but there is a high suspicion of disease [593–597,630,673]. Of individuals who test positive for gastrointestinal (GI) specimens, the median duration of RNA shedding in the GI tract is 12.5 days following negative respiratory tract specimens [630,672]. Less frequently, shedding in stool can be prolonged and has been documented up to 70 days post-symptom onset, or 33 days following clearance from the respiratory tract [673,674]. As in respiratory tract specimens, RNA detection does not necessarily imply infectious virions are produced, but SARS-CoV-2 has been cultured from stool specimens in some studies [673,674].

Like molecular methods, antigen testing can be used to detect SARS-CoV-2 proteins in the acute stages of the disease following the incubation period in upper respiratory tract specimens such as NP, nasal swabs, and possibly saliva. Antigen detection using immunoassays like lateral flow rapid diagnostic tests (RDTs) are often less sensitive than molecular methods [640,675] but can detect SARS-CoV-2 antigen reliably when the viral load is high in the clinical specimens (i.e., typically

from 1-3 days before the onset of symptoms to 5-7 days after symptom onset), whereas the likelihood of SARS-CoV-2 detection decreases in the second week post-symptom onset [675].

In contrast to RNA and antigen detection, immunologic responses take longer to appear, with antibodies typically begin to appear six days post-symptom onset, as viral RNA levels begin to decline [644]. Typically, the first detectable antibody in human blood is immunoglobulin M (IgM), followed by immunoglobulin G (IgG). However, concomitant rise of IgM and IgG immunoglobulin classes as well as IgG first seroconversion have also been observed [676]. Little data is also available for immunoglobulin A (IgA) detection, a marker of mucosal immune responses, but it is evident that both IgA and IgM decline rapidly over the course of infection [677]. The median seroconversion time for total antibody, IgM, and IgG was 9, 10, and 12 days post-symptom onset (or 15, 18, and 20 days post-exposure), respectively [677]. It is unclear how long IgG responses last, or whether they confer protection against subsequent SARS-CoV-2 re-infection [678]. The longest study on the antibody dynamics tracked IgG up to 115 days after symptom onset in sera and saliva [679]. Immune responses may vary depending on disease severity and host factors such as immunocompromising conditions or other medical comorbidities, and the value of immune responses will be dependent on the ability to provide neutralizing antibodies or cellular immunity capable of viral clearance. The applications and limitations of serology and other immunodiagnostics are discussed in more detail in later sections of this paper.

A.7.3. Specimen Pre-processing Requirements

While detection of SARS-CoV-2 from respiratory specimens can be performed using RNA or antigen detection, they sometimes require a pre-processing step like heat lysis or inactivation using guanidinium salts before nucleic acid extraction and amplification or testing, to ensure safe handling conditions, depending on local biosafety risk assessments [570,591,610,617]. Specimen types such as sputum may require mucolytic agents such as dithiothreitol (DTT), N-acetyl-L-cysteine (NALC), or proteinase K (PK) to reduce specimen viscosity prior to testing [680]. Other pre-processing steps would include centrifugation for specimens like stool [673], PK digests for tissues (e.g., lung biopsies), and specimen aliquoting into compatible tubes for testing (if testing from primary specimens containers is not possible). With any manipulation of the primary specimen (i.e., pre-processing steps), careful consideration should be undertaken to ensure there are no potential impacts to downstream testing (e.g., RNA or antigen stability). Of note, some pre-

processing steps, like specimen lysis, can be done in conjunction with nucleic acid extraction using automated instrumentation (discussed in the real-time RT-PCR section of this review).

Overall, the choice of the specimen and timing of collection is crucial for accurate detection of SARS-CoV-2, as are factors such as the severity of illness. Given that the performance characteristics of diagnostic methods depend on numerous variables as well as the method(s) used as a comparator and disease prevalence, a comprehensive synthesis of all method performance falls outside the scope of this review. However, general concepts for performance characteristics, important considerations, and a description of the technologies used for SARS-CoV-2 detection in the clinical setting or in development are presented in the following sections.

A.7.4. Molecular Methods for Viral RNA Detection

While no true reference standard exists for detecting SARS-CoV-2, nucleic acid amplification tests (NAAT) such as real-time RT-PCR are the methods of choice for SARS CoV-2 diagnostic testing [479,480,485,490,491,681,682]. Following sequencing of its genome [445], laboratory-developed tests (LDTs) for the detection of the SARS-CoV-2 were quickly developed, and protocols were circulated broadly by the healthcare regulatory bodies such as the World Health Organization (WHO) and US Centers for Disease Control and Prevention (CDC) [458,479,683–685]. Many commercial kits have since become available and were authorized for use through Emergency Use Authorization (EUA) by the entities such as the Food and Drug Administration (FDA) and Health Canada. Lists of authorized medical devices related to COVID-19 in Canada and the US are regularly updated online [464,465], and examples of them are summarized in Table A.1.

Table A.1. Example of the approved NAATs for emergency use by US FDA for detection of SARS-CoV-2 RNA. The full list is available in Ref. [464].

Device/assay (manufacturer)	Method	Target genes	Specimen	Authorized setting(s)	Time and throughput	LoD ^a	Ref.
cobas 6800/ cobas SARS-CoV-2 (Roche Molecular Systems, USA)	RT-PCR	ORF1ab + E	NS, NPS, OPS	H, M, H- Pooling	3 h for the first run results but 90 min per run in continuous mode/ 864 samples per 8 h	46 copies/ml	[686]
Abbott m2000/ RealTime SARS-CoV-2 (Abbott Diagnostics, USA)	RT-PCR	RdRP + N	NS, NPS, OPS, BAL	H	7 h per run/ 470 samples per 24 h	100 copies/ml	[687]
NeuMoDx 288/ NeuMoDx SARS-CoV-2 (NeuMoDx molecular, USA)	RT-PCR	Nsp2 + N	NS, NPS, OPS, BAL, saliva	H, M	1.3 h per run/ 288 samples per 8 h	150 copies/ml	[688]
Panther Fusion/ Aptima SARS-CoV-2 (Hologic, USA)	TMA	ORF1ab	NS, NPS, OPS, MTS, NPW, NPA, NA	H, Pooling	2.4 h per run/ 500 samples per 8 h	0.026 TCID ₅₀ / mL	[689]
LIAISON MDX/ Simplexa COVID-19 Direct (DiaSorin Molecular, Italy)	RT-PCR	ORF1ab + S	NS, NPS, NW, NA, BAL	H, M	1 h per run/ 8 samples per run	500 copies/ml	[690]
FilmArray/ BioFire Respiratory 2.1 (RP2.1) Panel (BioFire Diagnostics, USA)	RT-PCR	S + M	NPS	H, M	2 min hands-on time/ 1 h per run	160 copies/ml	[691]
ePlex/ ePlex SARS-CoV-2 (GeneMark Diagnostics, USA)	RT-PCR	N	NPS	H, M	2 min hands-on time/ 1.5 h per run	750 copies/ml	[692]
GeneXpert Xpress/ Xpert Xpress SARS- CoV-2 (Cepheid, USA)	RT-PCR	E + N2	NS, NPS, OPS, MTS, NW, NA	H, M, W	1 min hands-on time/ 45 min per run	0.02 PFU/ml	[693]
Accula Dock/ Accula SARS-CoV-2 (Mesa biotech, USA)	RT-PCR	N	NS, MTS	H, M, W	5 min hands-on time/ 30 min per run	150 copies/re action	[694]
ID NOW/ ID NOW COVID-19 (Abbott Diagnostics, USA)	NEAR	RdRP	NS, NPS, OPS	H, M, W	2 min hands-on time/ 13 min per run	125 copies/ml	[695]
Sherlock CRISPR SARS-CoV-2 kit (Sherlock Biosciences, USA)	RT-LAMP, CRISPR- Cas13	ORF1ab + N	NS, NPS, OPS, NPW, NPA, NA, BAL	H	1 h per run	6750 copies/ml	[696]

SARS-CoV-2 DETECTR Reagent Kit (Mammoth Biosciences, USA)	RT-LAMP, CRISPR- Cas12	N	NPS, OPS, MTS, ANS, NPW, NPA, NA	H	45 min per run	20000 copies/ml	[697]
NovaSeq 6000/ Illumina COVIDSeq Test (Illumina Inc., USA)	Next generation sequencing	98 targets on the virus	NPS, OPS, MTS, ANS, NPW, NPA, NA, BAL	H	3072 samples per 12 h	500 copies/ml	[698]

^a LoD of each assay is the lowest LoD reported on the Instruction for Use file of that assay, regardless of the specimen types. Abbreviations: LoD: limit of detection; RT-PCR: reverse transcription-polymerase chain reaction; TMA: transcription-mediated amplification; NEAR: nicking enzyme amplification reaction; RT-LAMP: reverse transcription loop-mediated isothermal amplification; NS: nasal swab; NPS: nasopharyngeal swab; OPS: oropharyngeal (throat) swab; BAL: bronchoalveolar lavage; MTS: mid-turbinate nasal swab; NPW: nasopharyngeal wash; NPA: nasopharyngeal aspirate; NA: nasal aspirate; NW: nasal wash; ANS: anterior nasal swab; H: laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests; M: laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform moderate complexity tests; W: patient care settings operating under a CLIA Certificate of Waiver; PFU: plaque-forming units; TCID₅₀/mL: median tissue culture infectious dose/ml.

Real-time RT-PCR

Among NAATs, real-time RT-PCR is the most widely used method for the detection of SARS-CoV-2. As shown in Figure A.3A, the sample workflow for SARS-CoV-2 real-time RT-PCR includes specimen collection, transportation of the samples to the laboratory, specimen lysis, purification of viral RNA through nucleic acid extraction, and real-time RT-PCR amplification, detection, and analysis. Prior to real-time RT-PCR amplification, specimens are lysed to provide access to the SARS-CoV-2 RNA, and a nucleic acid extraction is performed to remove potential inhibitors that could impede amplification of the target. Both the lysis/extraction and RT-PCR amplification can be performed sequentially through manual processing on individual instruments, or the entire process can be automated.

(i) Specimen lysis and RNA purification.

To release viral RNA from host cells and virions, specimen lysis can be performed using physical (e.g., heat, sonication, or homogenization), chemical (e.g., organic solvents, detergents, chelating agents, or chaotropic agents), or enzymatic methods (e.g., proteases) [491,680,699,700]. Lysis steps based on enzymatic digestion (e.g., proteinase K digestion) are common for nucleic acid extraction in clinical laboratories. Following specimen lysis, extraction of viral RNA is performed to remove cellular debris and contaminants that could potentially inhibit the RT-PCR reaction and

purify the nucleic acids [701–703]. In many automated instruments, silica-coated magnetic microbeads are used to capture nucleic acids, which can be sequentially transferred into different wash solutions by a robotic pipetting instrument with a magnetic head [699–703]. The efficiency of several extraction methods has been compared for detecting SARS-CoV-2, and the results favor commercial kits over manual methods like organic extractions containing guanidinium thiocyanate-phenol-chloroform [704].

It should be noted that while nucleic acid extraction is essential to achieve optimal sensitivity in molecular assays, recent studies have described extraction-free protocols for molecular testing for SARS-CoV-2 to circumvent the potential bottleneck of extraction if the supply of extraction reagents or consumables is limited (or to provide options for low-income environments) [705–713]. However, without a nucleic acid extraction to remove PCR inhibitors in clinical specimens, there is a notable reduction in sensitivity, but the extent is dependent on the method and target used for SARS-CoV-2 detection, the type and duration of the lysis/inactivation method (heat or chemical), input volume, the specimen type, the transport media, and the viral load in the specimen [704,708,714–716]. For example, a sensitivity of a 60-minute heat inactivation alone reached 100% sensitivity for specimens with moderate to high viral loads (Ct values between 20-30) but declined to 54% in specimens with (Ct values > 30) [715].

(ii) Target amplification and detection

The amplification in real-time RT-PCR involves two main steps. First, an enzyme called reverse transcriptase creates a complementary DNA (cDNA) from the viral RNA. The cDNA is then used as a template in a real-time PCR amplification reaction where fluorescence is produced as DNA amplification occurs [480,717]. The PCR portion of real-time RT-PCR contains a fluorescent probe or dye to generate fluorescence (e.g., dual-labeled hydrolysis probe or intercalating dyes that bind to double-stranded DNA like SYBR Green) [718–723]. Figure A.3B illustrates the principle of a typical real-time RT-PCR reaction using a dual-labeled hydrolysis probe. Overall, if amplification of the target genes occurs during the cycling through the denaturation, annealing, and extension stages, a fluorescent signal is produced that can be captured by the real-time thermocycler [458,480,724]. If the fluorescence crosses a defined threshold, the cycle in which it occurred is termed the threshold cycle (Ct). Ct values help interpret results as positive, negative,

or indeterminate (or equivocal), and each real-time RT-PCR method must validate its cutoff values, as they may differ between methods and instrumentation [479,490,725].

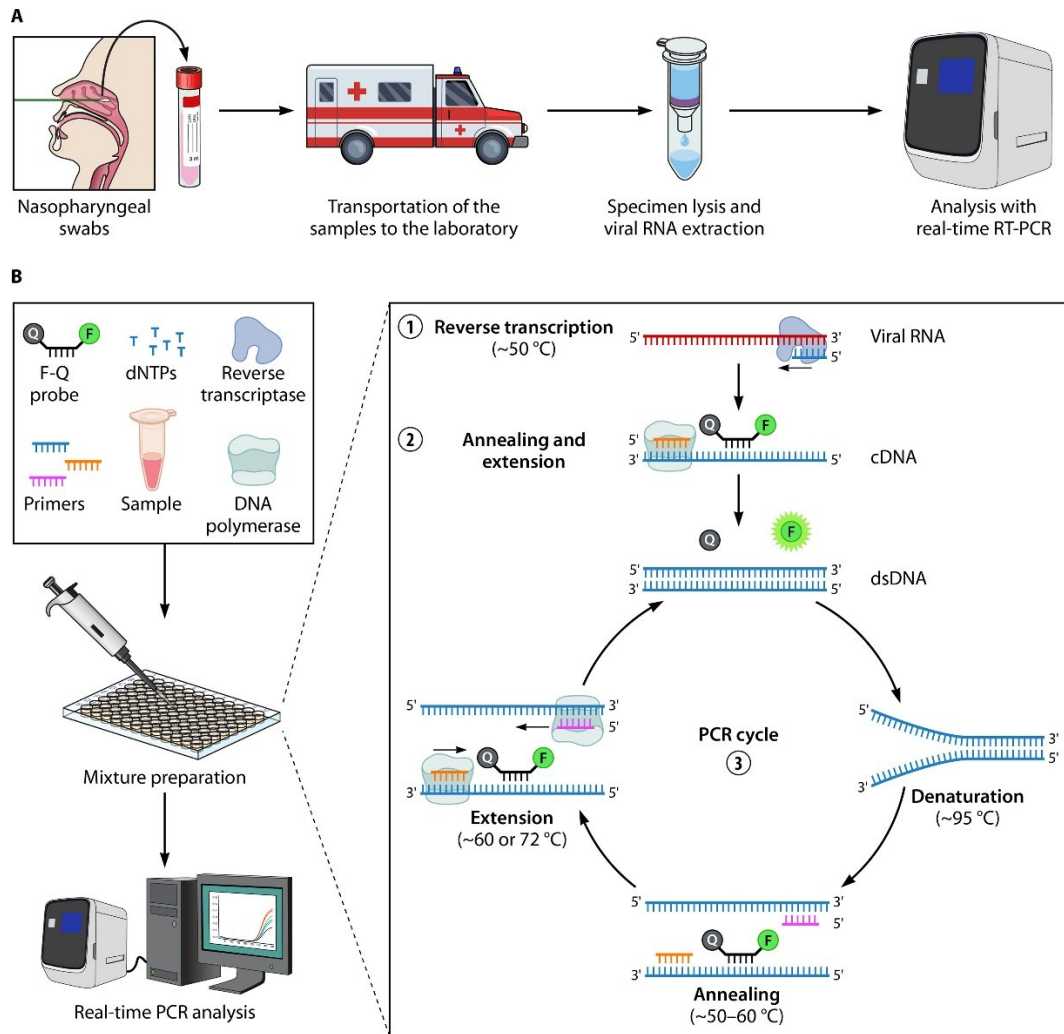


Figure A.3. Real-time RT-PCR analysis. (A) Typical steps required for the detection of SARS-CoV-2 with real-time RT-PCR. (B) Principle of real-time RT-PCR: 1) during reverse transcription, reverse transcriptase (RT) creates a complementary DNA (cDNA) from the viral RNA template, with the aid of the reverse primer (or random oligonucleotides). The RNase H activity of the RT digests the initial RNA template. 2) The DNA polymerase activity of RT (or of the Taq polymerase) completes the second DNA strand guided by the forward primer and cDNA. 3) The newly formed double-stranded DNA (dsDNA) is used as a template for the PCR portion of the assay. At the annealing stage, the reverse primer binds to the sense strand of dsDNA in a sequence-specific manner, and the forward primer and a dual-labeled probe bind to the antisense strand of the DNA. In this stage, the fluorophore (F) present on the probe is masked by the quencher (Q). During the extension step, the DNA polymerase extends the forward primer and, in the process, hydrolyzes the probe, resulting in the release of the fluorophore. Then, following excitation, fluorescence emission can be captured by the real-time thermocycler. With each round of PCR amplification, the dsDNA amplicon is multiplied by a 2-fold factor, with a proportional increase

in the overall fluorescent signal. After 30 to 40 cycles of amplification, the RT-PCR is complete. The PCR cycle at which the fluorescent signal crosses the threshold for positivity is called the threshold cycle (Ct), and Ct values are inversely proportional to the quantity of target present in the reaction.

The performance of real-time RT-PCR depends on a number of factors, including the specimen type, the timing of collection, the quality and quantity of viral RNA, the primer and probes designed and their viral RNA target, the reagents used for the RT-PCR reaction(s), the instrument and its operational parameters, and the signal/cutoffs used for result interpretation [458,479,490]. Typically, real-time RT-PCR assays demonstrate high sensitivity and specificity for SARS-CoV-2. In a systematic review and meta-analysis by Hellou et al., the pooled sensitivity for SARS-CoV-2 detection from 29 studies was 96.2% (95% CI: 91.0% to 98.4%) and a pooled specificity of 98.1% (95% CI: 95.9% to 99.2%) [543]. Sensitivity is of the utmost importance to ensure that cases in a population are identified, and specificity is important to ensure that no false positives occur. SARS-CoV-2 NAATs like real-time RT-PCR do not cross-react with other respiratory viruses, including human coronaviruses.

(iii) SARS-CoV-2 targets and data interpretation

Various targets have been used for SARS-CoV-2 real-time RT-PCR, but the genes encoding E, N, S, and ORF1ab are used widely [479,480,490,726–730]. Despite limited access to control materials early in the pandemic, LeBlanc et al. assessed the analytical performance of various LDTs and commercial RT-PCR assays and found that most of them had a high sensitivity with similar limits of detection (LoD) in the range of 3.4 to 4.5 log₁₀ copies/mL (11.2 to 141 copies/reaction) [479]. Similar proficiency testing across laboratories has been performed by others, demonstrating high sensitivity and specificity across different NAAT methods and instruments [731–733].

In the initial stages of the pandemic, dual- or multi-gene detection strategies were adopted for real-time RT-PCRs, to ensure assay specificity [479]. As the pandemic progressed and the disease prevalence increased, some laboratories implemented single-target detection of SARS-CoV-2 in LDTs to streamline workflow; however, many commercial methods continue to rely on detection using two or more targets [734]. In duplex or multiplex real-time RT-PCR assays, if the identification of any target is considered a positive result, sensitivity would be enhanced compared to the requirement of at least two targets to be positive for a SARS-CoV-2 result to be released as

such [479]. However, this strategy comes at a risk of decreasing specificity and potentially increasing the false positivity rate [479]. Regardless of the approach for testing, each target should have a validated range of values that defines a positive, negative, or indeterminate result, as well as conditions that would trigger an invalid result (e.g., processing or quality failures) [479,735]. Ct values can be used in real-time RT-PCR to define these cutoffs, and indeterminate (or equivocal) results arise for values falling between the Ct cutoff values of negative results and the reproducible Ct value cutoff for positivity. This is sometimes termed the diagnostic gray zone for result interpretation [479,736].

Low Ct values suggest more viral RNA was present in the specimen, whereas high Ct values represent specimens with lower virus burden, as more cycles were required to amplify the viral target. Therefore, Ct values are sometimes used as a surrogate for viral load. While low viral loads (indicated by high Ct values) could represent early or late disease, it could also be explained by non-specific reactions (i.e., false-positive reactions), poor collection techniques, specimen integrity issues during storage or transport, or a problem occurring during laboratory processing. As discussed in the timing of specimen collection section, some studies have evaluated the correlation between Ct values and infectivity [645,648–654]. There are some data to suggest that specimens that have SARS-CoV-2 RT-PCR results with Ct values of >24 cannot be effectively grown in tissue culture [649], yet other data has shown that SARS-CoV-2 can be recovered from cultured specimens with a Ct value >35, at a lower frequency [650,651]. While methodologies may have differed between studies to explain these differences (e.g., fresh vs. frozen specimens), it should be noted that the infectious dose required for human infection with SARS-CoV-2 is unknown and influenced by many biological and environmental variables. Given the variability that can occur in specimen collection, transport, and processing, there are no biological correlates accurately linking Ct values to infectivity or potential for transmission. Detectable virus by NAATs does not imply infectious virus. Of note, even if such a correlate existed, Ct value cutoffs cannot be applied universally to all NAATs, as they are method-, reagent, and target-specific, and to date, there is no international standard that can be used for calibration. Of note, other studies have investigated the role of Ct values in predicting the clinical course of COVID-19 or prognosis [737,738]; however, given the number of factors that could influence Ct values, along with the inability to standardize respiratory specimens, the role of Ct values to accurately predict clinical outcomes would likely be inconsistent, and further research is needed. While the interpretation of

Ct values requires careful consideration, it is clear that Ct values vary based on the viral burden, which itself varies throughout SARS-CoV-2 infection. Staging infection can provide epidemiological clues and can help with patient management. For example, low viral loads (i.e., high Ct values) are seen during pre-symptomatic, early, or late stages of infection, whereas low Ct values are seen between the early and late stages. If clinically indicated, patients with high Ct values should undergo repeat testing within 24 to 48 hours to determine if the Ct value is stable, rising, or declining to help stage potential exposures in contact tracing [603,604,667,739]. However, Ct value interpretation is complicated in asymptomatic infections, where the time of infection onset may be unknown. Therefore, to rule out potential false-positive results, repeat testing is recommended in patients with high Ct values suggestive of low viral loads [603,604,667,739].

(iv) Automation

LDTs and commercial assays for moderate to high throughput testing for SARS-CoV-2 require relatively expensive equipment and experienced personnel to obtain accurate and robust data, and the turn-around time for results can take several hours. Real-time RT-PCR assays are constantly being improved to increase specimen throughput, provide rapid specimen turn-around time, reduce hands-on time, and facilitate results interpretation and reporting. Automated high-throughput instruments are capable of performing over 1000 tests daily, with performance characteristics greater or equivalent to LDTs [603,604,702,740–746]. LDTs typically require separate nucleic acid extraction and amplification steps, but these processes can occur simultaneously with high throughput instruments, along with full traceability, and results can be directly reported through interfacing with the laboratory information system. One of the first high throughput instruments with a commercially available SARS-CoV-2 detection assay was the cobas 6800 instrument (Roche Molecular Systems, USA), but other highly automated instruments relying on NAAT technology are now available with similar performance, testing capacity, and workflow benefits. These include the Abbott RealTime SARS-CoV-2 assay on the m2000 instrument (Abbott Molecular, USA), Hologic Panther SARS-CoV-2 assay (Hologic, USA), the NeuMoDx SARS-CoV-2 assay (NeuMoDx molecular, USA), and the Becton Dickinson BD Max reagents (Becton Dickinson, USA) [747–753]. Advances have also been made for LDTs for SARS-CoV-2 testing, using semi-automated robotics to streamline specimen processing, nucleic acid extraction, RT-PCR setup and amplification, data interpretation, and interfacing for data reporting [746]. LDTs

for SARS-CoV-2 have also been adapted for other instruments, such as droplet digital PCR (ddPCR). In ddPCR, water-oil emulsions are used to partition nucleic acid samples into thousands of nanoliter-sized droplets, and PCR amplification is carried out within each droplet [754–757]. To date, the performance of ddPCR has been shown to be equivalent or slightly more sensitive than LDT comparators, but limited data is available for its use in clinical laboratories [754–757].

(v) Specimen pooling

Regardless of the NAAT used for SARS-CoV-2 detection, manufacturers of nucleic acid purification kits or RT-PCR reagents and consumables have been challenged with the rapid increase in testing demands that came with the global spread of SARS-CoV-2. While challenges to meet testing resources and limitations in the supply chain, research into alternative testing strategies have been explored [705]. A possible strategy to increase testing capacity and gain laboratory efficiencies is group testing (i.e., specimen pooling) [758]. While many pooling permutations are possible, its simplest application involves combining patient samples before testing and retesting individual specimens following the identification of a positive pool [759–763]. The optimal number of specimens within pools (i.e., pool depth) varies with disease prevalence and assay performance [758,762,763]. While larger pool depths may achieve higher efficiency, particularly for high throughput instruments, the trade-off is the accompanying reduced sensitivity, with the potential generation of false-negative results [758]. When prevalence is low, typically only a subset of specimens with low viral loads pass undetected, while testing capacity is increased and the cost of testing reduced [758,762,763]. In settings of high disease prevalence, the merits of pooling are lost given the high number of pools that need to be resolved. Other challenges for pooling include the increased human resource requirements for specimen registration and processing, but robotics and pooling software can help mitigate some of these issues [762]. For lower throughput analyzers like NAAT-based RDTs, pooling can also be considered [763]. Overall, while thorough validation and careful consideration of potential impacts of pooling should be considered before implementation on any instrument, pooling can offer an opportunity to clinical laboratories to increase testing capacity, save costs, and mitigate the supply chain limitations for laboratory testing [762].

(vi) *RT-PCR-based rapid diagnostic tests (RDTs)*

Unlike high-throughput automated instruments that are focused on large specimen volumes, rapid diagnostic tests (RDTs), as their name implies, are focused on speed. While this is acceptable for routine testing, rapid diagnostic tests (RDTs) have been developed to provide rapid results with easy-to-use testing, with minimal hands-on processing steps to facilitate training and testing. The first RDT based on real-time RT-PCR that obtained EUA from the FDA and Canada was the Xpert Xpress SARS-CoV-2 assay on the Cepheid GeneXpert platform (Cepheid Inc., USA), which provides results in about 45 min, with less than 5 min hands-on specimen processing time [462]. This NAAT-based RDT showed analytical and clinical performance characteristics often greater than LDTs and other commercial NAATs [536,603,604,741–743,747,764,765]. It should be noted that while Xpert Xpress is often referred to as a point-of-care (POC) test, this testing is not typically performed at the time and place of patient assessment and is more commonly performed in a laboratory setting; therefore, the term RDT would be more appropriate. The most current version of the Xpert SARS-CoV-2 assay is multiplexed with influenza A and B viruses, as well as the respiratory syncytial virus (RSV), which can present with similar respiratory symptoms [766]. More highly multiplexed assays like the BioFire Respiratory 2.1 Panel with SARS-CoV-2 (BioFire Diagnostics, USA) are also available that allow a syndromic approach with simultaneous detection of SARS-CoV-2 and several other respiratory viruses [767,768]. While syndromic testing is also being developed for larger instruments, such assays on RDTs are particularly useful for remote communities or resource-limited settings, or for testing of populations where rapid diagnosis would be of benefit (e.g., patients admitted to the ICU).

Other devices with a focus on potential POC applications have integrated RT-PCR with rapid (5–30 min) technologies such as digital microfluidics, visual lateral flow readouts, and portable instruments [690,692,694,769–773]. All these assays have the advantage of speed and simplicity but are prone to limitations such as low sensitivity, low specimen throughput, and minimal scalability [739]. For developing countries or other resource-limited settings where instrumentation is lacking, other cost-sparing testing alternatives are being explored. Arumugam et al. demonstrated the proof-of-principle of an RT-PCR that could be conducted in 12 min using a setup consisting of thin-walled PCR tubes, *sous vide* immersion heaters/circulators, and an end-point readout performed with a LED gel-viewing box [774]. Such creative and innovative solutions

from industry and academic settings help meet the global needs for SARS-CoV-2 laboratory testing, besides other NAATs rapidly being developed and validated.

Isothermal amplification technologies (IATs)

In efforts to develop portable and rapid diagnostic tests for SARS-CoV-2, NAATs other than RT-PCR have been investigated. Isothermal amplification technologies (IATs) are conducted at a constant temperature, eliminating the need for expensive equipment such as thermocyclers. The principles behind IATs rely on thermal or enzymatic denaturation of nucleic acids, followed by nucleic acid amplification reactions, and have been reviewed in detail elsewhere [681]. Isothermal NAAT technologies include transcription-mediated amplification (TMA), nicking enzyme-assisted reaction (NEAR), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and systems using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) (CRISPR-Cas). While most IAT methods have been applied to DNA, they can often be adapted to RNA amplification by adding an RT step (e.g., RT-LAMP and RT-RPA) [681,722,775,776]. Other IATs were designed for the intent of RNA amplification (e.g., TMA). Only TMA has been commercialized on a high throughput instrument, but other IAT technologies have been explored for uses as RDTs or potential POC applications (e.g., RT-RPA, RT-LAMP, NEAR, and CRISPR-Cas) [681]. The following sections will describe examples of IAT technologies and current and potential applications.

(i) Reverse transcription recombinase polymerase amplification (RT-RPA)

As seen in Figure A.4, the mechanism of RPA relies on homologous recombination [777]. RT-RPA uses a DNA polymerase to extend forward and reverse primers and make copies of each DNA strand (like PCR). However, to unwind and copy the DNA strands generated from reverse transcription, RPA requires the adenosine triphosphate (ATP)-dependent targeting activity of a recombinase complex, as well as the polymerase activity of a strand displacement DNA polymerase (e.g., *Bsu*) [777,778].

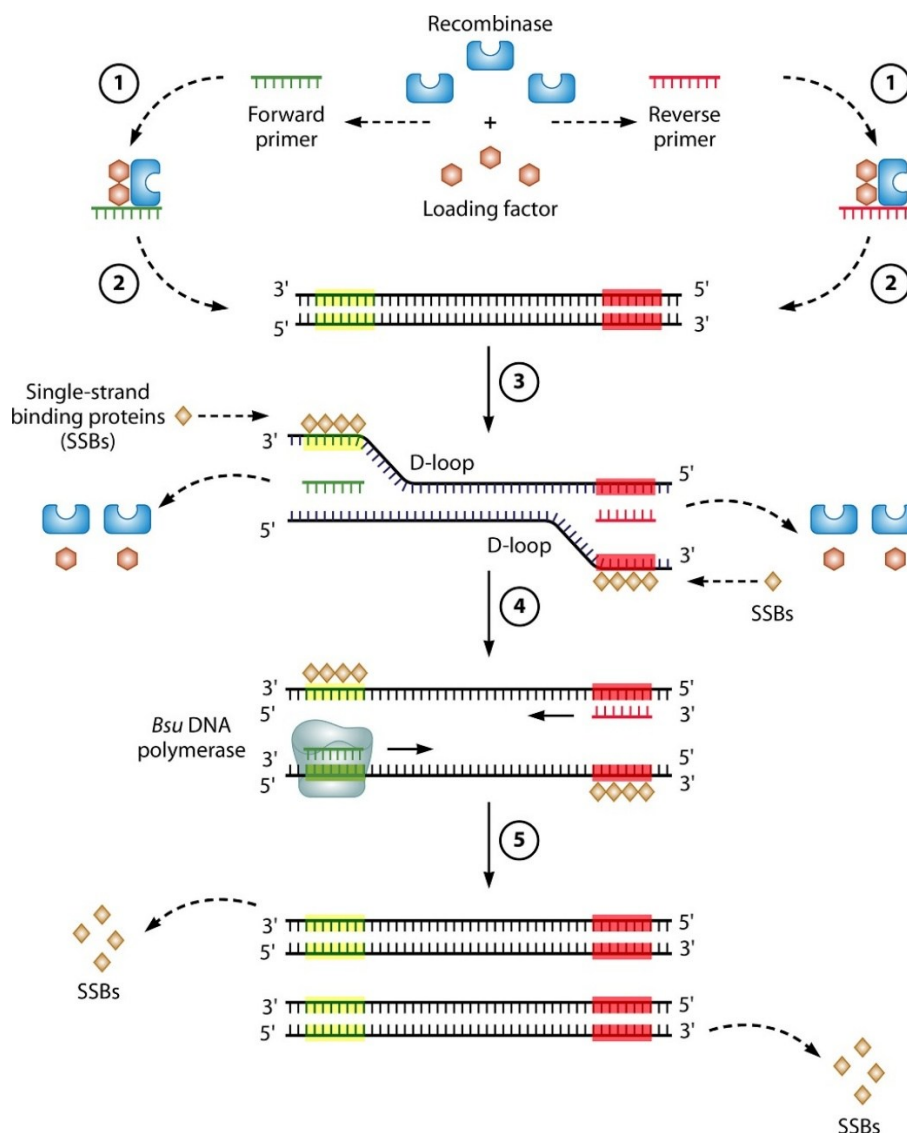


Figure A.4. Mechanism of RT-RPA. The RT-RPA reactions typically occur between 37 and 42°C in the following steps. 1) The reaction is initiated by binding of a recombinase (e.g., T4 UvsX) and loading factor (e.g., T4 UvsY) to each of the forward and reverse primers. 2) These recombinase/loading factor/oligonucleotide complexes search for homologous sequences in dsDNA, formed in the RT reaction from viral RNA (not depicted). 3) Once the sequence homology is found, the recombinase complex invades the duplex DNA, forming a structure called a D-loop in an ATP-dependent reaction where there is the unwinding of dsDNA and binding of the primer to its complementary sequence. Access to the primer-binding sequence is possible due to the stabilization of the opposite strand by single-strand binding proteins (SSBs) (e.g., T4 gp32). Subsequently, the recombinase and loading factor disassemble and are released to initiate other rounds of target recognition. 4) Following binding of the forward and reverse primers, these primers are extended at their 3' end using a strand displacement DNA polymerase (e.g., *Bsu*), and during the elongation process, there is further separation of the two strands. 5) Eventually, SSBs are displaced, and the replication of both strands is complete. This figure was inspired by the work in Ref. [777].

While the mechanisms for RT-RPA is relatively simple, the reaction components are fairly complex. Single-tube RT-RPA reactions include forward and reverse primers, core enzymes (e.g., RT, recombinase, recombinase loading factor, and a strand-displacement DNA polymerase), proteins like single-stranded binding protein (SSB), and a number of ancillary components such as dNTPs, salts, buffers, co-factors, crowding agents, ATP and an enzymatic system to generate additional ATP (phosphocreatine and creatine kinase [CK]). Once added, magnesium (Mg^{2+}) initiates the RPA reaction. Fortunately, various kits are now commercially available for RPA (e.g., TwistDx, United Kingdom), with variations for the probe used in the detection step [777]. For example, the TwistAmp exo probes are used for fluorescence detection through a mechanism involving exonuclease III, whereas a detection system designed for a lateral flow assay output can be incorporated using nfo probes and endonuclease IV [777]. Alternative fluorescence technologies have also been used such as fluorescence resonance energy transfer (FRET) probes or CRISPR-Cas technology [722,775].

Unlike real-time RT-PCR, RT-RPA does not require sophisticated instrumentation like thermocyclers, thereby simplifying the testing process. The ease-of-use of this isothermal technology makes RT-RPA an attractive candidate for point-of-care molecular tests. RT-RPA technology has been applied to the detection of other RNA viruses like Ebola [777]; however, to date, data presenting its use for the detection of SARS-CoV-2 is scarce [722,775,779]. Kim et al. used a modified version of RT-RPA to detect SARS-CoV-2 and achieved a sensitivity of approximately 4 copies/reaction in a 10 min reaction that used a lateral flow immunoassay (LFIA) readout. Their RT-RPA correctly identified all 18 contrived specimens generated by spiking heat-inactivated virus into NP or saliva [775]. A second publication by Xia and Chen described another modified single-tube version of RT-RPA introduced by GenDx called reverse transcription-enzymatic recombinase amplification (RT-ERA), as well as the whole-course encapsulated procedure for exponential amplification from RNA (WEPEAR) protocol [722]. The WEPEAR protocol contains all the reaction components necessary for RT-ERA, except the activator Mg^{2+} which is loaded into the microtube's lid. Following the RT reaction, the tube can be spun and mixed to initiate the modified RPA reaction. Using FRET probes for a fluorescence output or nfo probes for LFIA-based detection, the WEPEAR protocol achieved high sensitivity in the range of a single copy per reaction. Unfortunately, this method was only attempted on a single clinical specimen and would require further validation. Other applications of the RT-RPA for the detection

of SARS-CoV-2 involve the use of CRISPR-Cas technology, which will be covered in a later section (CRISPR-Cas technology).

(ii) *Transcription-mediated amplification (TMA)*

TMA is an IAT that amplifies RNA from an RNA template [480,780–782], and this technology has been applied to SARS-CoV-2 diagnostics on high-throughput analyzers [747,770,783,784]. Figure A.5 illustrates the principle of TMA.

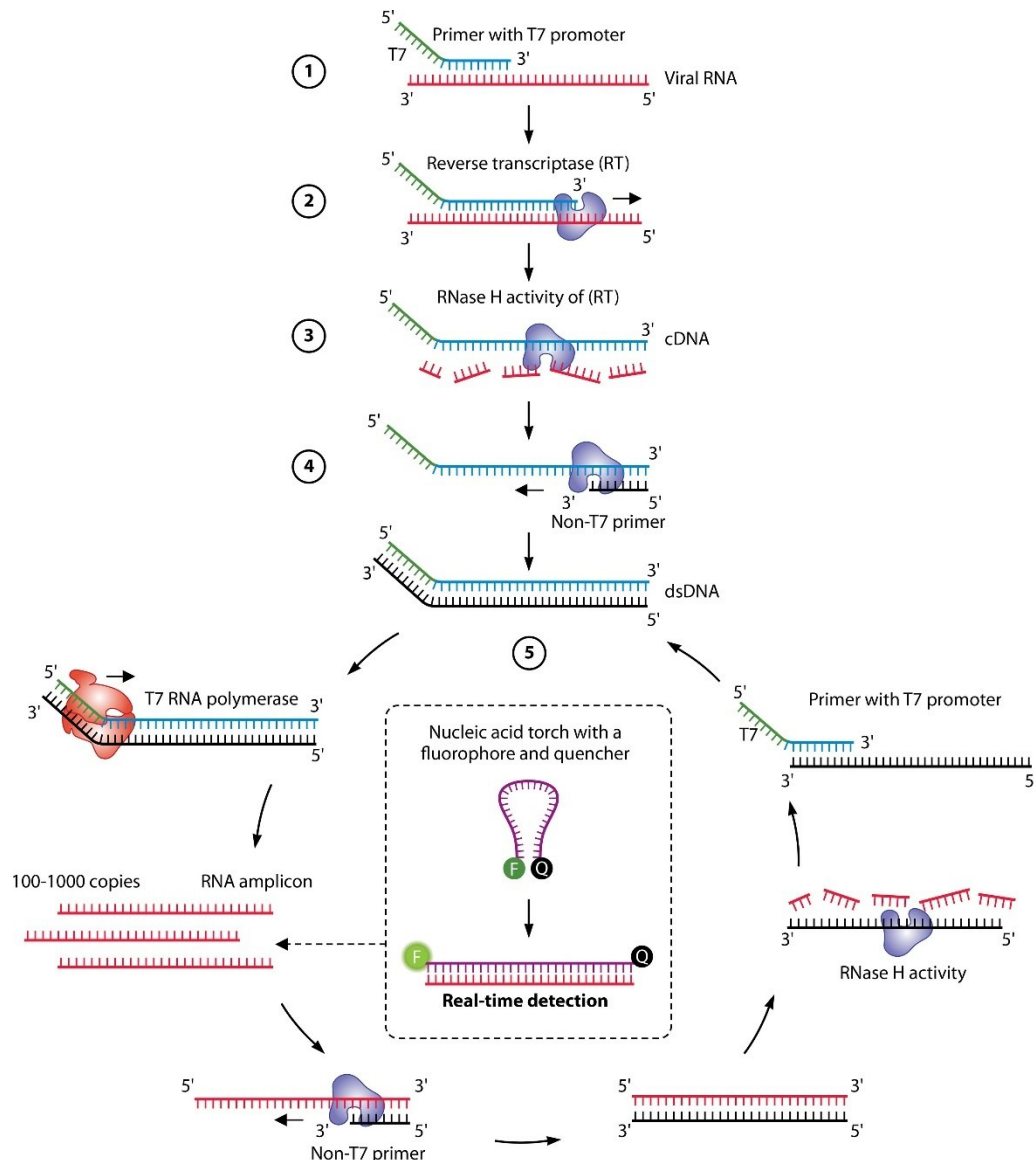


Figure A.5. Principle of TMA. 1) The reactions use a reverse primer that is complementary to the sequence of the RNA template, but the reverse primer also contains an overhang with a promoter sequence for T7 RNA polymerase at its 5'-end. 2) Reverse transcription is conducted by the RT; the newly transcribed cDNA includes both the target sequence and T7 promoter. 3) The RNA

template is digested by the RNase H activity of the RT. 4) dsDNA is produced by the DNA polymerase activity of the RT. 5) The produced dsDNA is used as the template for transcription mediated by the T7 RNA polymerase. RNA is thereby amplified several-fold and, through the activity of the same enzyme(s), can serve as the template for a new TMA reaction. As the cycle progress, exponential amplification ensues. Detection of the amplified RNA is usually accomplished using sequence-specific molecular beacons (“torch”) or hybridization probes targeting the single-stranded RNA (ssRNA). This figure was inspired by the work in Refs. [780,781].

The Aptima SARS-CoV-2 assay is performed on the Hologic Panther instrument, a highly automated instrument capable of processing over 1000 specimens daily [480]. Its principle combines a purification step using target capture, TMA for RNA amplification, and chemiluminescent probes for RNA detection. In the target capture, SARS-CoV-2 RNA is isolated from specimens using magnetic microparticles coupled to oligomers containing sequences complementary to specific regions of the target RNA molecules, as well as poly-deoxyadenosine residues. By modifying the temperature, sequential hybridization can occur between the RNA target and sequence-specific portion of the capture oligomers, and then a hybridization step occurs between the poly-deoxyadenosine region of the capture oligomer and the poly-deoxythymidine sequence that is covalently bound to the magnetic microparticles [785]. After the purification, TMA reactions occur, while detection is achieved through the hybridization of sequence-specific single-stranded oligonucleotide probes labeled with acridinium ester. A reagent is applied to generate a chemiluminescent signal that can distinguish between free and bound probes. A luminometer captures the resulting light emitted from bound probes, expressed as relative light units (RLU).

The Hologic Aptima SARS-CoV-2 assay has only recently been authorized by the FDA and Health Canada, but data on its performance are scarce. The Aptima SARS-CoV-2 assay showed higher analytical sensitivity than some LDTs using real-time RT-PCR, and the performance against other high throughput analyzers was equivalent [747,783,784]. Compared to the Hologic Panther Fusion SARS-CoV-2 assay (i.e., real-time RT-PCR on a highly automated instrument), the Aptima SARS-CoV-2 assay showed similar analytical sensitivity, with LoDs ranging between 62.5 and 125 copies/ml, and the clinical performance was equivalent [770]. Given the widespread use of Panther instruments in clinical laboratories for other pathogens (e.g., *Chlamydia trachomatis* and *Neisseria gonorrhoeae*) [786], the SARS-CoV-2 assay on this high-throughput instrument was highly anticipated.

(iii) *Nicking enzyme-assisted reaction (NEAR)*

The ID Now COVID-19 (IDNCOV) assay performed on the ID Now instrument (Abbott Diagnostics, Inc., USA) is an IAT that uses NEAR technology, and this RDT was recently authorized for use for POC testing in the USA and Canada. NEAR reactions are typically coupled to fluorescence detection following exponential amplification of DNA but can be used to detect an RNA template with the addition of a reverse transcription step [787,788]. NEAR reactions occur under isothermal conditions (at 60°C) and in several steps mediated by two main enzymes: 1) a nicking endonuclease, that recognizes specific restriction endonuclease sites in DNA (e.g., Nt.*Bst*NBI [5'-GAGTCNNNN^N-3']), but only nicks one strand; and 2) a strand displacing DNA polymerase like *Bst*, that can displace downstream DNA during synthesis at temperatures around 65°C (Figure A.6). Strand displacement is possible due to the fact that the *Bst* DNA polymerase lacks 5'→3' exonuclease activity common to other DNA polymerases (e.g., *Taq* polymerase).

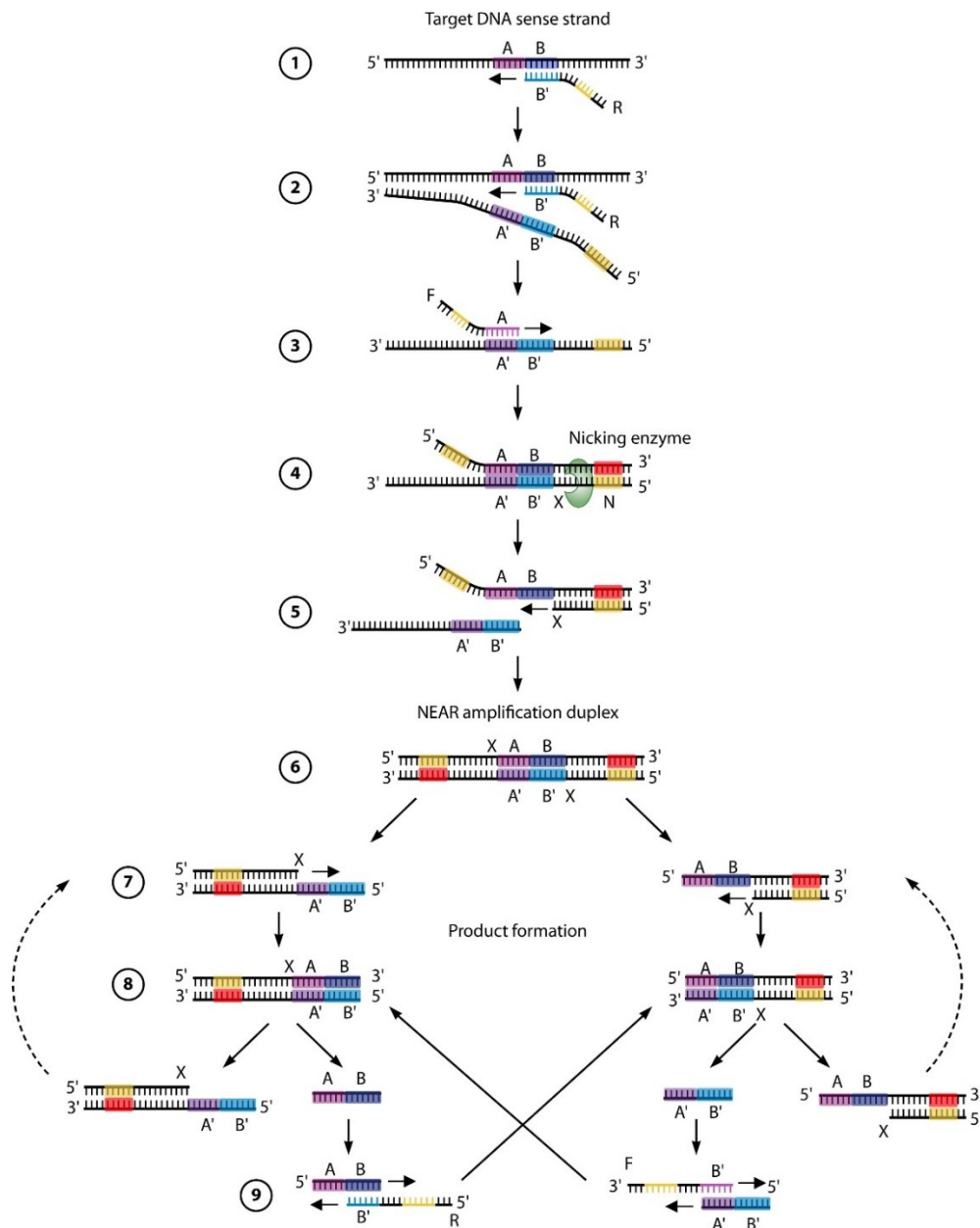


Figure A.6. Principle of NEAR technology. The NEAR amplification reactions occur at 60°C and can be broken down into two milestones: NEAR amplification duplex formation and product formation. 1) The target recognition region (B') of the reverse primer (R) binds to the complementary sequence (B) of the target DNA sense strand and is fully extended by the strand displacement DNA polymerase. 2) A second R primer binds to the template DNA and, during extension, displaces the elongated product of the first R primer extension. 3) The recognition region (A) of the forward primer (F) binds to its complementary sequence (A') in the R extension product, and F is extended to create a double-stranded nicking enzyme recognition site (N). 4) Nicking enzyme recognizes N and cleaves a single strand of DNA in a sequence-specific manner at the cut site (X). 5) This releases a fragment of the R extension product. The remaining fragment serves as a primer and is extended at its 3'-end. 6) This extension completes the double-stranded complex, termed the NEAR amplification duplex, which is the starting point for product formation.

7) Nicking enzymes bind to the nicking enzyme recognition sites on both ends of the NEAR amplification duplex and cleave at X. 8) The resulting single-strand nicks create two complexes, each consisting of a single-stranded target region flanked by a nicking enzyme recognition region. 9) Repeated nicking, polymerization, and strand displacement activities result in the amplification of the AB and A'B' target products. Cleaved complexes are regenerated, while the AB and A'B' products can anneal to R and F primers, respectively. In turn, the bidirectional extension of the primer and product each creates duplexes that lead to the generation of the opposite product upon cleavage. Product amplification continues until reagents or enzymes are depleted. This figure was inspired by the work in Ref. [788].

While the mechanism for nucleic acid amplification with NEAR may be complex, IDNCOV testing is simple and rapid. The assay has a processing time as low as 5 min for positive results with high viral loads and 15 minutes for specimens with lower viral loads or negative results. When compared to LDTs or commercial NAATs, many recent studies have demonstrated excellent specificity/negative percent agreement (NPA) near 100% but relatively poor sensitivity/positive percent agreement (PPA) between 48% and 70% for the detection of SARS-CoV-2, while other studies showed high specificity/NPA (~100%) as well as high sensitivity/PPA values above 90% [741,752,789–795].

The reason for the disparities in sensitivity/PPA between studies are likely multifold and include differences in patient population (setting, host factors, presence or not of compatible symptoms), the specimen type, the timing between collection and testing, the transport conditions used (dry swabs or transport media), the quality of specimens (prospective vs. retrospective), the spectrum of viral loads in the specimens evaluated (proportion of specimens with low viral loads), or differences in performance characteristics of the comparator method(s) [741,752,789–795]. For example, swab type affected IDNCOV performance, where NP swabs showed a PPA of 64% compared to 48% with nasal swabs [796]. Using residual positive and negative NP swabs collected in VTM, Mitchell et al. compared IDNCOV to the CDC real-time RT-PCR, and IDNCOV showed a sensitivity and specificity of 71.7% and 100%, respectively [789]. All false-negative results corresponded to specimens in which CT values were between 35 and 40, suggesting low viral loads. Smithgall et al. used residual NP swabs tested on the Roche cobas assay and found an NPA of 100% and 92.0% for INDCOV and Cepheid Xpert Xpress and the overall PPA of 73.9% and 98.9%, respectively [741]. However, they also noted that PPA varied with viral loads. When specimens were categorized by Ct values, both INDCOV and Xpert showed 100% PPA for specimens with medium to high viral loads (Ct values <30), but at low viral loads (Ct values >30),

the PPA for IDNCOV was 34.3% vs. 97.1% for Xpert [741]. In a recent study, Stokes et al. compared IDNCOV to an LDT and showed an excellent PPA of 89.1% [95% CI: 82.0% to 94.1%] for IDNCOV [795]. Notably, the PPA increased to 98.2% following the manufacturer recommendations for testing under EUA for symptomatic individuals tested ≤ 7 days post-symptom onset and within an hour of collection using the appropriate swab [795]. Overall, these studies not only demonstrate that the performance characteristics of a test is dependent on numerous factors, but also reflect the need for validations or verification of these factors in the setting and conditions where NAAT-based RDTs are applied [725,739].

(iv) Reverse transcription-loop-mediated isothermal amplification (RT-LAMP)

RT-LAMP is an IAT technology that is gaining interest for potential POC applications and is also being explored for routine diagnostic testing [480,490,491,681,797–799]. Like RT-PCR, RT-LAMP begins with reverse transcription of the target RNA into cDNA by RT, which is either done in a separate reaction or in the same tube as the LAMP reaction. The LAMP reaction can take place in a single tube at 60-65°C [711,800,801], and the process can be performed in as little as 20-60 min [801,802]. LAMP reactions consist of a strand displacement DNA polymerase (e.g., *Bst* polymerase), DNA template, dNTPs, typically from 4 to 6 primers, and depending on the LAMP permutation for signal detection, either a pH-sensitive colorimetric dye or a fluorescent dye [480,491,681,798,799,803,804]. The mechanism for DNA amplification using LAMP is summarized in Figure A.7.

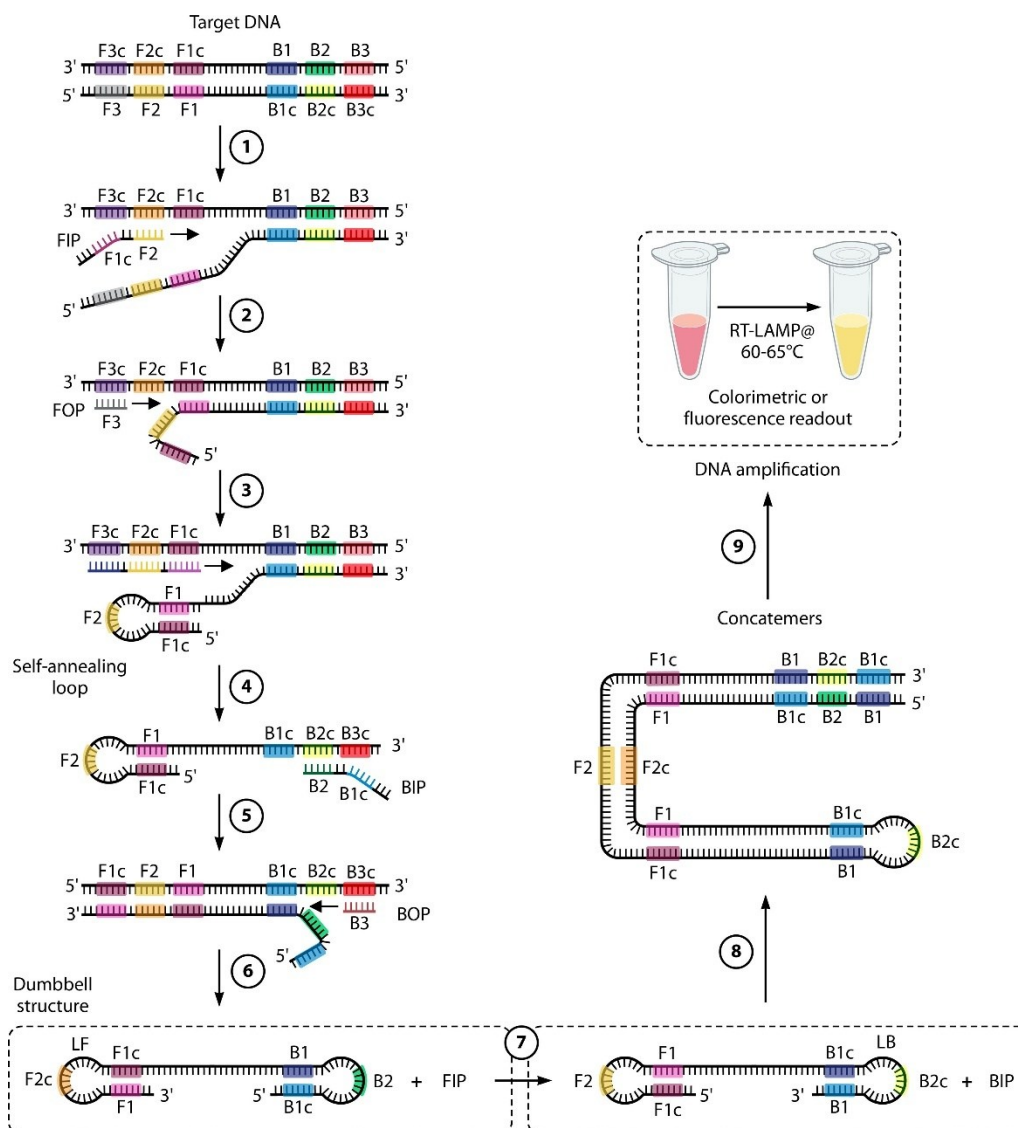


Figure A.7. Amplification of nucleic acids using RT-LAMP. Overall, there are four core primers that mediate all the processes in a LAMP reaction by recognizing six distinct regions of the target DNA through several steps. 1) After the conversion of the template RNA into dsDNA via reverse transcription (not shown), the LAMP reaction starts from strand invasion by the forward inner primer (FIP), which hybridizes through its F2 region to the F2c region of the target DNA. This initiates complementary strand synthesis using a strand displacement DNA polymerase. 2) The forward outer primer (FOP, also termed F3 primer) then hybridizes to the F3c region of the target DNA, and during extension, displaces the newly elongated strand from the FIP primer. 3) Given the FIP primer also contains an F1c sequence, the strand displacement triggered by the DNA polymerase and the FOP leads to forming a self-annealing loop in the 5'-end of the FIP-linked strand (regions F1 and F1c). 4) This single-stranded DNA with a stem-loop at its 5'-end then serves as a template for the backward inner primer (BIP), which hybridizes to the B2c region of the template DNA through its B2 sequence. 5) During elongation, the complementary strand opens the 5'-end stem-loop. Then, the backward outer primer (BOP, also termed B3 primer) hybridizes the B3c region of the target DNA, and its elongation displaces the BIP-linked complementary

strand. 6) The displacement of the BIP-linked strand results in a self-hybridization on both the 5' and 3' ends, leading to two stem-loops and the formation of a dumbbell-shaped DNA. 7-9) The amplification of the dumbbell structure with FIP leads to a concatemer and the formation of a second dumbbell structure that can be amplified with BIP. Amplification can pursue from the 3'-end of each dumbbell structure or with annealing of primers such as FIP and BIP. Additional loop primers (i.e., loop F [LF] and loop B [LB] primers) can also be used to increase the speed and sensitivity [480,798,799]. Visualization of the LAMP amplification is typically done by using pH-sensitive colorimetric or intercalating fluorescent dyes. This figure was inspired by the work in Refs. [803,804].

Variations of LAMP has been developed for potential POC applications with reactions that are monitored in one of three ways: 1) turbidity can be measured with a spectrophotometer at an optical density of 400 nm, as magnesium pyrophosphate precipitates in the solution as a byproduct of the LAMP reaction [480,805–807]. 2) Colorimetric detection can be performed using pH-sensitive dyes (e.g., cresol red or phenol red) that change color from the incorporation of dNTPs during amplification or using a metal indicator (e.g., hydroxynaphthol blue) that would assess the concentration of Mg^{2+} , used as co-factor for dNTP incorporation during DNA synthesis [797,808–810]. Color changes can be read by the naked eye or spectrophotometry [807,810]. And 3) fluorescence detection can be performed if an intercalating dye (e.g., SYBR green) is used in the LAMP reaction. When complexed with dsDNA, intercalating dyes can be excited to emit fluorescence, which can then be captured in real-time with a fluorometer or compatible thermocycler [802,808]. Alternative detection systems for RT-LAMP include CRISPR-Cas technology, and these will be covered in a later section of this review.

Fluorescence detection tends to be the most sensitive of the visualization methods, which make the LAMP technology amenable to real-time monitoring and high-throughput testing [797,802]. However, to our knowledge, no high-throughput instruments have adopted this technology to date. On the other hand, a commercial kit for real-time SARS-CoV-2 RT-LAMP (Variplex, Amplex Diagnostics, Germany) has been developed and compared to real-time RT-PCR. The commercial RT-LAMP kit showed moderate agreement to the real-time RT-PCR, with a clinical sensitivity of 76.3% [811,812]. Given its simplicity, RT-LAMP technology has also been used to develop rapid POC products [797]. Results are ready in 30 min using the RapiPrep COVID-19 (MicrosensDX, England), which integrates a magnetic bead-based RNA extraction with LAMP technology. However, a relatively low sensitivity of 80% and a specificity of 73% were observed when tested on 21 nasal swabs compared to real-time RT-PCR [813]. The authors suggested that while the

sensitivity and specificity were poor, the assay still had merit in some clinical applications such as algorithms using repeat testing over time. Other studies that used RT-LAMP demonstrated varying performance compared to LDTs based on real-time RT-PCR for commonly used specimen types [805,814]. The analytical sensitivity of most RT-LAMP assays was found to be in the range of 100 to 200 copies per reaction [808,815], while others have reported analytical sensitivities as low as 10 copies/reaction [802,816]. Altogether, this range is consistent with some LDTs and commercial real-time RT-PCR assays (e.g., cobas SARS-CoV-2 test) [479,729,816,817]. The variability in the performance of RT-LAMP assays could be attributed to differences in processing steps, specimen types, quality of the nucleic acid extraction, LAMP reagents, the viral target(s), the detection methods (manual versus automated), or the methodology (e.g., measuring turbidity, colorimetric, or using fluorescence), the viral load and patient populations tested, the number of specimens evaluated, the method used as a comparator, or other undefined or uncharacterized factors like inhibition rates [681,711–713,802,805,815,818–820]. Overall, RT-LAMP technology shows promise for large scale testing [821] and POC testing [711,729,816,817,822,823], but further optimization is still required.

(v) *CRISPR-Cas technology*

CRISPR and its Cas proteins are derived from prokaryotic defense systems against foreign nucleic acids [824–829]. When activated, Cas proteins can exhibit local DNase or RNase activity resulting in local cleavage (*cis*-cleavage) of the target DNA or RNA, as well as collateral damage (*trans*-cleavage) to neighboring single-stranded DNA (ssDNA) or RNA. A number of different Cas proteins have been identified that differ in nucleotide specificity for their *cis*-cleavage targets and in their ability to cause collateral damage to nearby nucleic acids [779,830–832]. The high degree of collateral damage caused by Cas12a or Cas13 can breakdown neighboring RNA or ssDNA, which can be exploited for detection. For example, dual-labeled ssDNA or RNA probes with a fluorophore-quencher combination can be used, and when the Cas12a or Cas13 system binds to the target DNA or RNA, the Cas proteins are activated to cleave the target and the probe. With a blue light generator, fluorescence is generated and visualized or captured by a fluorometer [833–835]. Alternatively, the RNA or ssDNA probe can be labeled with biotin, and the cleavage reaction observed with the aid of a specific immunochromatographic device (e.g., LFIA) and colorimetric detection [833–835]. Understanding the mechanism and permutations of CRISPR-Cas systems

have led to many technological advances in genome editing [836,837] and diagnostic applications such as the detection of RNA viruses (Figure A.8) [830–832,834,835,838,839].

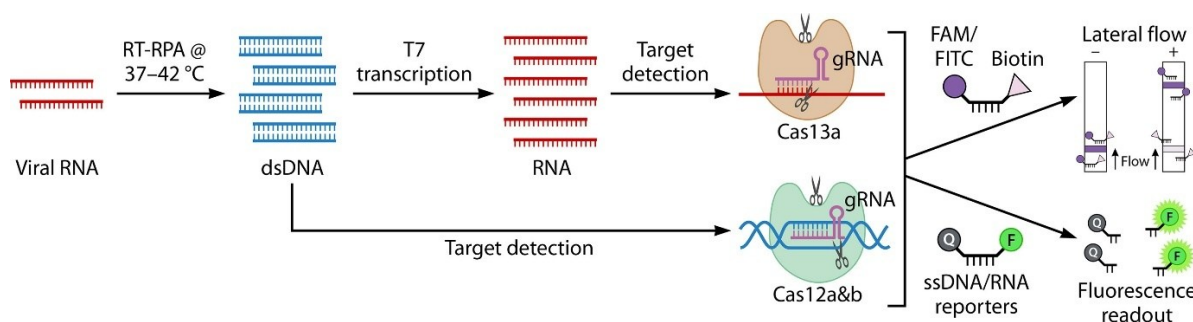


Figure A.8. Principle of CRISPR-Cas technology for viral RNA detection. First, the viral RNA is subjected to reverse transcription and amplification, e.g., in an RT-RPA reaction at 37–42°C, to generate dsDNA. The dsDNA can be targeted by guide RNAs (gRNA) directly in a CRISPR-Cas12 detection system, whereas RNA detection using the CRISPR-Cas13 system requires an additional T7 transcription step. When Cas12 or Cas13 is activated by recognition of gRNA, there will be cleavage of the target as well as non-specific cleavage of dual-labeled oligonucleotide probes. The probes are ssDNA or ssRNA for the CRISPR-Cas12 or CRISPR-Cas13 systems, respectively. The readout for either method can be colorimetric by incorporation of a fluorescein amidite (FAM)/fluorescein isothiocyanate (FITC)-biotin probes and using lateral flow dipsticks, or fluorometric readouts can be used by incorporation of a dual-labeled fluorophore (F)-quencher (Q) probes. Upon collateral cleavage, the unquenched fluorophore can be excited with blue light, and the resulting emission of fluorescence can be visualized or captured with a fluorometer. This figure was inspired by the work in Refs. [830,833].

The use of CRISPR-Cas as a diagnostic tool was proposed by two laboratories, which founded the Cas12a-based system named DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) [840], and the Cas13-based system termed Specific High sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) [841,842] (Figure A.8). Recently, a SHERLOCK-based COVID-19 test received EUA from FDA, which is the first EUA for any CRISPR technology. However, many variations of CRISPR-Cas12 and CRISPR-Cas13 systems have emerged for the detection of SARS-CoV-2 in a variety of clinical specimens, both in potential POC or high throughput testing formats [843]. These include SHERLOCK Testing in One Pot Covid test (STOPCovid), All-In-One Dual CRISPR (AIOD-CRISPR), Cas12b-mediated detection (CDtetection), CRISPR-assisted detection (CASdetect), and Cas13-based, Rugged, Equitable, Scalable testing (CREST) [835,840,843–847].

The composition and conditions of each stage of a CRISPR-Cas detection system can affect its speed and performance. For SARS-CoV-2 detection, the initial step in the CRISPR-Cas system

can be the lysis of the specimens, with or without an RNA extraction; however, purified RNA showed greater sensitivity [840,843,844]. For example, Broughton et al. demonstrated a DETECTR system that had an LoD of approximately 10 copies/ μ l using purified RNA, but removing the nucleic acid extraction step decreased the LoDs to 15000 and 500 copies/ μ l for contrived specimens consisting of spiked SARS-CoV-2 into $\geq 10\%$ UTM or $\geq 20\%$ PBS, respectively [840]. As such, the purification of RNA was recommended. Next, the time to results for CRISPR-Cas-based methods was low compared to real-time RT-PCR at 20 min [843], 40 min [834,840,845], 50 min [779], 60 min [835], or 70 min [844], but varied between method and detection system. All CRISPR-Cas methods described herein require conversion of SARS-CoV-2 RNA into dsDNA, which can be accomplished by RT-PCR, RT-LAMP, or RT-RPA. Huang et al. compared the CDC real-time RT-PCR to a DETECTR assay using either RT-PCR or RT-RPA with a fluorescence readout and achieved low analytical sensitivity (2 copies per sample, regardless of the method used for amplification) [779]. Cas12-based CRISPR systems are typically faster than Cas13-based methods, as Cas12 directly detects dsDNA, but the latter requires an additional transcription step to RNA (Figure A.8).

The methods used for SARS-CoV-2 results readout can also affect the speed and performance of the assays. While initially CRISPR-Cas assays used visual readout on commercial lateral flow assays, coupling the CRISPR-Cas technology to fluorescent reporters instead lead to more rapid results and sensitive detection [840,841,848]. For example, Joung et al. validated a SHERLOCK-based method that returned results in 70 min using the LFIA readout, while fluorescence testing was completed in 40 min [844]. Guo et al. demonstrated that the analytical sensitivity of CASdetec, a CRISPR-Cas12b based on RT-RAA and fluorescence output, was 10^3 copies/ml, whereas the sensitivity of DETECTR and SHERLOCK ranged between 10^4 and 10^5 copies/ml using an LFIA for detection [835]. Also, CRISPR-Cas systems have been adapted to one-tube reactions using fluorescence rather than amplification followed by detection using lateral flow methods, not only to reduce processing steps but also to decrease the potential for contamination [843–845]. To optimize the reaction in single-tube formats, the temperature for IAT must be compatible from the RT step to Cas-based detection. Ding et al. described a one-pot reaction at 37°C combining RT-RPA and a CRISPR-Cas12a system and could achieve detection of 1.3 copies of SARS-CoV-2 RNA [845]. With the high temperatures required for RT-LAMP ($55\text{--}65^\circ\text{C}$), Joung et al. developed a CRISPR-Cas12b system where a thermostable Cas12b protein was derived from *Alicyclobacillus*

acidiphilus that could maintain activity at higher temperatures [844,849]. A similar approach was used by Ali et al., who developed a single-tube RT-LAMP and CRISPR-Cas12a-based system able to reach analytical sensitivity (10 copies/reaction) comparable to the CDC real-time RT-PCR (5 copies/reaction) [843]. Given the many variables for CRISPR-Cas technology, optimization of this methodology for SARS-CoV-2 and other diagnostic testing is an active area of research.

While analytical performances of CRISPR-Cas technology showed some promise for detecting SARS-CoV-2, validation on clinical specimens has been scarce to date. Hou et al. showed that an RT-RPA CRISPR-Cas13a system with fluorescence detection was able to correctly identify 52 positive specimens for SARS-CoV-2, whereas an undefined real-time RT-PCR failed to identify five specimens with low viral loads [850]. Huang et al. compared the CDC real-time RT-PCR to a DETECTR assay using either RT-PCR or RT-RPA with fluorescence detection on 19 positive clinical specimens and identified all of them; however, three additional positives were detected [779]. These could represent false negatives for the real-time RT-PCR or false positives for the CRISPR-Cas12 assay (i.e., specificity would be 71.4% for the latter). The LoD for the CRISPR-based method was 2 copies/reaction compared to 5 copies/reaction for the CDC real-time RT-PCR, suggesting CRISPR-based detection may be able to identify SARS-CoV-2 at lower viral loads. Broughton et al. compared the CDC real-time RT-PCR to an RT-LAMP DETECTR system with LFIA or fluorescence readouts using 83 clinical specimens (41 positive; 42 negatives) and demonstrated a 95% positive agreement [840]. Of the 21 positive specimens for SARS-CoV-2 identified by the CDC real-time RT-PCR, Ali et al. using an RT-LAMP CRISPR-Cas12 system were able to identify 18 (85.7%) using the fluorescence readout, but weak or absent signals were noted with the LFIA detection [843]. Overall, this data shows variability in performance for CRISPR-based assays ranging from 80-100% sensitivity.

While CRISPR-based methods are showing promise for SARS-CoV-2 detection, research into this technology is evolving. Recently, an RT-LAMP-based CRISPR-Cas12 system was developed using an electric field gradient on a microfluidic device [851]. This allowed for on-chip, automated separation of nucleic acids from nasopharyngeal swab samples in 30 min, followed by CRISPR-based detection [851]. While further validation is required to fully understand the benefits of CRISPR-Cas technology, it has much potential for applications as POC devices or high-throughput testing platforms [779,843].

SARS-CoV-2 next-generation sequencing (NGS)

Understanding the genomic sequence of SARS-CoV-2 obtained from clinical specimens can help identify understanding the COVID-19 pandemic origins, delineate transmission events, unravel clues to pathogenesis, and monitor viral evolution over time [852–856]. Over the last few decades, DNA sequencing technologies have relied on modifications of Sanger sequencing, which was developed in the 1970s [857]. While Sanger sequencing technology is still used for small sequences (~0.5 to 1kb) such as single-gene targets, next-generation sequencing (NGS) technologies have allowed sequencing to be performed as massively paralleled reactions, allowing rapid access to complete genomes, at a scale and cost that is feasible for many laboratories [852–854,858–860]. For SARS-CoV-2 genomes of less than 30 kb, high-quality sequences can readily be obtained with NGS directly from clinical specimens, using strategies like amplicon-enrichment or bait-capture techniques to favor the sequencing of the viral targets [861]. NGS involves technologies such as sequencing-by-synthesis, sequencing-by-ligation, and ion semiconductor sequencing (e.g., nanopore sequencing), each with its own advantages and limitations [852–854,858–860]. The principle of each NGS technology has been reviewed elsewhere [852–856], and some of them are illustrated in Figure A.9.

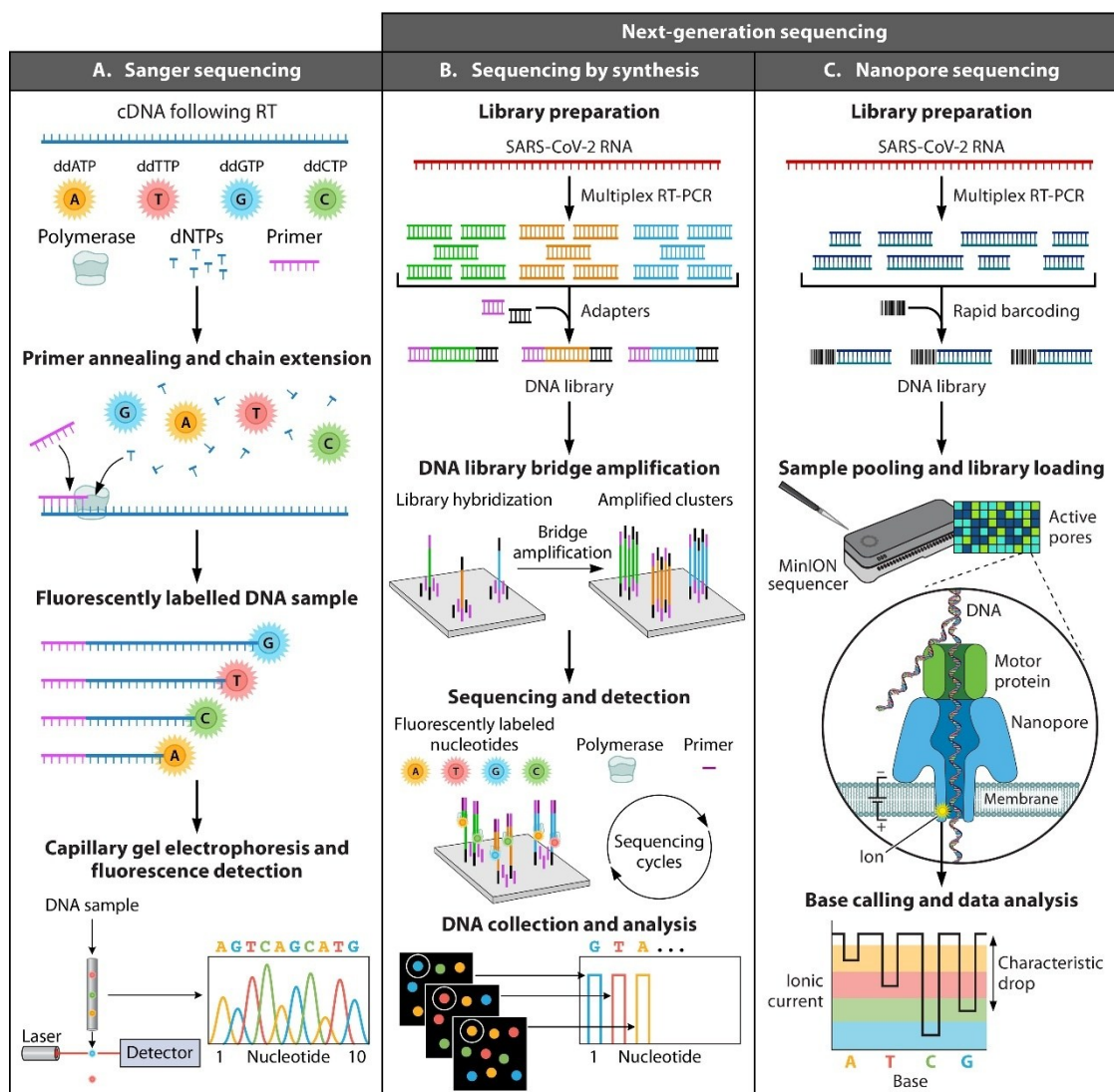


Figure A.9. Sequencing techniques for identification of SARS-CoV-2. (A) Sanger sequencing. First, SARS-CoV-2 RNA is often amplified through RT-PCR (not depicted). Sanger sequencing reactions can be undertaken to analyze either of the DNA strands, but only one strand per reaction can be assessed. The extension of the primer annealing to the template DNA occurs in the presence of DNA polymerase, buffer, cofactors, deoxynucleotides (dNTPs), and fluorescently labeled dideoxynucleotides (ddNTPs). The binding of the ddNTPs to the oligonucleotide strands will cease the extension, resulting in various DNA structures with different lengths. Next, the extended DNAs undergo a capillary gel electrophoresis system in which the shorter DNA strands move faster, resulting in the detection of the fluorescently labeled nucleotides in order of the size of the DNA strands. Finally, as DNA fragments are resolved and nucleotide-specific fluorescent signals are captured by a detector, a chromatogram is assembled to reveal the sequence of the template. (B) Next-generation sequencing (NGS) by synthesis. First, a library of millions of DNA fragments is created from the template (or enhanced by multiplex RT-PCR for SARS-CoV-2). Adapters are bound to the two ends of each DNA fragment. The adapters consist of a universal primer-binding site and a unique sequence (i.e., barcode) that can be hybridized to a specific sequence on the support (e.g., flow cell). Following hybridization, with complementary sequences of the adapters,

bridge amplification is used to amplify each DNA fragment at a defined physical position. In the sequencing and detection step, fluorescently labeled nucleotides are bound to the forward strands in the presence of a primer and polymerase, which results in the generation of fluorescent light that is detected by an analyzer in real-time. Many other NGS technologies are also available. For example, in (C) NGS by nanopore technology is presented. After creating a library of DNA fragments by multiplex RT-PCR and barcoding, the library is loaded onto a membrane containing nanopores. The nanopores are proteins that open the DNA double strand, and as each nucleotide is passed through the membrane, it causes a specific change in ionic current that can then be translated into the nucleotide sequence of the templates.

To date, a single commercial kit (Illumina Inc., USA) for NGS has been approved as a clinical diagnostic test under FDA EUA guidelines for COVID-19, which is based on sequencing-by-synthesis [862]. However, no data is available to date to describe its performance, advantages, or limitations compared to commonly used detection methods like real-time RT-PCR. Also, only a limited number of studies that have explored the use of NGS for SARS-CoV-2 detection for the purpose of diagnostic testing are available [861,863–865]. For example, using a laboratory-developed protocol for NGS, Bhoyar et al. compared NGS and real-time RT-PCR on 752 clinical specimens processed in duplicate on a single flow cell [865]. They demonstrated high concordance between the methods and a diagnostic increase in positivity of 5.7% with NGS (with detection of 6 cases testing negative by PCR, and 21 cases where PCR results were inconclusive). This study demonstrates the feasibility of processing 1536 specimens in a total of 17 h (11 h for sequencing and 6 h for analysis) [865]. In another study, a low-cost NGS sequencing approach was shown to achieve high sensitivity for detection of SARS-CoV-2 (84 genome units/ml), which is equal to or greater than some RT-PCR methods; however, this study only tested 10 specimens (five positives and five negatives) [863]. It is unclear whether high sensitivity would still occur if throughput would be increased to their proposed workflow of 192 specimens in 8 h. Bloom et al. showed 100% concordance between NGS and RT-PCR with a limited number of specimens (31 positives and 33 negative NP swabs) [864]. These authors propose NGS as a tool for population-based surveillance rather than individualized testing for medical decisions. While postulated to be able to achieve screening of thousands of samples on high-throughput NGS platforms, no data was provided to support the feasibility of this approach nor the impact of such a high level of specimen pooling. Overall, some data support the potential of NGS as a diagnostic tool for SARS-CoV-2, yet further analyses are required to understand its benefits and limitations.

Despite the potential for NGS as a diagnostic tool, the limitations of SARS-CoV-2 genome sequencing using NGS technologies should also be recognized. For example, NGS technologies are challenged with specimens with low viral load [861,866], as insufficient data or poor quality results are obtained for subsequent analyses. Efforts to increase sensitivity and quality issues are underway using target enrichment processes with NGS techniques such as multiplex PCR amplicon-based sequencing, hybrid capture-based sequencing, and ultra-high-throughput meta-transcriptomic sequencing [861,867]. Another limitation of NGS technologies is cost, which may be prohibitive for many diagnostic laboratories. Furthermore, the complexity of NGS workflow and requirement for sophisticated instrumentation and bioinformatics expertise may pose significant barriers to NGS access in many laboratories. If resources are limited, specimens could be prioritized and sent to referral laboratories to help inform public health responses and global surveillance initiatives.

While not routinely used for SARS-CoV-2 diagnostic testing in clinical laboratories, genome sequencing of SARS-CoV-2 positive specimens with NGS technologies has paved the way for numerous applications, including investigations on disease pathogenesis, epidemiology, virus phylogenetics, monitoring SARS-CoV-2 evolution, and the impact of viral evolution on diagnostic testing or interventions like therapeutics and vaccines [868–872]. For example, Meredith et al. used SARS-CoV-2 nanopore sequencing on PCR-positive specimens combined with epidemiological data to help identify nosocomial transmission events and inform infection control interventions [866]. They demonstrated the feasibility of rapid NGS in a healthcare setting by providing sample-to-sequence information in less than 24 h. From the discovery of SARS-CoV-2, NGS has been used to understand its origins and transmission. Initial phylogenetic analysis of SARS-CoV-2 genomes showed it was closely similar to human SARS-CoV, potentially used the same cell entry receptor (i.e., ACE2), and helped postulated the probable zoonotic origins of the virus (i.e., bats) [445,873–877]. Next, the first SARS-CoV-2 genomes were made available less than a month from the first recognition of disease reported from Wuhan, Hubei, China [445]. With rapid access to SARS-CoV-2 genome data, molecular methods like real-time RT-PCR were rapidly developed at the early stages of the COVID-19 pandemic and became the method of choice for SARS-CoV-2 detection worldwide. Through remarkable efforts from public health and researchers, SARS-CoV-2 genome sequences have been made available in public data repositories such as the Global Initiative on Sharing All Influenza Data (GISAID) (<http://www.gisaid.org/>) and

the National Center for Biotechnology Information (NCBI) GenBank database (<https://www.ncbi.nlm.nih.gov/>). This data helps provide a snapshot of global diversity and data that could be used for epidemiological investigations. In the initial stages of the pandemic, the high diversity of SARS-CoV-2 genomes was attributable to multiple independent importations of SARS-CoV-2 by travel overseas in countries of initial virus activity, and transmission routes could be investigated [866,878–882]. Following global spread and closure of international borders, sequence diversity was more limited, and sequencing of SARS-CoV-2 genomes was used in outbreak investigations and became particularly useful for cases in areas of unknown community transmission [866,878–882]. However, with natural evolution in the human host, or selective pressures from the recent introduction of SARS-CoV-2 vaccines or exploratory therapeutics (i.e., antivirals or convalescent sera), there is the increasing chances for the SARS-CoV-2 to further diversify and acquire mutations [883–887].

The genetic diversity of SARS-CoV-2 stems from naturally occurring mutations in its genome, which is common at higher frequencies in RNA viruses [888]. Some mutations might have no impact on SARS-CoV-2 protein sequences (i.e., synonymous substitutions), but these may affect the performance of diagnostic tests using NAATs if they occur in the target region for the assays [479,889–891]. From the various SARS-CoV-2 genomes that have been sequenced since its discovery, the M, E, and RdRp genes were shown to be fairly conserved compared to a higher divergence observed in some regions of the S genes [868,869,887,892–898]. Mutations in the genome of SARS-CoV-2 could also occur from point mutations, insertions, deletions, or recombination events that could affect protein sequence, structure, and function (i.e., non-synonymous substitutions). Both synonymous and non-synonymous mutations can be useful for epidemiological investigations, but it should be noted that non-synonymous mutations are of significant interest as they could have impacts in terms of disease transmissibility and severity or could help the virus escape from therapeutic (i.e., convalescent sera or antivirals) or preventative interventions (i.e., vaccines). Mutations in S are of particular concern as S glycoprotein epitopes are major targets for current and exploratory vaccines [470,899,900]. Multiple SARS-CoV-2 strain variants are circulating globally, but few are variants of concern (VOC) [887]. In the UK, a novel variant of SARS-CoV-2 [0B/501Y.V1, VOC 202012/01, or B.1.1.7 lineage] emerged with an unusually large number of mutations. This VOC has since been reported in several countries, including Canada and the US. It contains a number of mutations in the S gene (e.g., N501Y, 69/70

deletion, and P681H), and while these mutations have yet to show any impact on disease severity or vaccine effectiveness, some preliminary epidemiologic data suggest this variant is associated with increased transmissibility [887]. In Brazil, a SARS-CoV-2 variant from lineage B.1.1.248 was reported with an E484K S gene mutation associated with reduced neutralization capability by convalescent plasma [901–903]. Such mutations are concerning due to potential failures of therapeutic options or prevention strategies like vaccines that target similar viral protein epitopes. Furthermore, a novel VOC from lineage B.1.1.248 was identified with 12 mutations in the S gene, including both E484K and N501Y, suggesting the potential for increased transmissibility and immune escape [886,887]. Ongoing surveillance should be encouraged to identify novel SARS-CoV-2 variants and characterize the potential impacts of VOCs.

Overall, sequence-based surveillance of SARS-CoV-2 is important to ensure diagnostic tests accurately identify the virus and ensure there are no changes with novel variants in disease spread, disease severity, or effectiveness of vaccine or antivirals. Whether NGS will eventually become a common diagnostic tool remains to be determined.

A.7.5. SARS-CoV-2 Antigen Detection

Antigen detection methods, like NAATs, are used to detect active replicating viruses in the early stages of SARS-CoV-2 infection. Unlike NAATs that rely on the detection of viral RNA, antigen detection is based on the identification of SARS-CoV-2 proteins. The two main antigens in SARS-CoV-2 detection assays are S and N proteins. Antigen detection assays use similar technologies as serological methods. Like serology, high-throughput antigen-based testing can be performed on semi-automated or automated instruments using enzyme immunoassays (EIAs) technologies like enzyme-linked immunosorbent assays (ELISA) or chemiluminescence immunoassays (CLIAs). However, most antigen detections to date have aimed for methods allowing easy-to-use and rapid testing using portable devices, like LFIAs (also termed lateral flow immunochromatographic assays or lateral flow assays [LFAs]). The mechanism for LFIAs is shown in Figure A.10A, and other technologies (e.g., ELISA and CLIA) will be covered in the subsequent serology sections. This section summarizes the current knowledge on the clinical performance of antigen-based detection assays and their potential applications to support COVID-19 responses.

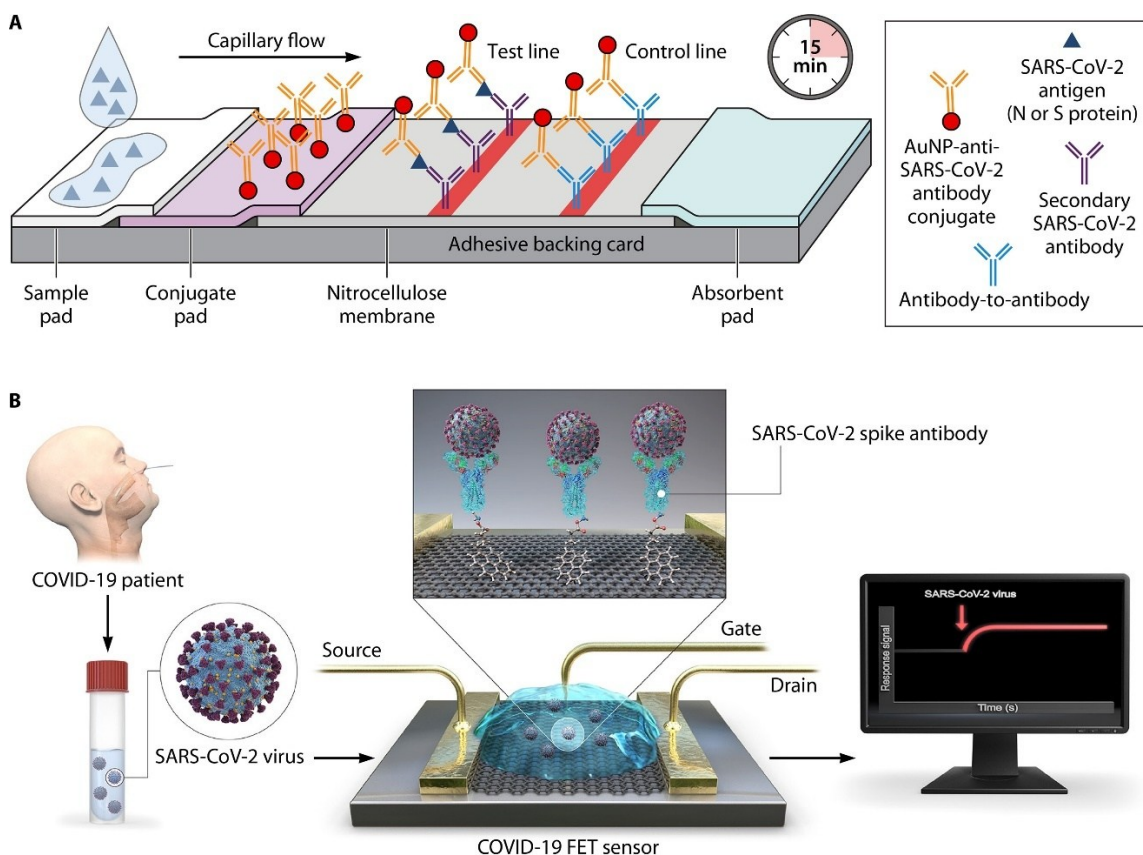


Figure A.10. Antigen testing for the detection of SARS-CoV-2. (A) Principle of a lateral flow immunochromatographic assay (LFIA). The design of LFIA for antigen detection is a qualitative immunologic reaction confined to a small portable device (e.g., cassette or dipstick) that can be performed in the laboratory or POC setting. Briefly, antigen in specimens (e.g., nasal, nasopharyngeal swabs, saliva) are placed in a well with a sample pad, and the fluid containing the antigen flows through the device via capillary action. The bottom of the well where the specimen is inoculated contains a sample pad, which is in contact with the conjugate pad used as a support for SARS-CoV-2-specific monoclonal antibodies (mAbs) that are labeled with colloidal gold nanoparticles (AuNPs) or other tags. If present, SARS-CoV-2 antigen (usually S or N protein) forms a complex with the mAbs bound to the AuNP, and the entire complex migrates via capillary action until it is captured by other SARS-CoV-2 antigen-specific mAbs immobilized on the nitrocellulose membrane (i.e., the test line). As antigen-antibody complexes are trapped at this location, they form a line that can be visualized by the naked eye or with the aid of a detector. Also, mAbs-AuNP particles, whether conjugated with antigens or not, continue to migrate until captured by an anti-mAb antibody at the control line. This ensures proper liquid flow through the device and the test validity. (B) Point-of-care detection of SARS-CoV-2 antigen using a FET-based sensor. Upon binding of spike (S) proteins to the anti-S monoclonal antibodies immobilized on the graphene sheet via the PBASE linker, a change in the voltage-ampere diagram reveals the presence of the virus. Panel B of this figure is adapted with permission from Ref. [904]; copyright 2020 American Chemical Society.

Hundreds of companies have been developing antigen rapid diagnostic tests (Ag-RDTs) for SARS-CoV-2 detection [675]. Ag-RDTs (e.g., LFIAs) are often promoted as POC devices for rapid testing and immediate management of patients in settings such as physicians' offices or clinics; however, these tests are not always performed in these settings. RDT is a more appropriate term if performed in a laboratory setting. At the time of this review, few antigen-based detection assays have received EUA approval in the US and Canada, but many more applications have been submitted as laboratory and/or POC tests [464,465,905,906]. Examples of SARS-CoV-2 Ag-RDTs that have received EUA for testing in laboratory or POC settings in the US or Canada include technologies relying on a colorimetric LFIA with a visual readout, an instrument-based antigen detection using a fluorescence-based LFIA, a microfluidic immunofluorescence assay, and a chromatographic digital immunoassay (see Table A.2).

Table A.2. Example of approved antigen tests for emergency use by US FDA for detection of SARS-CoV-2. The full list is available in Ref. [464].

Device/assay (manufacturer)	Method	Target antigen protein	Specimen	Authorized detection window	Authorized setting(s)	Time	LoD ^a	Ref.
BinaxNOW COVID-19 Ag Card Home Test ^b (Abbott Diagnostics, USA)	LFIA, visual readout	N	NS, ANS	7 d.p.o	Home, H, M, W	15 min	140.6 TCID ₅₀ /mL	[907]
CareStart COVID-19 Antigen test (Access Bio, USA)	LFIA, visual readout	N	NPS	5 d.p.o	H, M, W	10 min	800 TCID ₅₀ /mL	[908]
QuickVue SARS Antigen Test ^b (Quidel Corporation, USA)	LFIA, visual readout	N	ANS	5 d.p.o	H, M, W	10 min	7570 TCID ₅₀ /mL	[909]
Veritor System For Rapid Detection of SARS-CoV-2 (Becton, Dickinson and Company [BD], USA)	LFIA, instrument readout	N	NS	5 d.p.o	H, M, W	15 min	140 TCID ₅₀ /mL	[910]
Sofia 2 Flu + SARS Antigen FIA ^b (Quidel Corporation, USA)	LFIA, IFA, instrument readout	N	NS, NPS	5 d.p.o	H, M, W	15 min	91.7 TCID ₅₀ /mL	[911]
LumiraDx SARS-CoV-2 Ag Test ^b (LumiraDx Ltd., UK)	Microfluidics, IFA, instrument readout	N	NS	12 d.p.o	H, M, W	12 min	32 TCID ₅₀ /mL	[912]

Clip COVID Rapid Antigen Test ^b (Luminostics Inc., USA)	LFIA, ILA, instrument readout	N	ANS	5 d.p.o	H, M, W	30 min	88 TCID ₅₀ /mL	[913]
Ellume COVID-19 Home Test ^b (Ellume limited, Australia)	LFIA, IFA, smartphone readout	N	MTS	N/A	Home, H, M, W	15 min	6309 TCID ₅₀ /mL	[914]

^a LoD of each assay is the lowest LoD reported on the Instruction for Use file of that assay, regardless of the specimen types.

^b These assays do not differentiate between SARS-CoV-2 and SARS-CoV. Abbreviations: LoD: limit of detection; LFIA: lateral flow immunoassay; IFA: immunofluorescence assay; ILA: immunoluminescent assay; d.p.o: days post onset of symptoms; NS: nasal swab; ANS: anterior nasal (nares) swab; NPS: nasopharyngeal swab; MTS: mid-turbinate nasal swab; H: laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests; M: laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform moderate complexity tests; W: patient care settings operating under a CLIA Certificate of Waiver; TCID₅₀/mL: median tissue culture infectious dose/ml.

While EUA was granted for some Ag-RDTs, at the time of this review, limited data have been published on their clinical performance characteristics. The performance of antigen and molecular-based POC tests for detecting SARS-CoV-2 was the subject of a recent Cochrane review [640], but limited data was available to be summarized [817,915–920]. Briefly, the review summarized data from five studies, representing 943 samples. The average sensitivity of Ag-RDT was found to be 56.2% (95% CI: 29.5% to 79.8%), and the average specificity was 99.5% (95% CI: 98.1% to 99.9%). In comparison, NAAT-based RDT had an average sensitivity of 95.2% (95% CI: 86.7% to 98.3%) and specificity of 98.9% (95% CI: 97.3% to 99.5%). High specificity was observed for both molecular and antigen-based detection methods; however, poor sensitivity was noted for Ag-RDTs, but a high level of heterogeneity was noted between studies. The variability could be explained by factors including the methods used for SARS-CoV-2 detection, the comparator method during the evaluation, the patient populations assessed, SARS-CoV-2 prevalence, the timing of specimen collection, specimen types used, and the antigen stability during analyses.

Consistent with the low clinical sensitivity of Ag-RDTs, the analytical sensitivity was defined in some studies, which described LoDs of Ag-RDTs of approximately 1000-fold less than culture-based detection of SARS-CoV-2, and 10,000-fold less than NAATs [920]. Mertens et al. noted that when specimens with high viral loads were evaluated (defined as specimens with real-time RT-PCR Ct values <25), the sensitivity of Ag-RDT increased to 74.8% compared to an overall sensitivity of 57.6% when all specimens were considered [916]. Scohy et al. observed an overall

sensitivity of Ag-RDT of 30.2% [918]. When specimens were further characterized by real-time RT-PCR Ct values, the impact of Ag-RDT poor sensitivity was clear. With specimens with Ct values <25 (1.8×10^5 copies/ml), Ct values <30 (9.4×10^3 copies/ml), or Ct values <35 (4.9×10^2 copies/ml), the sensitivity of Ag-RDT was showed to be 100%, 70.6%, and 46.9%, respectively. Linares et al. showed that an Ag-RDT achieved 86.5% sensitivity (95% CI: 75.0% to 97.0%) if symptomatic patients with high or moderate viral loads were tested within seven days of symptom onset [906]. As seen with all other SARS-CoV-2 tests, the timing of the testing likely plays an important role in assay performance. Recognizing the relatively poor sensitivity compared to NAAT but the potential role for Ag-RDTs, the WHO interim guidance described situations where such a test could be considered [675]. The WHO supports the use of Ag-RDTs for conditions such as: 1) testing in areas where NAATs are not available (e.g., remote areas) or when the result turnaround times using NAATs are high; 2) in outbreak investigations, but the frequency of testing in these settings remains unclear; 3) in areas where the prevalence is high; and 4) for testing of asymptomatic contacts of positives cases.

Given the poor sensitivity of Ag-RDTs for detecting SARS-CoV-2, research is being performed to improve sensitivity using novel sensors and biosensors technologies [921]. So far, a few studies have leveraged the power of electronic and electrochemical methods to create fast and sensitive diagnostic devices for SARS-CoV-2 detection. For example, Seo et al. described a field-effect transistor (FET)-based biosensing device that detected SARS-CoV-2 at concentrations 2.42×10^2 copies/ml in clinical specimens, without sample pretreatment in approximately 3 min [904]. The FET sensor used SARS-CoV-2 monoclonal antibodies to spike proteins, which were coupled to graphene sheets through a 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE) linker (Figure A.10B). With the high conductivity and other properties of graphene, real-time dose-dependent detection of SARS-CoV-2 antigen can be achieved down to 1 fg/ml. While further optimization is needed to commercialize this technology, it has the potential to help increase the sensitivity of antigen detection. In another study, Mahari et al. developed an electrochemical device using a screen-printing technique. They immobilized antibodies against SARS-CoV-2 spike proteins on screen-printed carbon electrodes (SPCE) to detect the virus and developed an integrated in-house built portable device which could measure the changes in electrical conductivity upon the reaction between antigens and antibodies. The device was able to detect spiked SARS-CoV-2 antigens in saliva with an LoD of 90 fM within 10-30 s [922].

While FET and electrochemical sensors show promise to increase the sensitivity of antigen detection, the desire to achieve the highest sensitivity possible with RDTs has been the subject of recent debate [739,750,752,923,924]. Some authors have proposed that with repeat testing, the chance of identifying SARS-CoV-2 infected individuals increases [739,923–926]. This may hold true on a population level where testing may otherwise not have been performed (e.g., asymptomatic testing), and there would be fewer consequences if detection of the virus is missed. However, repeat testing and the possibility of false-negative results in an acute care setting poses many more challenges, particularly when decisions have to be made at the time of or shortly after the time of presentation. For existing or novel technologies, studies with more robust data are needed to better define the utility of Ag-RDTs in various settings. These should include prospective analyses that consider factors such as the timing of collection, time for symptom onset, symptomatic and asymptomatic patient populations, disease prevalence, stability, and following manufacturer recommendations for stability and transport (i.e., direct testing of specimens, not using specimens in transport media).

A.7.6. Serological Immunologic Methods for SARS-CoV-2 Detection

Serological assays detect antibodies specific to SARS-CoV-2 in blood sources like serum, plasma, or whole blood (including fingerstick and heel pricks sometimes used in POC testing), and the possibility of antibody detection in other body fluids such as saliva are being explored [927–929]. Given the typical time required to detect immune responses to SARS-CoV-2 is around one to two weeks, serological tests have limited utility for SARS-CoV-2 diagnostics in the acute stages of the illness, but could have value once immune responses have had time to occur [678,930–934]. Many serologic assays for detecting SARS-CoV-2 antibodies have been developed and commercialized, and a list of authorized medical devices related to COVID-19 in the USA and Canada are regularly updated online [464,465,928,935]. Despite access to serological assays, their interpretation is further complicated in the post-vaccine era for SARS-CoV-2, and the relevance of antibody detection must be considered in the context it is used and its limitations. Following a section describing serological methods, a discussion on the relevance and possible applications is provided.

Serological assays often use recombinant antigens to capture SARS-CoV-2-specific antibodies, with the N protein and the receptor-binding domain (RBD) of the S1 subunit of the S glycoprotein

being the most commonly used antigens. Serological methods can target one or more immunoglobulin isotype (i.e., IgA, IgM, or IgG) or total antibody, and theoretically, any of these isotypes could provide neutralizing activity against SARS-CoV-2 [936]. IgM and IgG are commonly used immunoglobulins in serologic assays for SARS-CoV-2, while IgA detection is less commonly used [935]. The value of IgA and IgM has shown some merits for the detection of early immune responses to SARS-CoV-2, but there has been some concern regarding the rapid decay of IgA and IgM in serum and saliva compared to IgG [679,937,938]. While more data is needed to fully understand serologic responses in different settings or patient populations, it is clear that IgA, IgM, and IgG all have the potential to generate neutralizing responses to SARS-CoV-2 [679,937,938].

The most common methods used are ELISA in 96-well plate format and CLIA for automated higher throughput instruments [486,935]. LFIAs are also available to provide rapid results. It is unclear whether detectable antibodies in commercial serology methods are associated with immune protection, as large population-level studies and quantitative immunoglobulin analyses are required to assess if detection of SARS-CoV-2 specific immunoglobulins correlates with immune protection. However, studies are underway to investigate the extent of neutralizing antibodies produced over time [679,937,938].

The performance of immunologic assays remains unclear as there is a lack of a gold standard for method comparison. Methods are often compared amongst themselves from a consensus standard, but true performance and relevance of antibody responses, and the optimal testing algorithm, requires further investigation [939]. For now, the applications of serology include a role as an adjunct to molecular testing to support the identification of SARS-CoV-2 in a patient suspected to have COVID-19 (i.e., persistent or progressing symptoms), but the absence of testing or repeated negative (or indeterminate) results obtained by NAATs. It should be noted that some patients may be antibody negative at the time of testing, but that does not preclude memory B cell activity or functional T cell responses with subsequent exposures to SARS-CoV-2. Some studies argue that immunologic methods could also be used in seroprevalence studies to aid in ongoing outbreak investigations (to identify cases beyond the detectable window of NAAT), to determine past exposures of populations to SARS-CoV-2 (e.g., healthcare workers or general population), or to assess attack rates in defined populations or geographical areas. However, correlations to neutralizing antibody titers would be required to fully understand serologic data, along with the

impact of prior infection with SARS-CoV-2 and other human coronaviruses, as well as the impact of vaccines. High sensitivity and specificity are desired, but with these many confounding factors, result interpretation is complicated [486,940]. The following sections will describe common methods used for SARS-CoV-2 serology, including ELISA, CLIA, and LFIAs, along with a discussion of the potential value and limitations of immunologic methods.

Enzyme-linked immunosorbent assay (ELISA)

While many permutations exist, ELISAs can be categorized into four main types: sandwich, direct, indirect, and competitive [941]. For the detection of SARS-CoV-2, indirect [647,942–945], modified indirect [647], and double-antigen sandwich ELISAs [643] are the most commonly used methods (Figure A.11A).

In the indirect ELISA method, SARS-CoV-2 recombinant antigens are coated on a solid support such as wells of a 96-well microplate [647,942–945]. Patient serum or plasma is added, and if present, SARS-CoV-2-specific antibodies will bind to the immobilized antigens. Following washing steps, a secondary antibody is then added that is specific to the isotype targeted (i.e., anti-human IgM, anti-human IgG, or anti-human IgA). This secondary antibody (or conjugate) is linked to a fluorophore that can generate fluorescence or conjugated to an enzyme for the colorimetric or chemiluminescent signal generation following the addition of a substrate. Usually, colorimetric methods are used in which optical density (OD) (i.e., absorbance) of the solution can be measured using a spectrophotometer and correlated to the concentration of the target antibodies. Other permutations of ELISA are also possible, but while these techniques all share similarities in terms of their signal generation mechanisms, each can vary in performance. Typically, the time to result of ELISA is 1-5 h [480], and more recently, POC applications of ELISA for the detection of anti-SARS-CoV-2 antibodies have been explored using microfluidics technology [946]. The performance characteristics and limitations of SARS-CoV-2 serology will be discussed in a later section.

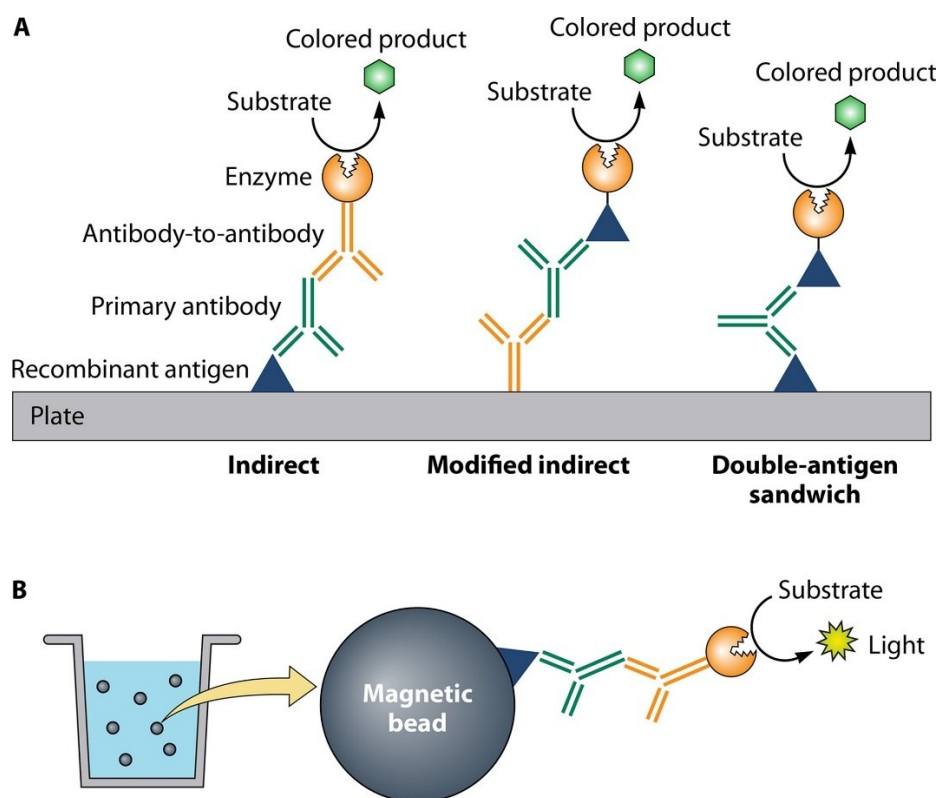


Figure A.11. Common serologic immunoassays for the detection of SARS-CoV-2-specific antibodies. (A) Common designs of ELISA methods including indirect, modified indirect, and double-antigen sandwich assays. (B) Magnetic bead-based CLIA. This figure was inspired by the work in Refs. [941,947].

Chemiluminescence immunoassay (CLIA)

CLIA is an immunoassay that is commonly used in highly automated serological instruments and is known for its high sensitivity compared to other serological assays [948]. Similar to ELISA, variations of CLIA have been applied for the detection of SARS-CoV-2 antibodies, such as indirect [949,950] and sandwich methods [676].

CLIA has two main differences with colorimetric ELISAs. First, the final reaction produces light, which is detected by a luminometer in relative light unit (RLU) rather than OD, which is an absolute value. To produce light, the conjugate uses an enzyme-substrate reaction such as AP and Lumigen APS-5 substrate. Second, the antigenic materials are often coated on magnetic microspheres in a liquid-phase reaction rather than a multi-well plate, which allows for easy separation of bound and unbound molecules by a magnet and faster reactions due to providing a large surface area and allowing the reactions to occur entirely in suspension [947,951,952] (Figure

A.11B). Some CLIA assays have been commercialized and used on high throughput automatic analyzers. For example, the LIAISON assay (DiaSorin, Italy) uses magnetic beads coated with SARS-CoV-2 S1 and S2 antigens to detect IgG antibodies. This assay is capable of providing up to 170 results/h in a fully automated manner.

Fluorescent microparticle immunoassays (FMI)

Recently, Norman et al. described an FMI-based single-molecule array assay that consisted of a mixture of four different color-coated beads coupled to S, S1, S2, and N proteins and used for the detection and differentiation of SARS-CoV-2 IgM, IgG, and IgA antibodies [953]. In this technique, an excess amount of beads is utilized in comparison to the number of antibody molecules in the samples in a way that either zero or one antibody molecule binds to each bead. After the reactions occur between the coated beads and antibodies and other reagents, the beads are loaded into an array of 216,000 femtoliter-sized wells for imaging, which allows for the detection of antibodies with single-molecule resolution. When tested on a set of 81 plasma samples, the technique showed 86% sensitivity and 100% specificity for the samples during the first week after symptom onset and 100% sensitivity and specificity for the samples taken after the first week post symptom onset [953].

Similarly, to facilitate the simultaneous detection of the IgG, IgM, and IgA isotypes of SARS-CoV-2-specific immunoglobulins, a multiplex SARS-CoV-2 antibody immunoassay based on Luminex technology was developed for detection on the Bioplex (BioRad Laboratories) automated flow cytometer [929]. This multiplex immunoassay was used to demonstrate SARS-CoV-2 antigen-specific antibody responses in 33 saliva and 206 serum samples from participants with RT-PCR-confirmed SARS-CoV-2 infection. Overall, this technology could detect prior SARS-CoV-2 infection with high sensitivity and specificity at ≥ 10 days post-symptom onset, but the performance varied with targets [929]. FMI technology allows for robust antibody profiles to be simultaneously detected in a single reaction. A commercial assay for the Bioplex 2200 has recently received FDA approval, but no data on this assay has been published to date.

Lateral flow immunoassays (LFIA)

As described in the antigen detection section, LFIAs can be used as an RDT or POC testing to detect antibodies against SARS-CoV-2 in blood or serum samples. The most frequently used antigens are recombinant S or N proteins to capture total antibody or detect and sometimes

differentiate IgM and IgG antibodies [935]. The LFIA devices are low-cost, portable, rapid (~15 min), and require only a few microliters of samples, making them suitable for fingerstick tests [248,954,955]. Figure A.12A illustrates an example of an LFIA used for the simultaneous colorimetric detection of SARS-CoV-2-specific IgM and IgG. Many different types of LFIAs have been developed to detect SARS-CoV-2, with variations in antigens used for antibody capture, immunoglobulin isotypes detected, and overall performance [935,956].

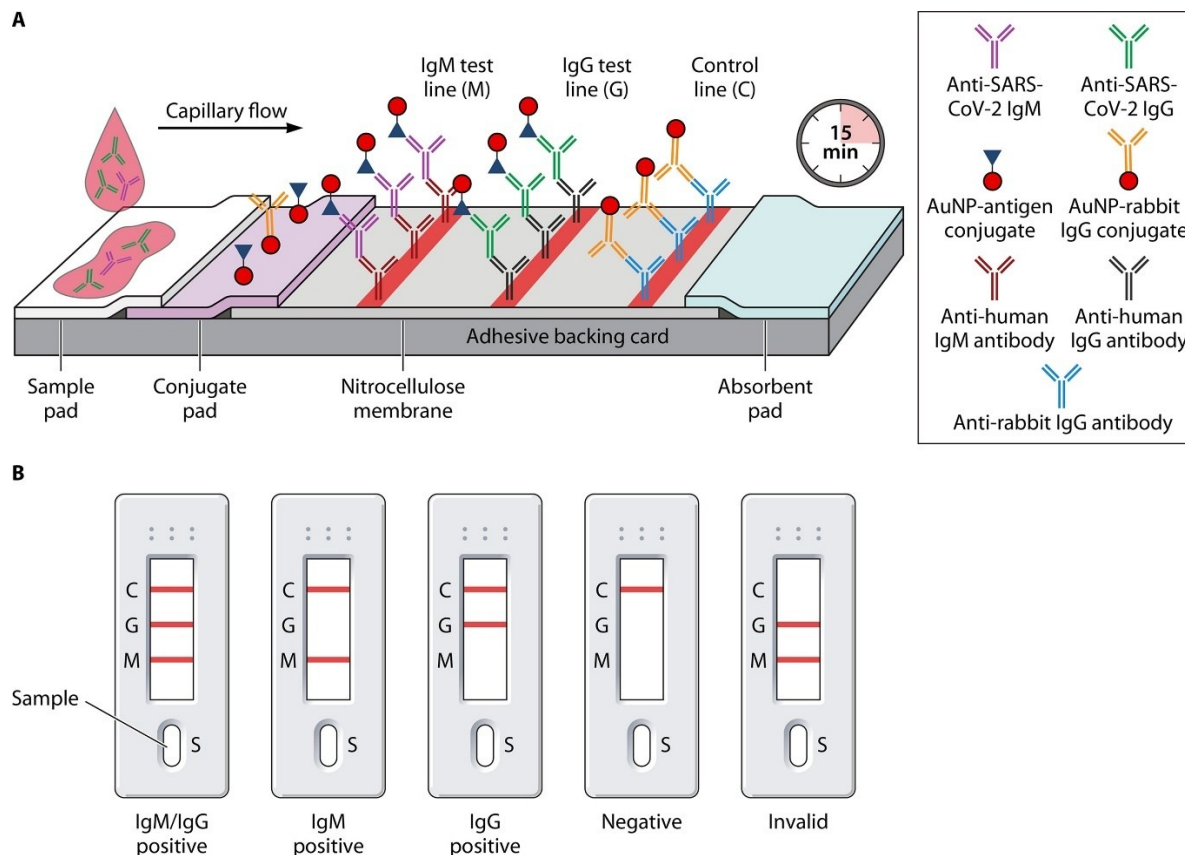


Figure A.12. Serologic lateral flow immunoassays for the detection of SARS-CoV-2 specific antibodies. (A) Schematic of a lateral flow immunoassay device for the simultaneous detection of IgM and IgG antibodies. Upon the addition of the sample, the liquid moves towards the pre-immobilized reagents through capillary action and reacts with them. If IgM/IgG antibodies are present in the sample, they bind and form a complex with the recombinant SARS-CoV-2 antigen conjugated with colloidal gold nanoparticles (AuNPs). The complex is then captured in one of the test lines by anti-human IgM/IgG antibodies, resulting in a pink color due to the accumulation of the AuNPs. There is also AuNP-rabbit IgG conjugate that will be captured in the control line, indicating a proper liquid flow through the device. The results will be observable in ~15 min. (B) Variations of results of the lateral flow assay device in a cassette format. This figure was inspired by the work in Ref. [954].

Performance of serological immunologic methods for SARS-CoV-2

In a systematic review and meta-analysis, the results of 40 studies up to April 30, 2020, were summarized, and differences in sensitivity between serological methods used for SARS-CoV-2 antibody detection, as well as antibody class were presented [486]. Data for IgA were scarce, and no conclusions could be drawn. For IgM detection, the pooled sensitivity (with 95% CI) for LFIA was lowest at 61.8% (50.8% to 71.8%), whereas ELISA was higher at 81.7% (71.8% to 88.5%), and CLIA was highest at 84.3% (70.7% to 93.0%). Similarly, the pooled sensitivity for IgG detection was 64.9% (53.8% to 75.4%), 80.6% (71.9% to 87.9%), and 93.5% (84.9% to 98.1%), for LFIA, ELISA, and CLIA, respectively. The pooled specificity was fairly similar between LFIA, ELISA, and CLIA methods, at 96.6% (93.8% to 98.4%), 99.7% (99.0% to 100.0%) and 96.6% (84.7% to 99.5%) for IgM detection, and 97.6% (96.2% to 98.8%), 98.9% (96.7% to 99.8%) and 97.8% (62.9% to 99.9%) for IgG detection, respectively. Other meta-analyses and systematic reviews yielded consistent conclusions for sensitivity and specificity for IgM, IgG, or total antibodies [928,957,958].

One important issue affecting the diagnostic performance of serological assays is the time post-symptom onset that the samples are taken for serological analyses, as the antibody profiles change over the course of the disease. In a meta-analysis and systematic review [486], the pooled sensitivity for IgM detection was low in the first week post-symptom onset at 25.3% (16.3% to 31.1%) for LFIA, but increased to 51.8% (30.3% to 69.6%) after two weeks, and 69.9% (58.4% to 79.9%) after three weeks. ELISA detection of IgM showed similar values for week one, two and three post-symptom onset at 26.7% (15.6% to 35.6%), 57.6% (15.9% to 88.2%), and 78.4% (54.1% to 91.9%). CLIA followed the same trend but with higher values at 50.3% (10.9% to 81.2%), 74.3% (16.1% to 99.4%), and 90.6% (51.8% to 99.4%), respectively. Next, the pooled sensitivity for IgG at weeks one, two and three post-symptom onset were 13.4% (4.7 to 29.6%), 50.1% (24.8% to 77.0%), and 79.7% (71.4% to 86.9%) for LFIA, 23.7% (12.7% to 38.1%), 65.3% (46.3% to 79.4%), and 82.1% (76.4% to 89.0%) for ELISA, and 53.2% (28.7% to 67.6%), 85.4% (48.1% to 98.1%), and 98.9% (86.9% to 100.0%) for CLIA, respectively [486].

Overall, the performance of serology assays varied between methods and was dependent on the timing of collection, and the applicability of these methods is limited to date. It is important to recognize that other factors such as antigens and the inherent characteristics of a test or instrument

used for antibody detection can also impact the performance of immunologic assays. Also, data summaries in recent meta-analyses and systematic reviews so far have shown high levels of heterogeneity and risk of bias and applicability between studies [928,957,958]. Thus, careful attention should be paid to the study design, their limitations, and whether the conclusions derived from these evaluations are justified. It remains unclear to what extent, the duration, and whether neutralizing antibodies will provide protection against subsequent SARS-CoV-2 infections, and what is the impact of prior infection with other human coronaviruses [930,931]. It is possible that prior infection with other coronaviruses or other conditions (e.g., pregnancy or chronic illnesses) may cross-react with SARS-CoV-2 serology assays, and robust evaluation of methodologies should include specificity analyses against related viruses and possible interfering substances [959,960]. The recent introduction of vaccine programs will further complicate the use of immunologic methods as diagnostic tests but may expand their use to determine whether individuals are immune once exposed to the SARS-CoV-2 through natural infection or following immunization. This remains an active area of research that requires comparisons to neutralizing antibody titers and cell-mediated immunity.

Neutralization assays and research areas of interests for serology

The efforts of the scientific community to help understand the host responses to SARS-CoV-2 is fundamental in understanding COVID-19 pathophysiology and interpretation of SARS-CoV-2 laboratory diagnostics [961–964]. There has been an extraordinary effort to rapidly develop serological methods in various formulations, and while some were licensed under EUA in the US and Canada, their use to make recommendations on a patient's current or future susceptibility to SARS-CoV-2 infection remains unclear [464,465]. Given the timing of the immune responses to SARS-CoV-2 infection, it is clear that serology would not be useful for diagnosis in acute phases of illness; antibodies would not be present in substantial quantities in this timeframe to allow public health interventions [570]. In fact, positive serology does not preclude viral shedding [645,965], and the absence of antibodies does not preclude the possibility of immunity. To date, recommendations for the use of serology include clinical research (e.g., seroprevalence studies) to help inform public health strategies or policies on a population-level [539,570,678]. For example, seroepidemiologic studies can support ongoing outbreak investigations or retrospectively assess infection rates in certain populations [570,966]. On an individual level, serology may have considerations for individual patients with the multisystem inflammatory syndrome or in situations

where suspect cases are repeatedly tested negative by NAATs, but symptoms persist (and the timing fits into a period of at least two weeks post-symptom onset) [539,570,678]. Future research is required to further understand the full value of SARS-CoV-2 serology, especially in the context of immunization with COVID-19 vaccines.

Key areas of research interest should include the relevance and duration of antibody-based immune responses to better understand whether antibody responses in commercial or LDTs correlate to the protective immune responses against subsequent SARS-CoV-2 infection, as well as the magnitude and duration of these responses. Understanding this correlation can be an important function to assess immunity on an individual or population level, or even to qualify blood donors desiring to become convalescent plasma donors that could subsequently be used in clinical trials [967,968]. This would require a quantitative analysis of seroconversion (detection of measurable antibodies following infection), which would not be possible with qualitative assays such as LFIAs but may be possible using semi-quantitative ELISAs (where antibody detection relies on a signal value that is normalized to a calibrator to establish a signal to cutoff [S/CO] ratio), or ideally using quantitative ELISAs. Quantitative detection of SARS-CoV-2 antibodies over time could be used to demonstrate seroconversion, and quantitative ELISAs have become commercially available (e.g., the liquid-based double antigen sandwich ELISA from Roche [Elecsys Anti-SARS-CoV-2 S]). Quantitative antibody analyses, paired with a fundamental understanding of how well serologic methods correlate to neutralizing antibodies (nAbs), may help establish a threshold where populations are protected from SARS-CoV-2 re-infection (known as a correlate of protection).

The establishment of a correlate of protection first requires an in-depth understanding of whether nAbs are produced, to what extent, whether they provide protection, and the duration of these responses, then analyzing these data in large population studies to correlate a level of nAbs that may confer protection against subsequent infection [969]. The quantification of nAbs in serum or plasma is performed using target- and immunoglobulin isotype-specific neutralization assays like plaque reduction neutralization assays (PRNT) [480,678,872,970]. Briefly, PRNT relies on the ability of nAbs to effectively prevent SARS-CoV-2 from infecting cultured cells that would otherwise cause cytopathic effects (i.e., cell death/lysis) [480,678,966,970–972].

Nonetheless, PRNT assays are known to be time consuming and laborious. To increase specimen throughput, microneutralization (MN) has been developed to detect nAbs to SARS-CoV-2 that rely on a similar principle as PRNT but are performed in 96-well formats and without the need for semi-solid overlays [966,972]. To further automate the detection of nAbs, a focus reduction neutralization test (FRNT) can be used where patient sera/plasma dilution can be assessed for their ability to reduce viral plaque formation, foci, or individually infected cells using immunocolorimetric staining and visualization using digital imaging [936]. Despite MN, FRNT assays are amenable to moderate throughput testing, and alternatives have been developed using fluorescence detection and luminescence to increase sensitivity. On the other hand, MN and FRNT share a similar challenge as PRNT: they all rely on SARS-CoV-2 propagation in cell culture, which requires laboratory facilities with higher levels of biological containment (i.e., Biosafety Level 3 [BSL3] in Canada or physical containment level 3 [PC3] by the WHO) [872]. These practices are not common outside reference laboratories [678]. To circumvent these biosafety requirements, pseudovirus-based neutralization assays (PBNAs) have been developed, where SARS-CoV-2 proteins are expressed on a surrogate virus backbone such as a lentivirus, retrovirus, or vesicular stomatitis virus (VSV) [971,973,974]. Other alternatives include the use of a protein-based surrogate neutralization ELISA (snELISA), which is based on the competition between ACE2 and nAbs for binding to recombinant antigens [975–981].

Few studies have looked at nAbs, and data on the extent and duration of nAbs over the course of SARS-CoV-2 infection is scarce [963,982–985]. Suthar et al. used FRNT to demonstrate that nAbs binding to SARS-CoV-2 S protein RBD domain is detectable within six days post-PCR confirmation [936]. While days post-onset would have better defined the antibody profiles over time, these authors showed that there is rapid isotype switching in nAbs from IgA and IgM to IgG1 and IgG3. In a limited dataset from Long et al., it was noted that there was a decline in nAbs over time in both asymptomatic and symptomatic individuals [676]. Similarly, Seow et al. performed a longitudinal study using sequential serum sampling and demonstrated that seroconversion was observed in 95% of cases, with nAbs present eight days post onset of symptoms [986]. They also noticed that the magnitude of nAbs responses depended on disease severity and waned over time, which was consistent with others [643,676,987–990]. In some cases, nAbs persisted up to 60 days, while others approached the limit of detection at this period [986]. In a more recent study by Isho et al., the duration of antibody responses was evaluated in both serum and saliva [679]. Antibodies

to S protein were detected in 90% of cases approximately 10 days post-symptom onset, and longitudinal studies showed the persistence of nAbs up to 105 days. Similarly, nAbs (defined using snELISA) reached a maximum from days 30 to 45. Interestingly, the sensitivity of anti-S or anti-RBD IgG in saliva was 89% and 85%, showing promise for non-invasive methods for seroepidemiologic studies and vaccine trials. The sensitivity of IgA, on the other hand, was 51% and 30%, and for IgM was 57% and 33%, respectively. It should be noted that the limited number of longitudinal studies seem to suggest that SARS-CoV-2-specific antibodies decrease over a two or three month period; however, the longevity of humoral immunity is not yet fully understood for SARS-CoV-2. It is possible that memory T cells may still be able to mount effective responses upon re-exposure to SARS-CoV-2 [964,991–993]. Research into the role of cell-mediated immune responses is needed to help unraveled knowledge gaps for SARS-CoV-2 immune responses [963,964].

Overall, whether derived from vaccines or natural infection, quantitative analyses of nAbs and establishing a correlate of protection would help inform public health and healthcare policies. Using neutralization assays with targets outside of those used in vaccines could still be of benefit to understand seroepidemiology to help better define populations at risk, which would be important for vaccine trials or evaluations of novel SARS-CoV-2-specific therapies like the use of hyper-IgG preparations or convalescent sera [975,978,994–996]. Like any other method for SARS-CoV-2 detection, serology research should consider antibody and cellular immune responses over time, have a population- and individual-level testing perspective, as well as account for factors like the presence or absence of symptoms, the timing from symptom onset (if present), the severity of illness, host factors (e.g., age and medical comorbidities), and any collection, transport, or processing steps that may affect the reliability of the analyte profiles over time. Additional research is still needed to meet these goals.

A.8. Conclusions and Outlook

Despite the recent availability of vaccines, with the ongoing and rapid spread of SARS-CoV-2 worldwide, laboratory testing remains the cornerstone of public health containment and mitigation strategies. Given the thoroughness of the data on methodologies in the body of the review, only key findings, some considerations for testing, and areas for improvement and successes are discussed below; however, a summary of testing modalities is presented in Figure A.13.

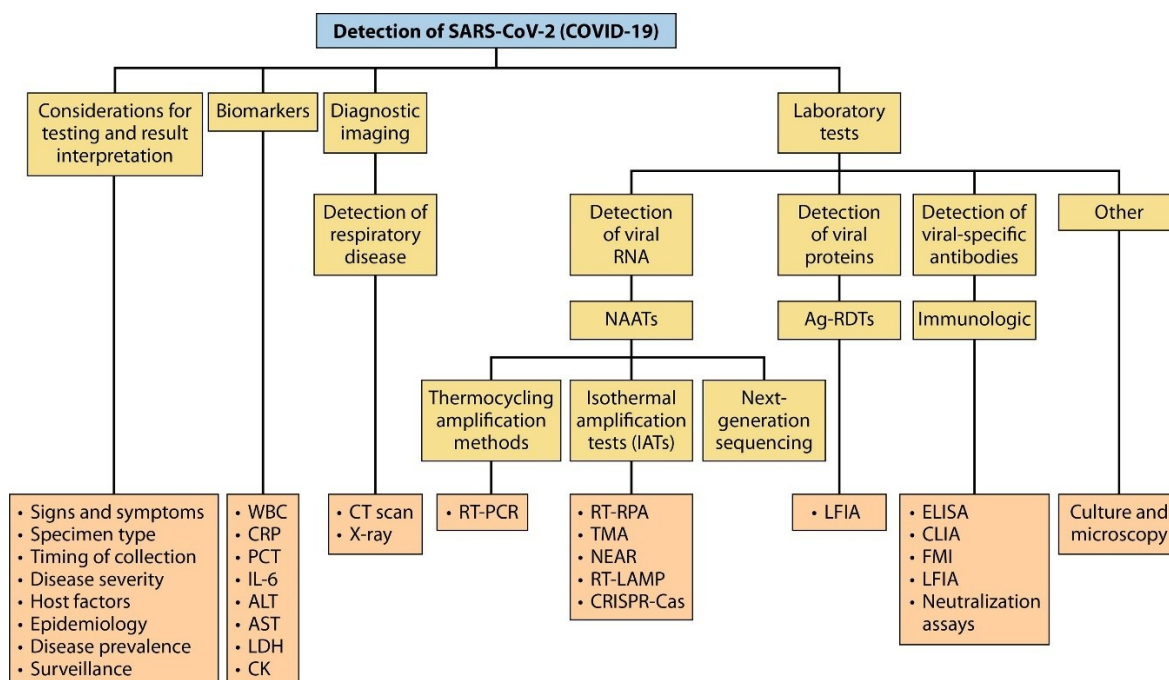


Figure A.13. Methods used for SARS-CoV-2 detection or identification of COVID-19 disease. Of note, the cell culture and microscopy are not used for clinical diagnosis, but for research purposes. Abbreviations: WBC: white blood cell; CRP: C-reactive protein; PCT: procalcitonin; IL-6: interleukin 6; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; CK: creatine kinase; NAAT: nucleic acid amplification test; RT-PCR: reverse transcription polymerase chain reaction; TMA: transcription-mediated amplification; RT-LAMP: reverse transcription-loop-mediated isothermal amplification; RT-RPA: reverse transcription recombinase polymerase amplification; CRISPR-Cas: clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas); NEAR: nicking enzyme-assisted reaction; Ag-RDT: antigen rapid diagnostic test; LFIA: lateral flow immunoassay; ELISA: enzyme-linked immunosorbent assay; CLIA: chemiluminescence immunoassay; FMI: Fluorescent microparticle immunoassay; CT scan: computed tomography scan.

While compatible signs and symptoms, routine laboratory testing for biomarkers of health or disease, and diagnostic imaging have roles to play in diagnostic investigations, none alone are sufficient for diagnosing SARS-CoV-2, and the reliance on specific laboratory testing is paramount. Many technological advances have been made to detect SARS-CoV-2, which include NAATs to detect viral RNA and immunoassays to detect viral antigens or virus-specific antibodies generated by the immune system in response to SARS-CoV-2. There is a growing list of commercially available methods for testing for SARS-CoV-2 [464,465], and while many have been validated, the results of any test should be interpreted with consideration of the context for which they are used and based on the sum of all diagnostic evidence. When using any method to

rule in or out SARS-CoV-2 infection, many factors should be considered, such as the timing and type of specimen collection, the anatomical site of sampling, the method and its expected performance characteristics, host factors like compatible signs and symptoms (vs. asymptomatic testing), risk factors for serious outcomes, and disease prevalence in the population [519,997–999].

NAATs like real-time RT-PCR quickly became the gold standard for diagnostic testing. However, apart from those listed above, other considerations for NAATs are inherent to the methodologies that are all based on the detection of SARS-CoV-2 RNA. For example, it is important to recognize that the performance of any molecular diagnostic method can be influenced by sequence mismatches between the method's targets and the different genome permutations of circulating SARS-CoV-2 lineages and variants. A possible strategy to reduce the chance of false negatives that could occur by target mismatches is the simultaneous use of molecular methods targeting more than one gene, as failure to detect a signal in one target gene may not preclude detection in another [479,601,891]. Alternative strategies could include the use of degenerate primers and probes, a strategy used with other RNA viruses that are prone to mutation [891,1000,1001]. Ongoing molecular surveillance using sequencing technologies should also be encouraged to monitor changes in SARS-CoV-2 genome sequences and COVID-19 epidemiology, particularly with an emphasis on variants linked to failures in diagnostic testing, with increased transmissibility, increased severity, or decreased susceptibility to convalescent sera or response to vaccines.

The primary reliance on NAAT for SARS-CoV-2 detection during the pandemic came with many challenges globally. With human resource strains and supply chain shortages, providing NAAT testing during the pandemic was challenged by factors such as PPE, human resource strains for sample collection and testing, swabs, and test reagent availability for NAAT testing. From a clinical perspective, alternatives to NP swabs traditionally used for respiratory specimens were rapidly validated, as well as the use of various transport media or swab-free options that are amenable to self-collection (e.g., saliva or saline gargles). Application of these specimens in the lab added complexity to laboratory workflow and required rapid validations to ensure compatibility with new or existing instrumentation.

To help meet capacity demands in the laboratory, laboratories were faced with the need to rapidly procure large quantities of supplies, acquire instrumentation, train additional personnel, and validate specimens, reagents, and equipment. Despite this, supply chain challenges and rapid

escalation of testing demands lead to the need for resource-sparing strategies for NAAT testing, including specimen pooling and extraction-free NAAT protocols. Such strategies come at the cost of a relatively reduced sensitivity, which is associated with the potential risk of missing detection of SARS-CoV-2 in specimens with low viral loads. It could be argued that small reductions in sensitivity may have little impact on the detection of most cases. Remnant SARS-CoV-2 at the outset of illness can persist for weeks and is unlikely to represent a period of communicability. On the other hand, missing detection of SARS-CoV-2 in a specimen with a low viral load at the early stages of illness may potentially lead to further virus spread. These testing strategies should be carefully considered prior to implementation.

SARS-CoV-2-specific NAATs evolved over time to facilitate testing and streamline laboratory workflow. For example, to further streamline testing for respiratory viruses, some methods have now been multiplexed to simultaneously detect SARS-CoV-2, influenza viruses A and B, and other respiratory viruses like respiratory syncytial virus (RSV). In non-pandemic years, influenza and other viral etiologies of respiratory tract infections represented a leading cause of death in North America, particularly among hospitalized patients with community-acquired pneumonia [1002–1008]. Interestingly, while co-circulation and co-infection with other respiratory viruses were reported, there was little activity for non-SARS-CoV-2 respiratory viruses [1009]. It is unclear whether public health interventions (e.g., travel restrictions, social distancing, hand washing, or PPE like masks) resulted in this decline, or whether other factors inadvertently contributed to biases in data such as fewer individuals seeking routine medical attention or the lack of testing for influenza and other respiratory viruses due to competition of resources used for SARS-CoV-2 testing [1009–1013]. Concomitant diagnostic testing using multiplex technologies could provide an option for syndromic testing that would ensure surveillance for SARS-CoV-2 and other important respiratory viruses like influenza and appropriate interventions as needed (e.g., antivirals).

The public and political pressure for laboratory testing evolved with public health indications, and laboratory testing continues to guide public health policies as restrictions ease or escalate throughout the ongoing pandemic [484,940]. One area of significant advancement in NAATs methodologies include the use of automation to minimize hands-on processing time and increase specimen throughput. With the high demand for laboratory testing, automation is an important consideration to avoid the possibility of staff repetitive stress injuries. Over the course of the

COVID-19 pandemic, the scale and demand for laboratory testing has been unmatched by other pandemics. Concerns over SARS-CoV-2 prompted testing of both symptomatic and asymptomatic individuals, in the context of public health case contact tracing and surveillance purposes. This includes testing for SARS-CoV-2 in both healthcare settings where patients are at increased risk (e.g., hospitals and long-term care facilities) and situations where testing would not otherwise have been performed (i.e., professional sports teams, public events, prior to or post-travel, and various workplaces). Thus, innovative, dynamic, and adaptable approaches were required to meeting the testing demands, which are covered extensively in this review.

Other areas are recognized as crucial to increase laboratory testing capacity but were not covered in the review such as supply procurement, distribution, and management, the coordination of training or recruitment of increased human resources for specimen collection, testing, and processing and registration. Also, the rapid validation of laboratory tests and collection devices to meet regulatory requirements despite availability through EUA from regulatory bodies, as well as the development of an information technology infrastructure to continuously improve laboratory information management system (LIMS) in laboratory testing workflow and dissemination of near real-time laboratory data to various stakeholders from local to national levels, and dissemination to the public through various media formats are of great value. While digitization was not an absolute requirement, the interconnectivity of data ensured transparency and up-to-date information as decision support tools for recommendations, policies, and guidelines [490,1014–1016]. All of these have been identified by the WHO as key factors to control the COVID-19 pandemic [1017].

To further enhance testing capacity and to provide access for rapid SARS-CoV-2 testing options, both antigen- and NAAT-based RDTs have been developed and are now readily available [464,465]. These can support rapid laboratory and POC applications, screening of large patient populations, or rapid deployment for assessments of target areas. NAAT-based RDTs rely on real-time RT-PCR or isothermal amplification methods (e.g., RT-RPA, RT-LAMP, and NEAR), and like antigen-based RDTs, can provide rapid results without complex instrumentation. The ease-of-use of these portable devices allows access to SARS-CoV-2 diagnostics in settings that may have been prohibitive for traditional laboratory NAATs. Throughout the literature, the main limitation noted for RDTs is their reduced sensitivity compared to traditional NAATs, but the evaluations of their performance do not always reflect conditions in which the RDT was licensed under EUA,

and the comparator method and distribution of expected specimen results can have a large impact on assay performance [725,741,789,790,906]. Even if these issues are not considered, and all SARS-CoV-2 detection results were considered of value, a possible strategy to mitigate the relatively reduced sensitivity of antigen- or NAAT-based RDTs, is to increase the frequency of testing in the patient population over time, to increase the chances of identifying individuals in who fall in a period of high viral shedding (which would less likely to be impacted by reduced sensitivity) [739,750,752,923,924]. On the other hand, implementation of such strategies has challenges of its own due to the limited scalability of RDTs, and the balance between sensitivity and testing frequency to achieve optimal SARS-CoV-2 detection in the target population would need to be defined [739]. Moreover, if, for example, EUA defined the use of RDTs as within seven days of symptom onset, this excluded their use for testing of asymptomatic individuals. While much development is ongoing to enhance existing RDT technologies, or explore novel methodologies [490,904,922,1018–1020], how RDTs can effectively be used in practice is the subject of ongoing debate, and further research is needed to understand what setting they would best be of benefit.

For serology, currently available commercial assays are based on ELISAs, CLIAs, and LFIAs, and are designed to detect SARS-CoV-2 antibodies. However, the performance of serologic assays varies across the different technologies, the timing from disease onset, and comparator methods. Clinical validation of serological methods are ongoing, but recent meta-analyses and systematic reviews so far have shown high levels of heterogeneity and risk of bias and applicability [928,957,958]. For example, with the time required to mount anti-SARS-CoV-2 antibodies during seroconversion (or possibly absence of seroconversion in mild disease), serology has limited value in identifying SARS-CoV-2 in the early stages of illness. Serology has value for seroepidemiologic studies to help with ongoing outbreak investigations, can aid in the diagnosis of suspect cases for whom NAAT testing was persistently negative or not performed, or help with conditions in children and adolescents like multi-system inflammatory syndrome (MIS-C) where NAAT test results may be negative [539,570,678,1021–1025]. Much research is needed to expand the knowledge on the use of immunologic methods, particularly in the context of the recent availability of vaccines.

Key areas of research interest for serology should include the relevance, magnitude, and duration of protective antibody responses, particularly faced with a population that may have been exposed

to SARS-CoV-2 or other human coronaviruses or vaccinated against SARS-CoV-2. Detection of different antibody isotypes is possible with commercial or laboratory-developed serological assays, and recent quantitative methods have now become commercially available; however, much is yet to be learned on how these methods correlate with protective immune responses. Establishing correlates of protection will require longitudinal studies with parallel assessments of quantitative levels of nAbs and cell-mediated immune responses. Various neutralization assays have been established to assess and quantify antibodies against SARS-CoV-2 in serum or plasma (e.g., PRNT, MN, or surrogates assays like PBNA and snELISA), but data is scarce when it comes to longitudinal studies undertaken to fully understand the differences or similarities between commercial assays for the qualitative detection of antibodies, quantitative antibody analyses over time, and the relevance to the level and duration of protective nAb titers. While the longevity of humoral immunity is not yet fully understood for SARS-CoV-2, some data suggest that antibody levels against SARS-CoV-2 wane over three months. Notwithstanding these observations, it is possible that memory T cells may still be able to mount effective responses upon re-exposure to SARS-CoV-2, and the role of cell-mediated immune responses may also provide additional benefits [963,964]. Further research is needed, as a greater understanding of the immune response to SARS-CoV-2 is fundamental in making informed recommendations for the use of immunological methods to assess current or future protection against the virus, to determine meaningful endpoints in the development and evaluation of effective vaccines, or to assess effectiveness following immunization with existing vaccines.

Overall, methods based on RNA, antigen, or antibody detection, as well as diagnostic imaging, all have a place in our response to SARS-CoV-2, but ongoing research is crucial to further optimize and apply all these testing modalities. The emergence of the SARS-CoV-2 pandemic has created a wave of innovative and creative thinking, and more and more creative methods and platforms are introduced with goals to increase the armamentarium of diagnostic methods for SARS-CoV-2 detection. Understanding the advantages and limitations of each method used for SARS-CoV-2 detection, as well as the development of novel methods, will help us unravel the unknowns of disease pathogenesis, epidemiology, and transmissibility and help us develop interventions to mitigate and contain its spread. There will always be a need for laboratory testing and collaboration between clinical laboratories, public health, infection prevention and control, and many others who contribute to the efforts to contain the spread of COVID-19. On the other hand, it is also recognized

that maintaining large investments into the rapid deployment of translational research and such a high degree of laboratory testing for COVID-19 will likely not be sustainable from an economic perspective, and justifications for such investments will be more difficult if cases decline significantly with vaccines. However, the lessons learned from SARS-CoV-2 could potentially be used in the preparedness for potential future pandemic threats, thus strengthening global health and surveillance systems.