

New molecular methods for point-of-care infectious disease diagnosis

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“If one of us, any human being, is suffering like a dog right now, is sick or hungry, it affects us all. It must concern us all, because ignoring human suffering is always an act of violence, and one of the most cowardly.”

Dr. Gino Strada

Founder of Emergency

To Kyrios,

Abstract

This thesis focuses on a multidisciplinary project to develop molecular methods for point-of-care (POC) diagnosis of infectious diseases. First, I describe an approach followed to develop a test that aims to discriminate bacterial infections from viral infections. The test is based on evidence that gene patterns detected in the blood of patients by isothermal amplification technologies used in conjunction with an electrochemical platform are specific enough to distinguish viral from bacterial infections. For the first time, a two-gene host blood RNA signature has been translated into a rapid (<25 minutes) and portable laboratory-on-a-chip platform, confirming that the approach holds significant potential for the development of quantitative diagnostics.

Second, and in response to the rapid evolution of the COVID-19 pandemic and the need for rapid, sensitive and specific diagnostic methods, I focused on the translation of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection to the optimised lab-on-a-chip platform to deploy a test with minimal time, expertise and personnel requirements.

Third, a novel method that rapidly, efficiently and cost-effectively extracts nucleic acid (NAs) from a clinical sample has been developed and combined with an additional detection method based on colourimetric isothermal amplification providing a potential solution to detect pathogens within 30 minutes from sample to result. This thesis proposes novel approaches for the development of POC tests for disease diagnosis that may soon be considered in clinical practice.

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Declaration

Statement of Originality

The work presented in this thesis describes my research under the supervision of Prof. Michael Levin, Dr Jesus Rodriguez-Manzano, Dr Myrsini Kaforou and Prof. Pantelis Georgiou. Ideas and results from the others' work are appropriately acknowledged with the standard referencing practices employed in this discipline. The material in this thesis has not been submitted for any degree at any other academic or professional institution. Whole blood transcriptomic-wide profiling of large cohorts of febrile children has been conducted by Prof. Levin's group and the data were made available to me by my supervisors.

Ivana Pennisi

London, August 2022

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Ivana Pennisi

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List of abbreviations

ACC	Accuracy
AUC	Area Under the Curve
CMOS	Complementary Metal-Oxide-Semiconductor
DB	Definite bacterial
DF	Dragonfly
dLAMP	Digital LAMP
DNA	Deoxyribonucleic Acid
dPCR	Digital PCR
dsDNA	Double-stranded DNA
DV	Definite viral
eLAMP	Electronic LAMP
FluA	Influenza A
FluB	Influenza B
HC	Healthy control
HRV	Human rhinovirus
ISFET	Ion-Sensitive Field-Effect Transistor
LAMP	Loop-Mediated Isothermal Amplification
LoC	Lab-on-a-chip
LoD	Limit of Detection
LW	Lacewing
MDR	Multidrug-Resistant
mRNA	messenger RNA
NAAT	Nucleic Acid Amplification Test
NAE	Nucleic Acid Extraction
PCR	Polymerase Chain Reaction
QG	Qiagen
qLAMP	Quantitative LAMP
qPCR	Quantitative PCR
RNA	Ribonucleic Acid
RNA-seq	RNA sequencing

RSV	Respiratory Syncytial Virus
RT-	Reverse Transcriptase- (a prefix, e.g., RT-PCR or RT-LAMP)
SEN	Sensitivity
SL	SmartLid
SPE	Specificity
TB	Tuberculosis
TPP	Target Product Profile
TTP	Time-to-Positive
WHO	World Health Organization

Scope of the Thesis

This thesis aims to develop and translate innovative molecular methodologies to diagnose infectious diseases at the POC, which will support patient management. Although this thesis was originally intended to focus on the use of host-transcriptomics (Part I) for differentiation of viral and bacterial infections, the clinical emergency of the COVID-19 pandemic expanded it to direct-pathogen detection (Part II). Therefore, to guide the reader through the two different research questions I focused on during the time of my PhD, I have divided the thesis into Part I, based on a transcriptomic approach, and Part II, based on a direct pathogen approach. The two main innovations described in this thesis resulted in the generation of intellectual property (IP) in the form of two patent applications. The first innovation (described in Part I, Chapters 2 and 3) involves the use of a microchip-based method to detect the presence of an RNA host response, indicating the likelihood of a specific diagnostic outcome. The second innovation (described in Part II, Chapters 5 and 6) focuses on a new sample preparation methodology developed during the SARS-CoV-2 pandemic, which enables nucleic acid extraction in less than three minutes without the need for laboratory equipment or electrical power.

Thesis Structure

The thesis is structured in seven chapters with a common topic, bridging the “diagnostic gap” by employing new molecular methodologies to enable the detection of infectious diseases.

Chapter 1 contains a general introduction to the diagnostic landscape for infectious diseases, highlighting the limitations existing in the diagnostic of infectious disease and presenting different approaches to overcome the diagnostic gaps.

Following a general introduction to the diagnostic landscape (Chapter 1), the core of this thesis is separated into Part I and Part II. Part I (Chapters 2 and 3) focuses on how the developed molecular methods can detect host-transcriptomic responses to determine a diagnostic outcome for discriminating bacterial infections from viral infections on a low-cost lab-on-a-chip platform. The study is performed on a paediatric population in close collaboration with the Paediatrics Infectious Disease Team led by Prof. Levin and Dr Kaforou.

This is followed by Part II (Chapters 4, 5 and 6), which explores the COVID-19 diagnostic scenario and describes the novel and rapid molecular methods to detect respiratory pathogens in an adult population at the point of need. Chapter 7 provides conclusions, ongoing work, and future work. Given the broad contents of this thesis, each chapter is self-contained (i.e., including an introduction and sections on materials and methods and on results and discussion), so that the reader can easily follow its contents. To provide visual clarity of the thesis’s content, Figure 1 summarises how the thesis is organised.

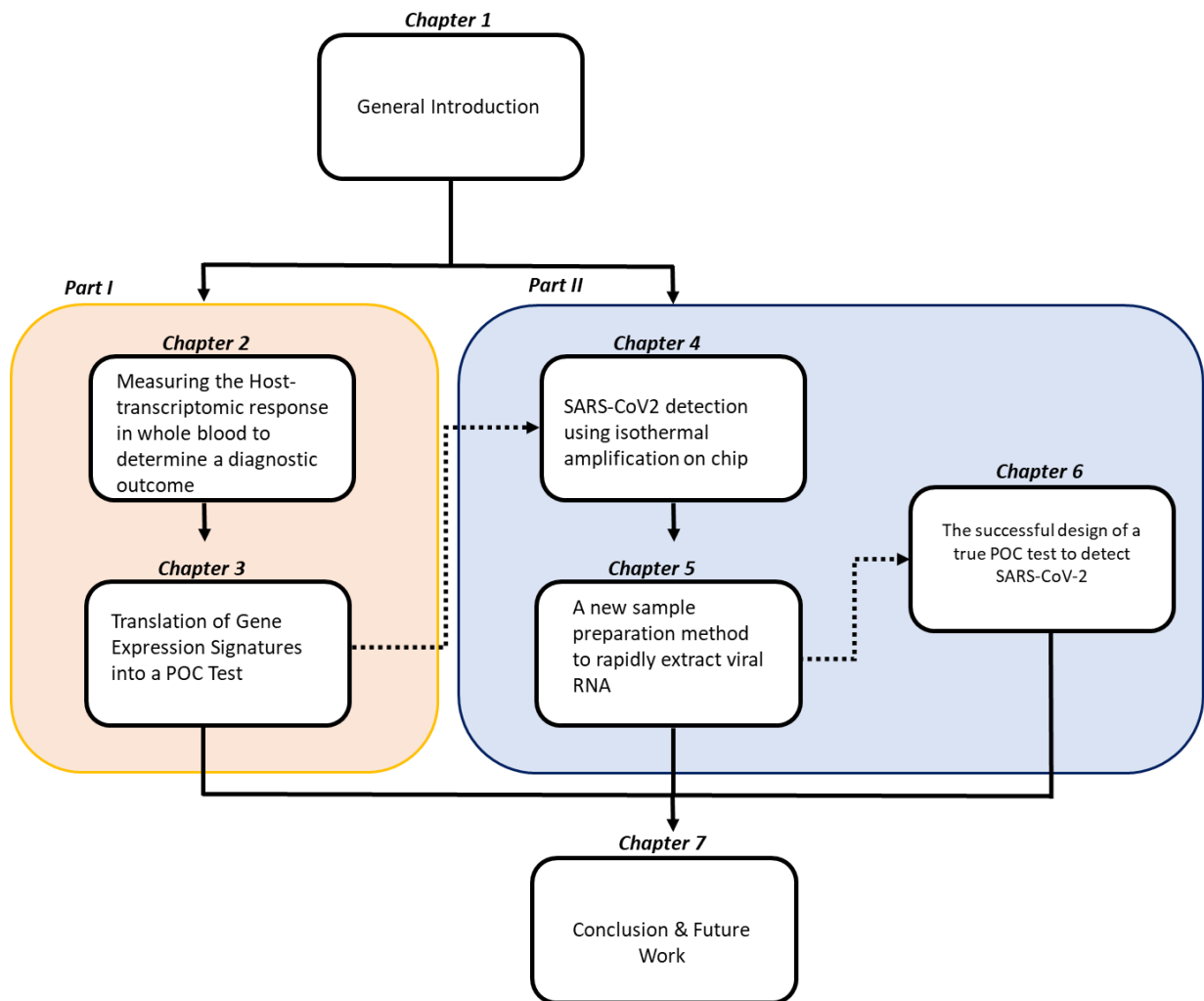


Figure 1. Thesis organisation.

Research Objectives and Contributions

The work described in this thesis aims to address the following research questions:

(i) Selection of optimal RNA biomarkers to distinguish bacterial from viral infections on microchip technology

Research question: Since clinical features cannot reliably distinguish bacterial infections from viral infections, improved access to rapid diagnostic services may facilitate prompt clinical decision-making and avoid the misuse of antibiotics. It has been shown that bacteria and viruses stimulate host inflammatory cells to express common and different genes, but can RNA biomarkers be employed to rapidly distinguish bacterial from viral infections on a chip-based technology?

Contributions: First, whole blood gene expression profiles were studied in bacterial and viral patients to select the gene candidate biomarkers. Second, novel LAMP assays to target specific bacterial and viral genes were developed and studied. Third, cross-platform validation was employed to study the performance of each gene candidate, allowing the choice of a minimal two-gene signature (*EMR1* and *IFI44L*). Validation of the LAMP assay was performed using clinical isolates from a clinical study (the Inflammatory and Infectious Disease Study (IRIS) study).

(ii) Translation of gene expression signatures into a microchip-based platform

Research question: Microchip-based technologies in combination with isothermal chemistry offer a promising alternative to conventional laboratory-based nucleic acid amplification tools due to their portable and affordable nature. How can an amplification reaction be translated onto a semiconductor-based platform?

Contributions: A LAMP-based assay was optimised to achieve a low limit of detection (LoD), therefore increasing the sensitivity of the assay to enhance the detection and discrimination of the two-gene signature on a lab-on-a-chip (LoC) platform. A pH-LAMP approach (low-buffered protocol) compatible with microchip-based technology was used in combination with an in-house developed electrochemical LoC platform and the results were validated in a commercial fluorescence-based instrument. Both platforms showed comparable results, confirming that the two-transcript signature can be detected using an LoC semiconductor-based sensing platform combined with isothermal amplification chemistries. These contributions led to publications (*Pennisi et al. "Discrimination of bacterial and viral infection using host-RNA signatures integrated in a lab-on-a-chip technology". Biosensors and Bioelectronics 2022*); (*Pennisi et al. "Translation of a host blood RNA Signature*

distinguishing bacterial from viral infection into a platform suitable for development as a Point-of-Care Test". Jama Pediatrics, 2021) and one patent application (*Pennisi et al. "A method for determining a diagnostic outcome," WO2022043341A1, 2020*).

(iii) SARS-CoV-2 detection into a handheld platform at the point of care

Research questions: In the COVID-19 pandemic, a high transmission rate and an increase in cases have been prominent characteristics. Identifying and isolating patients, containing its spread, and guiding clinical management are urgent needs that need to be addressed. Is it possible to diagnose patients with SARS-CoV-2 using the LoC platform? How will the LoC platform perform compared to the gold standard PCR instrument?

Contributions: Reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assays were tested on benchtop instruments and on-chip using a new two-well manifold to reliably detect the presence of SARS-CoV-2 on the LoC platform.

The study was run by our team at the Centre for Bio-Inspired Technology (CBIT) using clinical samples from Imperial College Healthcare NHS Trust, (Charing Cross Hospital) to validate the performance of our LoC platform compared to the gold standard real-time instrument. The results led to a publication (*Rodriguez-Manzano et al. "Handheld Point-of-Care System for Rapid Detection of SARS-CoV-2 Extracted RNA in under 20 min", ACS Central Science, 2021*) demonstrating that patients with SARS-CoV-2 infection can be reliably detected on the handheld platform in only 20 minutes with comparable sensitivity and specificity as provided by a benchtop commercial instrument (PCR).

(iv) Development of a new sample preparation method to rapidly extract viral RNA

Research questions: Nucleic acid extraction methodologies are a fundamental step for achieving highly sensitive and specific target amplification, but they are laborious, expensive, and time-consuming. Can a simplified, power-free, rapid and inexpensive method be developed to overcome the limitations of the existing commercial methods? How might such a frugal approach perform compared to the established gold standard methodologies?

Contributions: A novel magnetic-based sample preparation method was developed to extract NA from clinical specimens in less than three minutes. The extraction method presented here underwent two clinical validations and was compared to the Qiagen (column-based) extraction kit, the MM extraction kit and the PROmate (magnetic-based) extraction kit, showing that it can favourably compete with conventional laboratory-based extraction techniques that require expensive pieces of equipment and electricity and that introduce an additional time of over 60 minutes in the process. These contributions led to a patent (*Cavuto et al. "Lid assembly for a sample tube, method of*

using the same to collect magnetic beads, and sample processing kit”, GB2102573.9, 2021), a paper (I Pennisi, M Cavuto, et al. “A simplified, power-free sample preparation method to allow direct detection of SARS-CoV-2 RNA from nasopharyngeal samples”. In preparation) and a commercial kit named DF Sample Preparation Kit that has recently obtained permission to be sold on the European market under CE mark requirements.

(v) Development of a sample-to-result diagnostic test named DF to detect respiratory infections

Research questions: Can the SmartLid extraction method work in combination with a colourimetric LAMP reaction? What will be the performance of SmartLid-extracted NA when detected in colourimetric LAMP compared to the gold standard molecular methods? Can the DF methodology be used as a true POC test in diagnostic environments?

Contributions: The SmartLid extraction method has been optimised to be compatible with colourimetric LAMP detection without impacting the analytical sensitivity and specificity of the LAMP assay. A 30-minute sample-to-result test was achieved using the SmartLid sample preparation step combined with 25 minutes of incubation in a simple, affordable heat block with a set temperature of 63°C.

A step-by-step process on how to perform the test including an objective readout of the test result was developed to guide the user by implementing a companion app with integrated image processing. The DF sample-to-result test has been clinically validated and approved as a diagnostic test to detect SARS-Co-V-2, Flu A, Flu B, RSV and RHV. These recent contributions will be part of future manuscripts.

List of publications

Patents

1. Ivana Pennisi, Jesus Rodriguez-Manzano, Myrsini Kaforou, Michael Levin, Pantelis Georgiou et al. "A method for determining a diagnostic outcome," WO2022043341A1, published 2022-03-03.
2. Matthew L. Cavuto, Ivana Pennisi, Jesus Rodriguez-Manzano, Pantelis Georgiou, "Lid assembly for a sample tube, method of using the same to collect magnetic beads, and sample processing kit" GB2102573.9, Filed Feb 23, 2021.

Accepted/Published Peer-Reviewed Journals

1. I Pennisi, A Moniri, N Miskourides, L Miglietta, N Moser, et al. Discrimination of bacterial and viral infection using host-RNA signatures integrated in a lab-on-a-chip technology. Biosensors and Bioelectronics, Volume 216, 2022, 114633, ISSN 0956-5663, <https://doi.org/10.1016/j.bios.2022.114633>.
2. Digital Diagnostics for Africa Network. The potential of digital molecular diagnostics for infectious diseases in sub-Saharan Africa. PLOS Digital Health 1 (6), e0000064.
3. BW Wormald, N Moser, NM deSouza, KT Mantikas, Kenny Malpartida-Cardenas, Ivana Pennisi, et al. Lab-on-chip assay of tumour markers and human papilloma virus for cervical cancer detection at the point-of-care. Scientific reports 12 (1), 1-8 2022.
4. B Wormald, J Rodriguez-Manzano, N Moser, I Pennisi, TEJ Ind, K Vroobel, et al. Loop-Mediated Isothermal Amplification Assay for Detecting Tumor Markers and Human Papillomavirus: Accuracy and Supplemental Diagnostic Value to Endovaginal MRI in Cervical Cancer Frontiers in Oncology 11, 747614 2021.
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1. I Pennisi*, M Cavuto*, L Miglietta, R Reid, O Stringer, KM Cardenans, N Moser, P Georgiou and J Rodriguez-Manzano. A simplified, power-free sample preparation method to allow direct detection of SARS-CoV-2 RNA from nasopharyngeal samples. In preparation for PNAS.
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detection and discrimination of seven human-infective *Plasmodium* species validated with samples from Ghana.

3. I Pennisi*, R Reid*, M Cavuto, S. Frankin, J Herberg, M Kaforou, A Cunningham, A. Holmes, M Levin, P Georgiou and J Rodriguez-Manzano. Rapid extraction of viral respiratory pathogens from saliva samples using SmartLid. In preparation for the Journal of the American Chemical Society.
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Chapter 1

General Introduction

Chapter 1 is a general introductory chapter setting the background for the rest of the thesis. The focus is on the diagnostic landscape of bacterial and viral infections in both adults and children with particular emphasis on the existing challenges and gaps in diagnostics, especially in low-resource settings. Pathogen-based detection and the potential of measuring host-gene expression to establish disease diagnosis are then highlighted as the main clinical research aims of this study. Electrochemical-based detection and power-free sample preparation are discussed in this thesis as approaches to overcome the limitations in diagnostic tests and efficiently provide a fast and reliable diagnostic solution.

Diagnostic landscape of bacterial and viral infection

The concept of infectious diseases did not exist prior to Pasteur and Koch's 1876 discovery that anthrax is caused by *Bacillus anthracis*. After explanation of the etiology of anthrax in the late nineteenth century, the scientific identification of bacteria, viruses, fungi, algae, helminths, and protozoa as pathogens of specific diseases became common [1], [2].

Through advances in tools such as microscopy, microbial cultivation, immunologic and molecular methods and experimental animals, knowledge of the nature of microbes has advanced more rapidly [3]. The field of infectious diseases is broad and unique in the clinical context with its ability to cure and prevent disease, identify specific disease causes (microbes), and deal with diverse outbreaks [4]. In the event that a patient seeks medical attention as a result of illness, the doctor searches for evidence that will enable the diagnosis of the illness, which in turn leads to treatment that will ameliorate or cure the condition. It is the first step in the treatment and eradication of infectious microbial diseases to diagnose the infection [3].

As early as the beginning of the twentieth century, observation and physical examination were the most important and basic methods used to study patients. Nevertheless, during the last century, practical medicine began to be enriched with new diagnostic methods alongside the development of basic clinical diagnostic methods [3]. Antonie van Leeuwenhoek, also known as “the father of microbiology”, changed the course of infectious diseases when he enabled the visualisation of the microbial world through his microscopy study officially acknowledged by the Royal society in 1677 [5].

Although better Gram stains, microscopes and agar media are now available, standard culture and microscopy techniques continue to be the first line of diagnosis for infectious diseases. Among these are urinary tract infections (UTIs), the differentiation of gram-positive from gram-negative pathogens in sputum smears as well as the detection of *Mycobacterium tuberculosis* [6] [7]. The advent of new methods for recovering pure cultures of bacteria and eventually viral tissue culture enabled the development of susceptibility tests of bacteria against specific antimicrobial agents, thereby contributing to the study of the antibiotic resistance phenomena [4]. However, one of the major disadvantages of culture-based assays is their time-consuming nature [8]. As shown in Table 1, culture growth takes longer than other diagnostic tests.

Due to advancements in biochemistry, it has become possible to detect specific metabolic products of microbes, which has significantly reduced the time necessary to identify the pathogen of interest. Analytical detectors (spectrophotometer, fluorometer, radioactive counter) have enabled the detection of antibodies by enzyme immunoassay and enzyme-linked immunosorbent assay [5]

Nowadays, serological diagnosis has become an increasingly important part of patient care. Serological assays are useful screening tools for monitoring the risk of certain chronic infections, evaluating the immune status, and determining whether specific treatments are required in connection with organ transplantation [6]. They are also used to investigate the etiology of acute viral infections or latent infections reactivated. An antibody test for the diagnosis of a virus-specific infection is based either on the detection of virus-specific IgM antibodies or on the presence of significant increases in specific IgG antibodies. Several diagnostic applications of measuring IgG antibody activity to specific antigens have been developed to distinguish between serological responses due to acute infections and those resulting from chronic or past infections [9].

With modern antibody panels, it is possible to detect multiple antigens and antibodies within a few hours, which significantly reduces the diagnostic time. Therefore, these panels are becoming a more and more common tool in clinical diagnostics. Despite increasing attention has been given for public health purposes and for their rapid nature, these assays are not more sensitive than culture-based tests, nor do they provide information on antimicrobial susceptibility, limiting their value in diagnostics [9].

As pathogens have evolved rapidly over the last few decades and new diseases have emerged, it has become increasingly important to identify appropriate diagnostic techniques in order to manage infectious diseases more effectively [6]. In any population, the success rate of a specific diagnostic technique varies according to a number of factors, such as the type of pathogen, the availability of resources, the technical expertise, the severity of the disease, and the extent of an epidemic [6]. The landscape of diagnostic methods is rapidly evolving, as we have witnessed during the ongoing COVID-19 pandemic. Infectious diseases are still a leading cause of death worldwide, particularly in low and middle-income countries (LMIC), and especially in young children. Even though deaths from tuberculosis and HIV/AIDS have fallen significantly in recent years, in LMIC they still represent the leading cause of death with 1,208 thousand TB deaths annually, followed by HIV/AIDS with 675 thousand deaths annually. Malaria represents another infectious disease at the top of the causes of death with 411 thousand cases every year. Measles, Cholera and hepatitis A/B cause fewer than 200 thousand deaths per 1,000 people every year. Mortality estimates are provided by the World Health Organization (WHO) [1] (Figure 2).

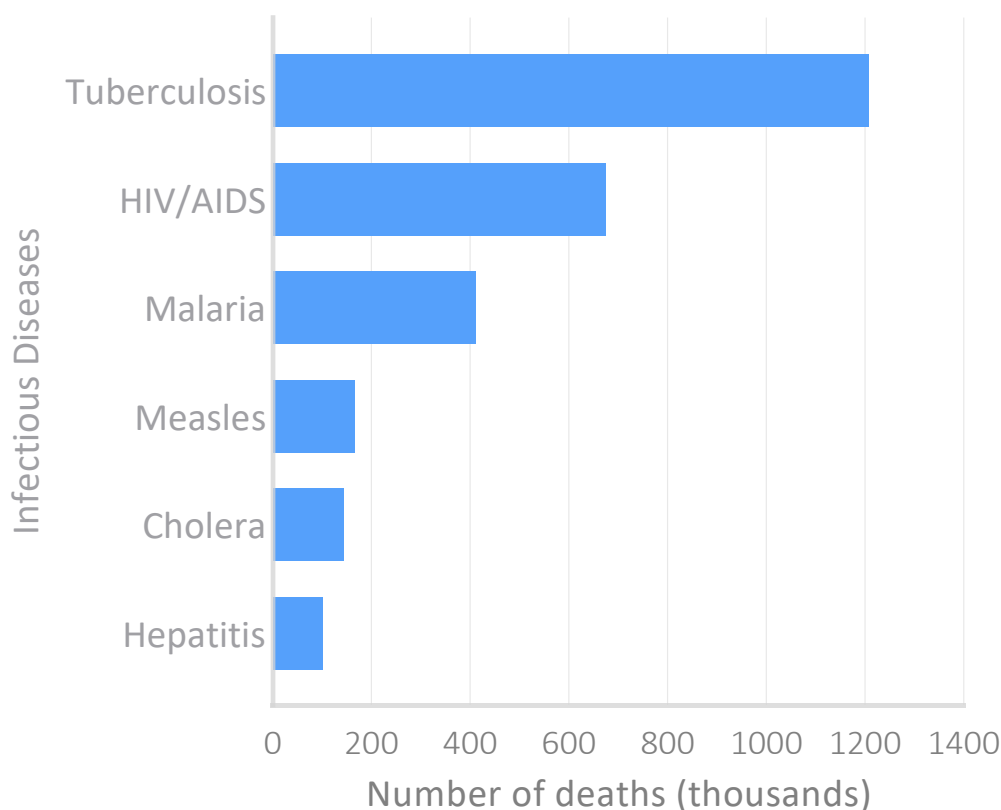


Figure 2. Leading causes of death worldwide from infectious diseases annually as of 2019 [10].
Illustration generated using GraphPad Prism9.

Infectious diseases such as Tuberculosis, HIV/AIDS, and Ebola remain major concerns for public health, with most of the cases representing a major cause of death in sub-Saharan Africa [11] where limited access to vaccines and diagnostic tests are the key drivers [1].

In some of the LMIC the diagnostic approach is still based on signs, symptoms and patient history, this approach is not sufficient to determine the aetiology of disease and the presence of pathogens as they usually have similar clinical presentations, highlighting the need for reliable POC diagnostic tests. However, over the last two decades, molecular amplification technologies have revolutionised the diagnostic field, allowing for the detection and often quantification of a greater number of pathogens [12]. As an example, rapid malaria tests or molecular diagnostics for quantifying viruses like HIV, hepatitis B and C viruses have revolutionised the development of antiretroviral drugs that can be used to manage and treat viral infections [6]. Nucleic acid (NA) amplification techniques such as polymerase chain reaction (PCR) are based on the detection of pathogen-specific DNA or RNA sequences in the sample and are considered the best methods to detect pathogens that are difficult to grow in vitro or in laboratory conditions [5]. The use of PCR techniques in conjunction with different pre-treatment processes can detect almost all types of pathogens [12]. Diagnostic advances in infectious diseases such as Tuberculosis have been made possible by automated NA amplification tests

such as GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) [13]. As of today, Xpert MTB/RIF and Xpert XDR are the most widely used automated NA amplification test for tuberculosis providing analytical sensitivity and high specificity for individuals not previously treated for tuberculosis as well as drug susceptibility testing for drugs resistance. Despite the fact that diagnostics for children are not always available in many low resource settings, the increased accessibility of the aforementioned diagnostic tools represents a promising route to improving healthcare outcome [13].

The Matrix-Assisted Laser Desorption-Time of Flight Mass Spectrometry (MALDI-TOF) Mass Spectrometry is another widely accepted molecular technique, often used in research laboratories when more confirmation data of a pathogen is needed [14]. A special matrix is used to treat samples that contain bacteria or fungi. The matrix will absorb the energy from the laser and further ionize and vaporize the samples. In order to accelerate the ionized molecules towards the detector, a high voltage is applied to them. As these molecules travel, they are separated according to the time it takes them to reach the detector, which is determined by their mass-to-charge ratio (m/z). It is the pattern of masses for a particular pathogen that determines its fingerprint mass spectrum [14]. Besides being a powerful confirmatory method for identifying pathogens and their associated mutations, its use is still time consuming.

A new pathogen-detection technique, aptamer-based immunoassay, is emerging as a viable alternative to antibodies when used for bacteria detection. The term aptamer refers to synthetic DNA or RNA molecules having secondary structures that are able to bind to specific targets with high affinity and stability. While aptamers are easy to label at a reduced cost, they cannot be directly labeled with enzymes, which is why fluorophores attached to DNA sequences are used to detect pathogens [15].

While molecular diagnostics can provide accurate results, they are still limited to laboratory use and cannot be operated in low-resource settings [16]. The main limitation is due to their reliance on sophisticated instruments based on expensive detection methods, which require highly skilled personnel for analysis [17]. The complex process of diagnosis and lack of adequate POC diagnostic test which pose many challenges to clinicians and doctors when making diagnostic decisions are reported in the research field as “diagnostic gaps” [18], [19] especially in LMIC, where they represent the reason for disease underdiagnosis or misdiagnosis negatively impacting the patient care [20].

According to the WHO, providing accessible, early diagnosis is imperative to prevent morbidity and mortality, particularly in infants and children living in LMIC [21], [22] Public health surveillance could be dramatically improved with increased access to quality screening and early detection. The development of new diagnostic methodologies suitable for diverse clinical settings and community care is essential to accelerating treatment initiation and improving health outcomes [23].

The COVID-19 pandemic has highlighted the need for accurate, reliable diagnostic tools for population testing in all clinical care settings in an unprecedented way. On 31 December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was reported for the first time to the WHO. This virus, recognised as the aetiologic agent of COVID-19 disease, was identified as the cause of the ongoing pandemic [24]. Since it was first recognised, there has been an exponential rise in the number of cases globally. As of 3 July 2022, 550,218,992 confirmed cases of COVID-19, including 6,343,783 deaths, have been reported to the WHO (Figure 3) (<https://covid19.who.int/>). Thus, the high transmissibility of the virus, especially among asymptomatic carriers, may be one of its distinctive characteristics.

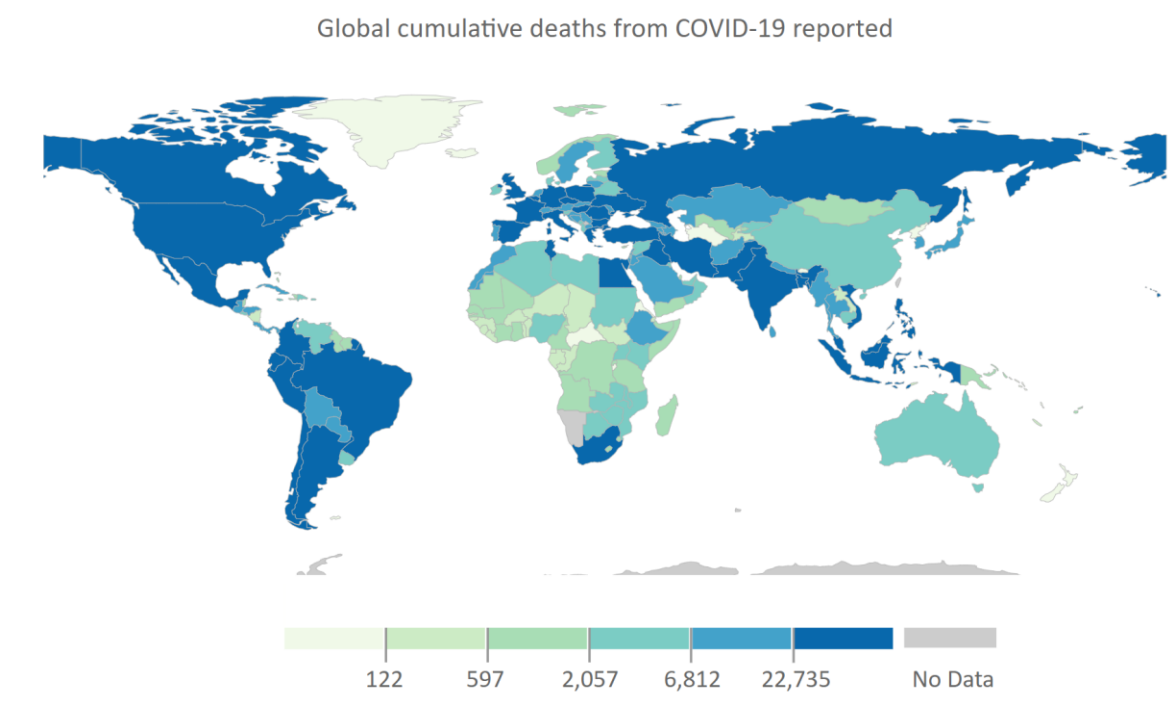


Figure 3. SARS-CoV-2 cumulative deaths reported from the WHO Coronavirus Disease (COVID-19) Dashboard. The data include deaths as of 3 July 2022.

The COVID-19 pandemic represents a global public health challenge. Since the virus began to spread it has impacted nearly every aspect of life not only on health but also on the environment, economy, education, and human psychology as reported in *Miyah et al* [25]. The Centre for Disease Control and Prevention (CDC) has been implementing public health interventions to reduce disease dissemination while engaging policies for disease detection and prevention [24].

To contain the epidemic, reduce disease transmission, and minimise the spread of the disease, the identification and isolation of contagious cases are crucial. As expected, an unprecedented number of SARS-CoV-2 testing schemes have been deployed widely across the world. The reference standard for diagnosing SARS-CoV-2 was quickly established as the detection of viral RNA by NA molecular analysis to assess a patient’s viral load [26], [27]. However, the long turnaround time (>12

hours) when employing reverse transcription polymerase chain reaction (RT-PCR) as molecular detection method makes difficult its use for timely decision making in the ED [28]. On the other hand, antigen or antibody tests, commonly known as rapid diagnostic tests (RDTs), which target the virus antigen or antibody by immunoassay directly from the clinical specimen, have become a common method and easy alternative solution due to the simple, rapid (< 30 min), and inexpensive technique. However, RDTs show a lower diagnostic sensitivity (40.0%–82.0%) compared to molecular tests (>90%) according to a study conducted by Reynard [28] testing different lateral flow devices and because of their imperfect sensitivity (leading to false-negative results), RDTs cannot be used to “rule out” COVID-19 in patients who are symptomatic [29].

The diagnostic process

A diagnostic process begins when a patient experiences a health problem, following which information is gathered and interpreted in order to determine a diagnosis. As part of the information gathering process there may be a clinical history interview, physical examination, consulting other clinicians as well as conducting diagnostic testing which over the past 100 years has become a critical feature of the evaluation of patient [30], [31].

Typically, a clinician would consider several diagnostic hypotheses to explain the patient's symptoms and diagnostic testing may occur during this phase to help the list of potential diagnoses to be refined as the diagnostic process progresses [32].

Diagnostic tests can be highly beneficial when they help identify a condition earlier than it is clinically evident, thereby having a positive impact on both health care workers and patients by reducing the time-to diagnosis and accelerating the therapeutic approach; for instance, a tumor may be detected by routine diagnostic biomarkers analysis, indicating the presence of a tumor-related biomarker before symptoms appear [32]. However, choosing the most appropriate diagnostic test is not an easy task.

According to a report published by the Centers for Disease Control and Prevention (CDC), there are currently thousands of diagnostic tests available and many more are expected to become available [33]. The availability of new molecular diagnostic technologies may constitute an essential part of the health care system, but unless they are used appropriately, they may add complexity to the diagnostic process which may negatively impact the healthcare worker.

Choosing the most appropriate test and interpreting its result requires an understanding of the patient's signs and symptoms, medical history, and a suspicion or pre-test probability of a disease [34], [35]. Generally, a pre-test probability refers to the probability that the patient will have the disease prior to receiving the test results. Upon receiving the results of the test, the post-test probability is used to determine whether a person has a high probability of contracting a disease. The

results of this measurement may be used to determine whether to accept a diagnosis of disease, rule it out, or order additional tests, which makes them important from a clinical perspective. It is important to use appropriate diagnostic tools and interpretation of diagnostic tests to avoid misinterpretation. When a diagnosis is accurate and made promptly, a patient has the best chance for a positive health outcome, as clinical decisions will be tailored to the patient's specific condition [36].

Consequences of misdiagnosis

A precise diagnosis is crucial to identify the aetiological agent causing the disease and can help to accelerate the right treatment, ensuring appropriate medical care [36]. The WHO emphasised a global priority in patient safety is to raise awareness about strategies that could be implemented to reduce diagnostic errors [37], [38]. If a diagnosis is missed, delayed, or incorrect, patients are at risk for omitted diagnostic investigations, inappropriate treatments, as well as increased financial and psychological stress [39]. A wide range of errors such as these reflect the limitations of the healthcare system. For example, in certain LMICs, diagnosis represents a tremendous challenge due to a lack of access to healthcare resources and testing facilities. This can lead to disease spreading, worsening of patient's symptoms, and risks of developing further health complications and, in the worst case, death [39],[40]. As a consequence of failing to diagnose appropriately, antibiotics are frequently overprescribed, contributing to the phenomenon of antibiotic resistance, which has become a major worldwide public health concern and requires international intervention to counteract its emergence [41], [42], [43]. Due to the importance of reducing the consequences of misdiagnosis, public health organisms enhanced the importance of developing reliable diagnostic tools as the first step of the diagnostic process. The availability of improved diagnostic tests can contribute significantly to the reduction of diagnostic errors, as well as provide guidance for regulating diagnostic treatments [44].

The diagnostic need

The WHO ASSURED and REASSURED criteria

In 2003, The WHO special programme for Research and Training in Tropical Diseases (WHO/TDR) published a set of criteria to describe the ideal test that can be used at all levels of the health care system in LMIC to guide treatment and clinical management decisions for infectious disease. The acronym that describes the seven criteria is "ASSURED", and it has become widely used as the dogma to develop an ideal test that can be employed at the POC [22].

ASSURED criteria emphasise the importance of accuracy, accessibility, and affordability. Currently, accurate diagnostic systems require sophisticated and expensive laboratory equipment and

are not easy to perform outside of laboratory settings. The consequence is that they are neither accessible nor affordable to patients at the lower levels of the health care system. Furthermore, for the community who have limited access to laboratories, the results may take extended periods and delay the appropriate timing of treatment.

The **ASSURED** acronym stands for:

- **Affordable** - Affordable and cost-effective diagnostics remain critical in a limited environment, given the economic conditions of many countries. Therefore, it is essential to have affordable diagnostic tools, so that a greater proportion of the population can have access to testing services.
- **Sensitive** - The test should minimise the rate of false negatives, as this would impact the results of diagnostic screening of the population.
- **Specific** - The test should minimise the rate of false positives. The ideal scenario would be to develop a POCT test with sensitivity and specificity that meets the TTP profile (TPP) indicated for the specific test or that meets the laboratory gold standard assay utilised and approved whenever possible.
- **User friendly** - The test should be easy to perform and require minimal training of personnel.
- **Rapid and Robust** - Results should be available within 60 minutes after sample collection, enabling patient management and treatment during the same visit, thus shortening the time from sample to result. Robustness refers to the ability of the test to overcome supply chain limitations without requiring additional transport and storage conditions.
- **Equipment-free** – This refers to the detached nature of the kit from laboratory equipment and its ability to be operated on small portable devices and ideally avoid the use of electricity. Completed tests are easy to dispose of and made of recyclable materials.
- **Deliverable to end-user** - Referencing the ability to ensure the technology reaches the end-users in resource-limited settings. Therefore, the organisational structures and relationships established must guarantee the coordination of selecting, procuring, shipping, storing, distributing, and delivering a new health technology to ensure it reaches resource constraint settings.

The original ASSURED criteria remain the core to developing a POC test although recently the advance in technologies has given the opportunity to further improve diagnostics by incorporating two additional criteria of real-time connectivity (**R**) and ease of specimen collection and environmental friendliness (**E**) to the original ASSURED, creating a new acronym, **REASSURED** [22].

Real-time connectivity - Wants to effectively ensure the real-time monitoring of the results, or a rapid result provided to the patient once the test has been completed. This would also allow the decentralisation of the result reading; the data would be shared in a common database, avoiding the

risk that incorrect data are recorded or transmitted. Data shared in a common database would also be beneficial for epidemiological surveillance, allowing a great amount of information to be stored to monitor the spreading of infectious disease and enhancing real-time decision-making, even across global health scenarios.

Environmental friendliness - To ensure that the increase in diagnostic tests being performed does not impact the environment, especially in limited resource settings where determined materials cannot be recycled releasing toxic fumes when burned.

The REASSURED criteria provide clear guidelines to help the development of POC devices, and significant efforts have been made to achieve similar characteristics, but challenges and limitations remain, and integration of sample-to-result systems at the POC has not been achieved yet [45].

Limitations in diagnostics and consequences

Limitations in microbiology

Significant limitations remain in the field of diagnostics. Bacterial and viral infections are not easily differentiated on the initial presentation of a patient. Both pathogens can induce similar clinical symptoms, making clinical history an imprecise tool for determining aetiology [46]. In the microbiology field, the gold standard methods for confirming bacterial infection are culture-based methods [47]. Depending on the pathogen, these assays can take days or even weeks to confirm the presence of the pathogen [48]. Furthermore, when the bacteria are present in an inaccessible body site or when antibiotics have been previously administered the result may come back negative [40].

One example that describes the limitations when using the pathogen-based detection approach is *Mycobacterium tuberculosis* (MTB), the aetiological agent of tuberculosis and representing one of the leading causes of death from an infectious disease globally, with an estimated 1.5 million (15%) deaths [49]. MTB is a slow-growing bacterium, so relying on culture method assays would require up to four weeks or more to grow [50]. Microscopy examination of the sputum specimen is difficult to obtain, especially in children. Most smears and cultures tend to be negative, leaving the diagnosis reliant upon clinical grounds, which is unreliable as symptoms are also common to other respiratory conditions (fever, weight loss, night sweats, cough) [8]. *In-vitro* and *in-vivo* immunodiagnostic tests such as the tuberculin skin test (TST) and the interferon gamma release assay (IGRA) are also used, but they can provide indirect evidence of past or current exposure to MTB [51]. The TST test assesses the exposure to mycobacteria but cannot discriminate between active and latent TB infection and has poor specificity. The IGRA tests such as the Quantiferon tests, are more specific for MTB (as the antigens are specific to MTB) but cannot distinguish current from latent or past infection [52].

The tests mentioned (shown in Figure 4) represent a limitation for rapid and reliable diagnosis, causing less than half of all cases of active TB to be promptly identified and treated, and similar diagnostic scenarios are common in viral and bacterial infections. As a result of these diagnostic challenges, the search for reliable biomarkers remains open [53], [54].

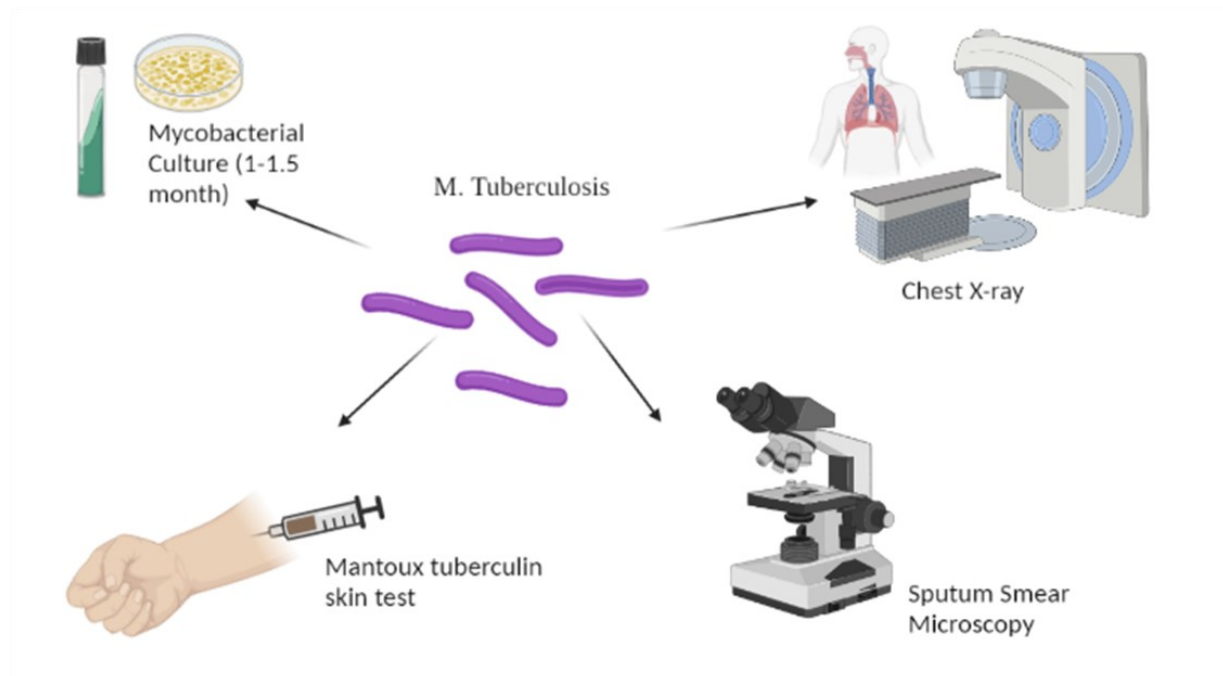


Figure 4. Laboratory diagnosis of *Mycobacterium tuberculosis*.

Illustrations show some examples of tests used in the laboratory to diagnose the presence of MTB. Illustration generated using Biorender.com.

Viruses and bacteria can often co-exist, being the cause of co-infection. This co-existence, or incidental occurrence of virus and bacterial infection, adds further complexity to the decision to treat an illness with antibiotics and thus leads to overtreatment with antibiotics for fear of missing bacterial infection [55]. Microbiologic diagnosis in most cases is time-consuming, and its success in providing accurate results is highly dependent on the type of specimen collection and pathogen that needs to be identified. Many viral infections share similar clinical symptomatology and their detection as a cause of severe disease represents a major step to identifying the best treatment, especially in high-risk groups. The diagnostic techniques for the diagnosis of viral infections include molecular detection by PCR and antigen detection that will be discussed later, serology to detect antigens and antibodies and tissue culture [56]. Serology tests blood samples for viral and bacterial antigens and antibodies (IgG and IgM) found in the blood that the immune system synthesises in the presence of a pathogen in order to diagnose diseases and determine the status of an individual's immune system. Tissue culture allows the pathogen to propagate in a controlled environment outside of a living organism and uses cell culture to study the pathogenic effect that the virus can use to impact the cell [56].

When assessing which diagnostic test is most suited to a certain scenario, factors such as the patient's stage of infection must be considered. During acute infection, the viral load is generally high

but may significantly decrease to levels that are not detectable as the infection is cleared. On the other hand, antiviral antibodies can take weeks to develop. For example, the levels of IgM and IgG antibodies produced against a virus can help to determine whether an infection occurred recently, but they are not present at the beginning of an infection [56]. Viral antibody and antigen detection methods are fast, but they require specialised equipment and, depending on the state of the disease, they might not be able to detect the viral antigens. This would result in low sensitivity of the assay, requiring other tests to verify the result. The success of the analysis depends on the nature of the sample itself; for example, viscosity or contaminants may affect the analysis, providing inconclusive results [57]. The low sensitivity of protein biomarkers compared to culture-based approaches is one of the major limitations of using protein biomarkers. In addition to the complexity associated with their use in industrial applications, their isolation and purification are expensive, making this application more challenging. On the other hand, culture-based methods, as discussed, can be extremely slow and require highly skilled technicians. Their cost, however, can also be high and they may not provide definite identification of the virus. Additionally, not all pathogens can be readily cultured in the laboratory [55].

In general, similar challenges in microbiology laboratories have pushed for new molecular approaches to diagnose and treat infectious diseases. Molecular tests are widely used in diagnostics as they can identify viral and bacterial pathogens reliably and accurately. Although promising, the implementation of new molecular technologies remains limited when the identification of the target is as challenging as MTB. This challenge motivates our research group to find alternative approaches to pathogen-based detection such as the use of biomarkers [58].

Limitations in NA detection methods

Molecular methods that are considered gold standards worldwide for identifying the causative pathogen are limited and expensive [59]. The most accurate amplification method to assess the presence of a target is the qPCR, but it requires a large, expensive instrument and a trained user who can process the sample and interpret the results [60]. Some successful qPCR devices have been developed to be employed in rich laboratory settings at the point of need to rapidly detect host genes or genomic DNA and RNA directly from pathogens [61]. Cepheid's Gene Xpert system, for example, detects pathogen DNA and RNA within 45 minutes, including a sample preparation module that can be inserted into the machine and has a fully automated system that processes and examines four different targets at the same time within each cartridge [61]. Roche's Cobas Liat has been used for viral and bacterial detection at the POC [61]. The system uses a benchtop multiplex RT-PCR that enables the screening of many pathogens. Another system developed by bioMérieux is the FilmArray

test, which integrates all the steps from sample preparation to nested multiplex PCR to sample detection within one hour [62].

Other molecular tests such as microarrays and RNA sequencing (RNA-seq), mostly employed as untargeted approaches (hypothesis-free) in well-resourced settings, are time- and cost-consuming and require highly skilled personnel [44]. These approaches, mostly used for genome-wide profiling purposes, present analytical sensitivity and quantification capabilities superior to other molecular tests (RT-PCR) with detection of amplicon specific reads even at the lowest tested spiking concentration. However, they require laboratory infrastructures and more complex post-experimental bioinformatics data analysis. Therefore, none of these methods represents the ideal concept of a POC test, limiting their widespread deployment while enhancing timing, cost, and complexity [21].

Limitations of sample preparation

Another limitation in the diagnostic field is related to the sample type, which can also introduce bias, especially from collection to analysis [63]. The clinical sample must be collected and stored appropriately to avoid sample contamination/degradation. Before analysing the target of interest, effective upstream preparation is required to ensure successful detection [64]. NA extraction is a determinant step in obtaining a reliable performance of the amplification product, but this is an extremely lengthy process consisting of instrument-based protocols. Therefore, the applicability at the POC is extremely limited [65].

When analysing gene expression analysis, the purification step is even more complex because studies are based on RNA isolated from whole blood [64]. Genomic DNA and other molecules that may affect downstream applications must be removed. Mature RNA is intracellular; therefore, cell lysis and purification steps are essential to separate RNA from sample and the cell [66]. RNA in general has the limitation of being less stable than DNA and degrades quickly if not manipulated under certain conditions [67]. For example, endogenous RNases can quickly degrade RNA; therefore, careful handling and storage of samples is critical to preserve its integrity. Techniques available for RNA or DNA purification take hours and are complex, far from being used at the POC or in limited-resource settings [63].

Approaches used to develop diagnostics

The motivation of my thesis is to combine different approaches to tackle the diagnostic limitations described, developing reliable, low-cost, and rapid diagnostic solutions to detect pathogens. Table 1 provides a summary of the diagnostic methodologies previously described and

their characteristics compared to the approach proposed in this thesis at the point of need. As shown, culture-based assays are the most inexpensive techniques (£1-6), they are still considered the “gold standard” for accessing the viability of pathogens; however, the state of some microorganisms can limit their detection capability [12]. Additionally, culturing can be very time-consuming (requiring 2 to 40 days), depending on the organism being cultured as well as expert personnel for culturing and result interpretation. Keeping in view the limited accuracy and slow determination of microorganisms, clinical diagnostics have been focused on culture-independent techniques [68].

On the other hand, RDTs such as antigen lateral flow-based assays have emerged for the rapid (15-30 min), handheld, easy to perform, and on-site detection of pathogens especially useful in limited settings although they lack in sensitivity compared to the reference standard (RT-PCR) as previously mentioned [28], [69]. Molecular NA amplification tests are reliable techniques in terms of analyte’s quantification and accuracy providing a remarkable advancement in pathogenic diagnostics [70]. However, they are considered expensive (£30-300) and difficult to implement at the POC. The approaches proposed in this thesis have been designed to overcome the limitations in molecular detection of NA. The aim is to achieve a sample-to-result turnaround time of 30 minutes at minimum cost (<£10) and with minimal physical intervention while providing accurate results comparable to the PCR standards in terms of diagnostic sensitivity and specificity.

Method	Analyte	Test time	Cost per test	QUANT	POC
Culture assays	Live virus or bacteria	1–40 days	£1–6	YES	NO
Rapid Diagnostic Tests	Antigen	15–30 min	£10–20	NO	YES
NA Amplification Tests	DNA or RNA	15min–12h	£30–300	YES	NO

Table 1. Overview of diagnostic techniques for the detection and identification of infectious diseases. QUANT = Quantitative; POC = Point-of-care.

Approach number 1: Isothermal amplification technology

NA amplification technologies (NAAT) are used in the field of molecular biology and recombinant DNA technologies as leading methods in detecting and analysing small quantities of NAs [71]. The most popular technique for NA amplification is represented by the PCR. However, it requires specialised equipment such as thermocyclers as the biochemical reaction is driven by precise regulation of three distinct elevated temperatures, long detection times (>1.5 hr) and highly trained personnel. Novel developments in molecular biology of DNA synthesis allowed DNA amplification

using isothermal methods such as transcription-mediated amplification (TMA), NA sequence-based amplification (NASBA), signal mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated amplification (LAMP), and helicase-dependent amplification (cHDA) [71] (Figure 5). In isothermal amplification of nucleic acids, nucleic acid sequences are accumulated rapidly and efficiently at constant temperatures. Various isothermal amplification techniques have been developed as alternatives to PCR since the early 1990s. It has been demonstrated that these methods of isothermal amplification can be used to detect targets such as DNA, RNA, cells, proteins, small molecules, and ions [71].

When using methods based on RNA transcription such as TMA, NASBA, and SMART (Figure 5, a), an RNA target is converted to ds cDNA with a promoter region through reverse transcription, followed by RNase H degradation of the original strand and DNA polymerisation initiated by a second primer. RNA polymerase amplification creates products that feed back into the original reaction, promoting the formation of more RNA and cDNA transcripts cyclically. NASBA requires three enzymes (RT-DNA pol, RNase H, and RNA pol); TMA requires two enzymes because the RT-DNA pol has intrinsic RNase H activity. SMART utilises a three-way junction with target and extension probes to initiate linear RNA polymerisation-based amplification [72].

Methods based on DNA replication with enzymatic duplex melting/primer annealing are HDA and RPA. In HDA, a helicase enzymatically “melts” dsDNA in RPA (Figure 5b), and a recombinase-primer complex scans dsDNA for the target site and facilitates primer binding. In both HDA and RPA, single strand binding proteins stabilise the separated strands; the rest of the reaction sequence is analogous to PCR [73].

Figure 5c illustrates the methods based on strand displacement using polymerases only from a linear target, through the use of outer primers. LAMP [74] involves six recognition sites on the target DNA; the 5' overhangs of the inner primers recognise sequences in the amplicon, which leads to the generation of a dumbbell structure. CPA [75] involves four recognition sites on the target DNA, and the inner primers lead to cross-priming after the first round. SMART-AMP [76] includes five recognition sites on the target DNA and a fifth booster primer; single nucleotide polymorphism discrimination is facilitated by the MutS protein.

Methods based on strand-displacing polymerisation (shown in 4, d) can be from inherently circular targets in RCA or from padlock probes that are circularised through the action of a ligase in RAM. In both cases, continuous amplification can be initiated through a second primer [32].

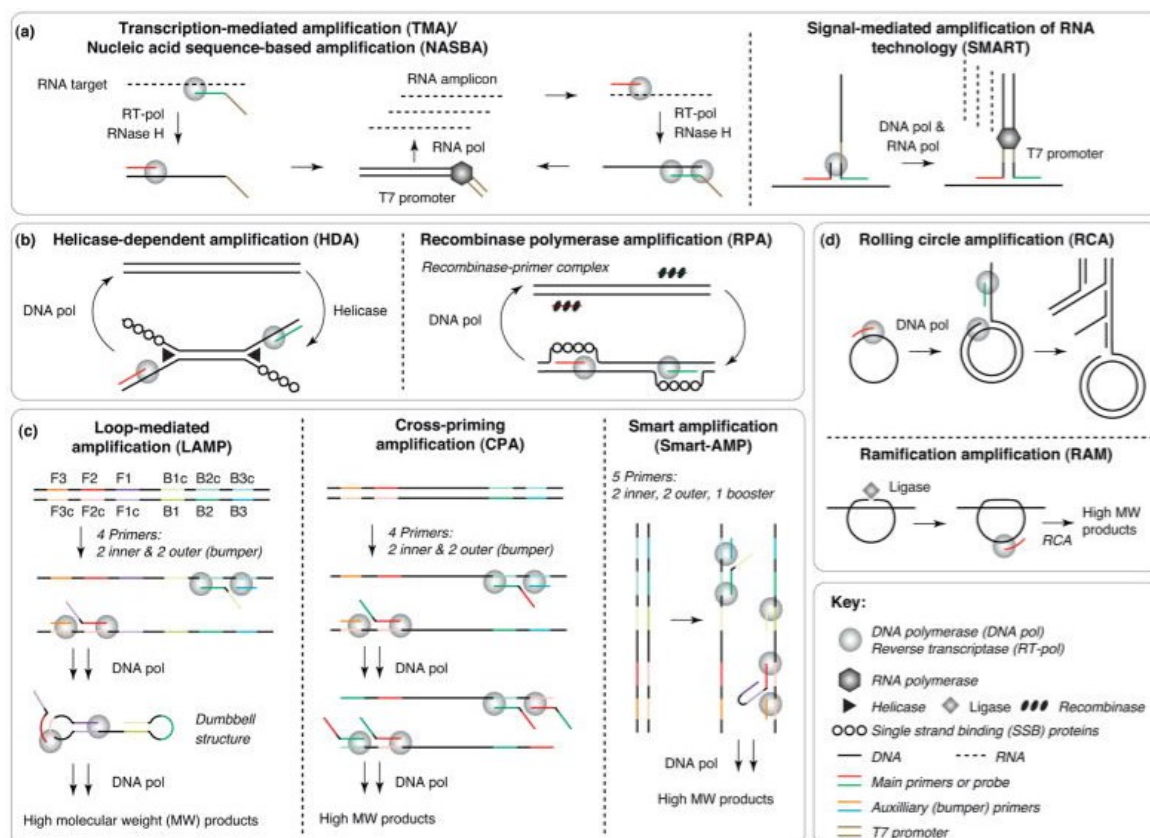


Figure 5. Overview of isothermal NA amplification reactions. Reprinted with permission from Niemz A et. Al [71].

Among the isothermal NAAT, LAMP is emerging as a popular method due to its simplified thermal management, offering exponential amplification of DNA and RNA targets based on the refolding and generation of self-priming structures that can be used as new templates, utilising one single enzyme with self-strand displacement activity allowing an overall accuracy comparable to the gold-standard PCR technique. Rodriguez-Manzano and Georgiou's group within the EEE Section has extensively employed the LAMP method to successfully detect pathogens such as *Plasmodium falciparum*, *Klebsiella pneumoniae*, and SARS-CoV-2 [77], [78], [79]. The LAMP method was used in this thesis as the predominant detection chemistry in conjunction with the proposed POC methods, and more details are provided in Chapter 2.

Approach number 2: Host gene-based biomarkers detection

Whole blood gene expression signatures were used to identify several bacterial and viral infections or pathogen-specific diseases such as malaria, dengue virus, respiratory syncytial virus infection, Kawasaki disease, TB, and SARS-CoV-2 infection [80]–[83]. This strategy has become increasingly popular due to its ability to quantify a candidate biomarker and provide a comprehensive diagnostic answer, as opposed to directly targeting the pathogen [84]. Many studies have shown that

the conserved nature of the host response offers novel approaches for diagnostic development in infectious diseases, allowing specific infections to be identified by the pattern of genes activated during the inflammatory response [80].

When a pathogen infects the organism, the host response, through the activation of different biological pathways, offers a complementary strategy to identify the infection. Differential gene expression analysis allows to identify the key genes that undergo changes in expression. Key genes can act as markers of disease holding the potential to be quantified and consequently used for the diagnosis of infectious diseases [61] (Figure 6).

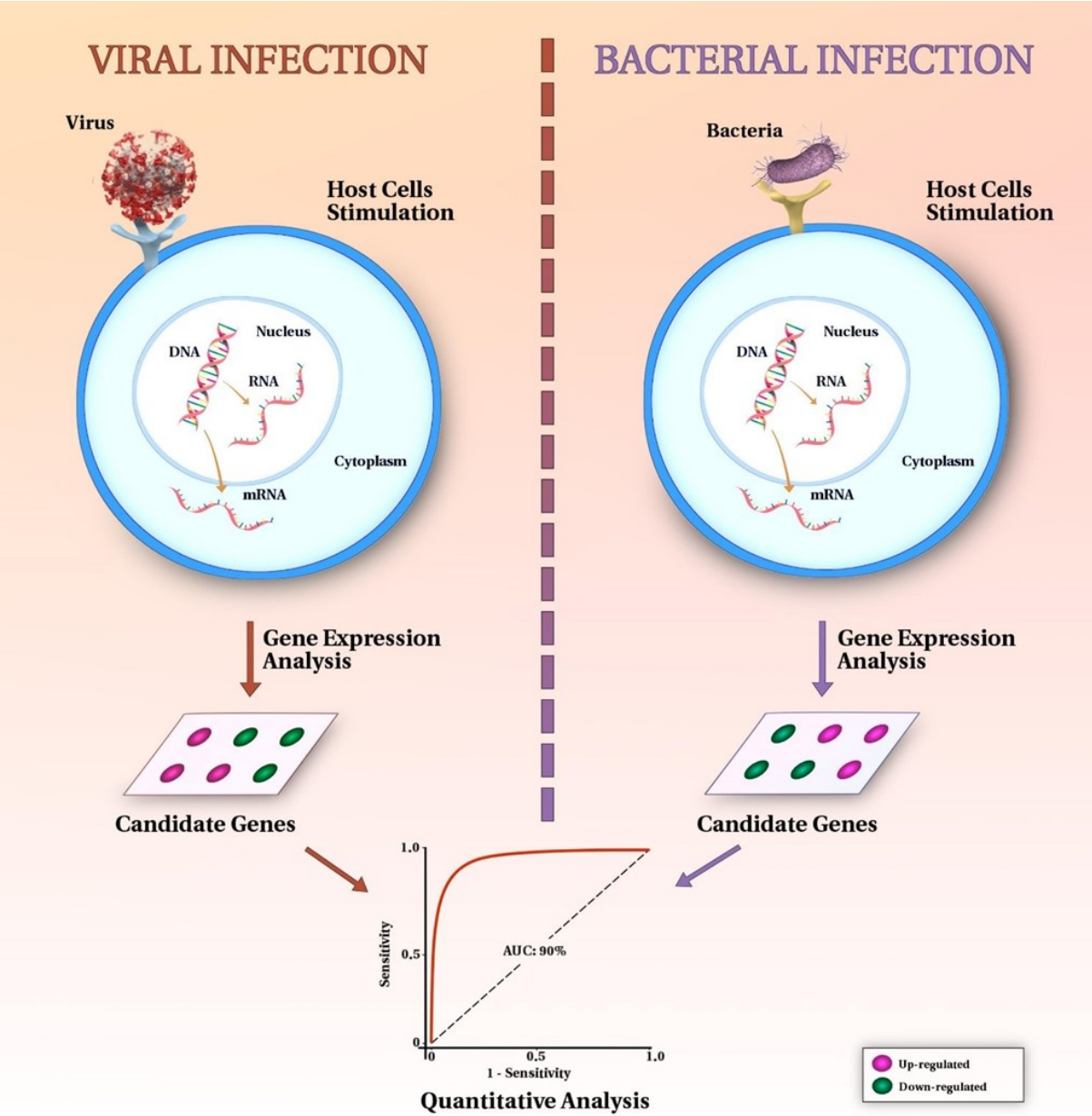


Figure 6. Bacterial and viral pathogens stimulate the host inflammatory cells, providing specific changes in the host's blood gene expression.

Different host-transcriptomic responses can be quantified in whole blood and quantified using molecular methods, allowing measurement of the diagnostic performance (e.g., Area Under the Curve) for the selected signatures to discriminate among infectious diseases. Illustration generated using Biorender.com.

Detection of gene expression signatures requires clinically usable technology for reliable and reproducible measurements of gene expression. In the early 1990s, researchers began studying the entire transcriptome (sum of RNA transcripts) and since the late 1990s, technological advancements have made transcriptomics a widely accepted field of study [85]. An early method of transcriptomic analysis based on sequencing was serial analysis of gene expression (SAGE) where transcripts were quantified by matching fragments of transcripts to known genes. The use of these methods was, however, largely superseded by high throughput sequencing of entire transcripts, which provided additional information concerning transcript structure, e.g., splice variants [85], [86].

In the mid-1990s and 2000s microarrays and RNA-Seq emerged as the dominant contemporary techniques. As early as 1995, microarrays were described for measuring transcript abundances through hybridization of probes to an array of complementary probes [87]. By using microarray technology, it was possible to simultaneously assay thousands of transcripts at a greatly reduced cost per gene and with a significant labor saving.

Until the late 2000s, spotted oligonucleotide arrays and Affymetrix high-density arrays (Santa Clara, California) were the methods of choice for transcriptional profiling [88]. As a result of advances in fluorescence detection, low abundance transcripts can now be measured more accurately and with greater analytical sensitivity due to high-throughput sequencing technologies. The RNA-Seq technique refers to the sequencing of transcript cDNAs, in which abundance is derived from the number of counts for each transcript [89].

The use of RNA-Seq began to gain popularity after 2008, when new technologies developed by Illumina (San Diego, CA) enabled the recording of millions of transcript sequences. With this yield, it is now possible to quantify the entire human transcriptome with accuracy [89]. Currently, the two main methodologies employed in order to generate RNA transcript data rely on the sequencing of individual transcripts (ESTs or RNA-Seq) and hybridization of transcripts to arrays of nucleotide probes (microarrays) [90].

The RNA host biomarkers, specifically blood signatures, which will be described in Part I of the thesis, can achieve high diagnostic accuracy in distinguishing bacterial from viral infections. Their accessibility when detecting pathogens that are confined in remote parts of the body is remarkable [61]. From a practical perspective, accessing a blood sample is much easier than obtaining an infected tissue. Clinical markers of disease severity have been seen to be correlated with changes in host expression, generating a transcriptomic severity score that can also play a role in helping classify patients according to their clinical conditions and monitoring disease status and response to therapy [61].

In Levin's research group, many studies using host-gene expression signatures have been undertaken to establish the feasibility of whole blood transcripts in identifying pathogens. Anderson and Kaforou [58] used genome-wide analysis of RNA expression in host blood to identify a 51-transcript signature distinguishing TB from other diseases in African children with a risk score showing a sensitivity of 82.9% (95% CI, 68.6%- 94.3%) and a specificity of 83.6% (95% CI, 74.6%-92.7%) for culture-confirmed TB compared to the sensitivity of the Xpert MTB/RIF assay for molecular detection of *M. tuberculosis* DNA, supporting the use of RNA expression signatures in helping distinguishing TB from other diseases [52]. *Herberg et al.* [83], [91], in a cross-sectional study that included 370 febrile children, used a two-transcript host RNA signature for discriminating children with bacterial infections from those with viral infections, with a sensitivity in the validation group of 100% (95% CI, 85%-100%) and specificity of 96.4% (95% CI, 89.3%-100%) when compared to reference-standard clinically assigned diagnoses [91].

In light of the current pandemic, more recent studies have validated blood transcriptional signatures to detect viral infections, including COVID-19 [83]. Using whole-blood RNA-seq Li, Ho Kwong et al. identified three-gene signatures discriminating bacterial from viral infections with infections with AUROC of 0.976 (0.919–1.000), sensitivity 97.3% (85.8-99.9), and specificity of 100% (63.1-100) achieving higher benefit in decision curve analysis than either CRP or leukocyte count for discriminating viral infections from all other infections. When SARS-CoV-2-positive participants were included, the signature discriminated 35 bacterial infections from 34 SARS-CoV-2-positive COVID-19 infections with AUROC of 0.953 (0.893-0.992), sensitivity 88.6%, and specificity of 94.1% providing proof of principle that small RNA transcript can differentiate viral infection including COVID-19, from other emergency presentations of infection in adults [83].

Host-based approaches appear to be suitable candidates in the diagnostic field for accuracy when screening thousands of different targets and they are currently being translated into diagnostic tests [92]. Part I of the thesis focuses on the use of the gene expression approach to discover its potential to be translated into a POC test for discriminating bacterial and viral infections with the intent to provide early diagnosis and appropriate therapy.

Approach number 3: Electrochemical-based detection of NA amplification

It has been discussed how the main molecular-based methods employed to enable quantitative and sensitive detection of NAs are limited by the requirement of bulky instruments, costs and complex data analysis [69], [70]. Isothermal amplification techniques previously described can be transferred on microchip-based technologies as they do not rely on fluorescence-based detection and can be easily used in combination with chemical sensors where specific analytes are converted into measurable molecules that can provide a quantitative result [93],[94]. Electrochemical-based

detection of NA on-chip is an emerging technology and the integration of the sample preparation and DNA array detection in the so-called 'lab-on-a-chip' configuration has the goal of fully integrating multiple processes on a microfluidic platform. The ability to perform all the steps of the biological assay on a single self-contained microchip promises significant advantages in terms of speed, cost, sample/reagent consumption, contamination, efficiency, and automation [94]. Such miniaturisation of the analytical instrumentation enables transportation of the laboratory to the sample source, as desired for POC testing. On these credit-card-sized micro-laboratories, on-chip fluid manipulations (Figure 7) have been demonstrated to transport microliters samples through a network of microchannels [94]. The on-chip approach using electrochemical detection in combination with LAMP chemistry has been described in this thesis as a successful method to detect infectious diseases for POC applications [95]. The electrochemical-based detection method is considered a promising tool for fast target detection because of its large-scale integration, low-cost manufacturing, and high diagnostic sensitivity when compared to the reference standard RTqPCR as well as capabilities for multiplexing purposes [93].

Part I of the thesis discusses the use of host-gene transcripts to discriminate bacterial and viral infections by employing the electrochemical-based approach, and Part II provides evidence of how the approach was successfully used to identify the presence of SARS-CoV-2 in clinical samples.

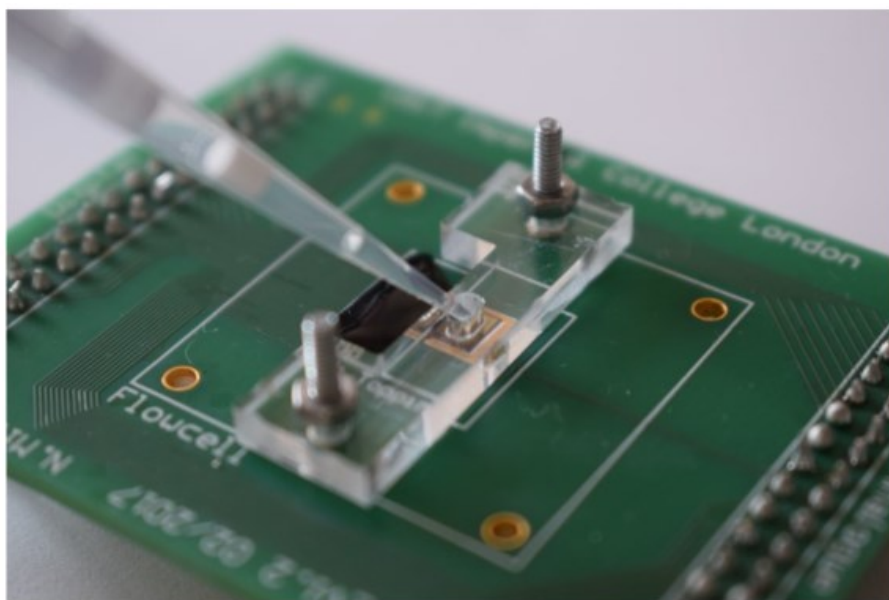


Figure 7. Example of a setup of on-chip reaction for NA detection. Loading sample and reagents on the top of the chip. A chamber to contain the reaction has been made by laser cutting an acrylic manifold screwed on top of the green cartridge. Chip designed by N. Moser.

Approach number 4: Electricity-free sample preparation

NA extraction (NAE) is still a lab-based procedure considered crucial for the successful detection of the analyte of interest. However, the complexity of current NA isolation methods makes

them unfeasible to use at the POC [96]. Extracting NA from clinical specimens requires careful handling to avoid degrading the NA and labour-intensive extraction processing. Commonly, samples are either applied to columns that require a series of centrifuging steps to purify the molecule of interest or bound to functionalised magnetic nanoparticle surfaces to go through all the purification requiring vortexing steps. To overcome the sample preparation limitations, a novel low-cost (<£10), simple, and accurate (SEN >90%) methodology has been developed to extract DNA/RNA via magnetic nanoparticles in less than three minutes without the need for electricity or cold-chain conditions. In Part II of the thesis, this methodology and its application in the diagnostic field are described.

Part I

Chapter 2

Validation of a two-gene signature on clinical samples using traditional diagnostic platforms

Part I of the thesis consists of Chapters 2 and 3 and focuses on how molecular methods presented in Chapter 1 can quantify host-transcriptomic responses to determine a diagnostic outcome for discriminating bacterial from viral infections on a low-cost LoC technology.

This study was performed on a paediatric population in close collaboration with the Section of Paediatric Infectious Diseases at St. Mary's, led by my supervisors Prof. Levin and Dr Kaforou and the department of EEE, led by my supervisors Dr Rodriguez-Manzano and Prof. Georgiou.

The conceptualisation of the work, the laboratory work, and the statistical analysis have been my contributions. To distinguish between bacterial and viral infections in children, I selected an optimal two-transcript host RNA signature (*IFI44L*, *EMR1*). The candidate gene combinations derived from datasets based on well-phenotyped cohorts (IRIS, EUCLIDS and PERFORM studies) were made available to me by the St. Mary's Paediatrics Infectious Disease group.

This chapter focuses on two main approaches:

1. The potential of using the host-gene expression approach to differentiate the two infection groups in children.
2. The development of RT-LAMP assays as an important step in the translation process and an alternative to state-of-the-art technologies (qPCR and microarray).

Concepts in Part I of this thesis resulted in the following journal articles and patent:

- **I. Pennisi**, J. Rodriguez-Manzano, A. Moniri, M. Kaforou, J.A. Herberg, M. Levin and P. Georgiou, "Translation of a host blood RNA Signature distinguishing bacterial from viral infection into a platform suitable for development as a Point-of-Care Test", JAMA Paediatrics, Jan 2021.
- **I. Pennisi**, J. Rodriguez-Manzano, N. Miscourides, A. Moniri, N. Moser, D. Habgood- Coote, M. Kaforou, J. A. Herberg, M. Levin and P. Georgiou, "A method for determining a diagnostic outcome", (Assignee: Imperial Innovations Limited). Filed 24-08-2020.
- **I Pennisi**, A Moniri, N Miscourides, L Miglietta, N Moser, et al. Discrimination of bacterial and viral infection using host-RNA signatures integrated in a lab-on-a-chip technology. Biosensors and Bioelectronics 2022 (accepted for publication).

Introduction

Why is the diagnosis of bacterial and viral infections important in children?

Diseases of bacterial aetiology and discrimination of bacterial and viral illnesses represent a challenge for medical teams worldwide, especially in febrile children [61]. Children under five are at greater risk of contracting infectious diseases in comparison to adults, especially in LMIC, where nutritional deficiency and unhygienic conditions are also a burden [61], [97]. The use of symptom-based examinations cannot be considered sufficient to differentiate between bacterial and viral infections, since symptoms are often similar in most cases, complicating the diagnosis [61]. The focus of rapid diagnosis of infectious diseases of children in the last decade has shifted from conventional laboratory techniques (antigen detection, microscopy and culture) to that of molecular diagnosis of infectious agents [98]. Several studies have shown that specific infections can be identified by the pattern of host genes activated during the inflammatory response [58].

Bacteria and viruses activate or inhibit different signalling pathways in the cells they infect and give rise to different host transcriptional signatures [21]. Many of the newly evaluated gene biomarkers, especially in combination, have achieved high accuracy in distinguishing between bacterial and viral infections [99]. In our research group, approaches based on host-derived RNA signatures have been successfully demonstrated to play a key role in the diagnosis, prognosis, or treatment of disease [62], [61], [91], [100]–[102]. These studies have suggested that specific infections can be identified by the pattern of host genes activated or suppressed in blood response during the inflammatory process. In this chapter, a subset of genes is identified and used as the basis for a diagnostic test.

Host-gene expression mechanism

All cells have the same genetic information, stored in deoxyribonucleic acid (DNA). Even though DNA is the same across the cells of an organism, the specific genes that are expressed, their level of expression, and their isoforms vary both between cells and between time points. Gene regulation defines the gene expression landscape of each cell [103]. DNA is made of nucleotides, each of which is composed of a monosaccharide sugar, a phosphate group, and a nitrogenous base (adenine, guanine, thymine, cytosine-AGTC). The sugar of the nucleotide and the phosphate of the next are joined by covalent bonds forming a strand. Hydrogen bonds bind the bases of adjacent strands to one another, bringing the two strands together and forming a double helix. The order of the nitrogenous bases (gene) holds the information of the genetic code and the generation of

functional products, and this encoded information is transferred to a ribonucleic acid (RNA) molecule through transcription.

Transcription factor proteins (TFs) in eukaryotes repress/suppress or activate the transcription of a gene. TFs can either recruit an enzyme (RNA polymerase) or block its binding to the promoter area of the gene of interest, to start or interrupt the transcription process. RNA polymerase is essential in the synthesis or production of the primary RNA molecule. If the gene that undergoes transcription encodes for a protein, the product of transcription is messenger RNA (mRNA) which, after processing and editing, will convey the genetic information to the ribosome [104]. For a protein to be produced, the mRNA exits the nucleus and undergoes post-transcriptional processing steps. One of these steps consists of splicing introns (addition of 7-methylguanosine to the 5' end and polyadenylation of the 3' end). The splicing of the introns (Figure 8) allows the mature mRNA to represent only the exonic, or coding, region of a gene. In gene expression, the mechanism of alternative splicing is crucial as it regulates isoform expression cell-specifically or time-specifically [59].

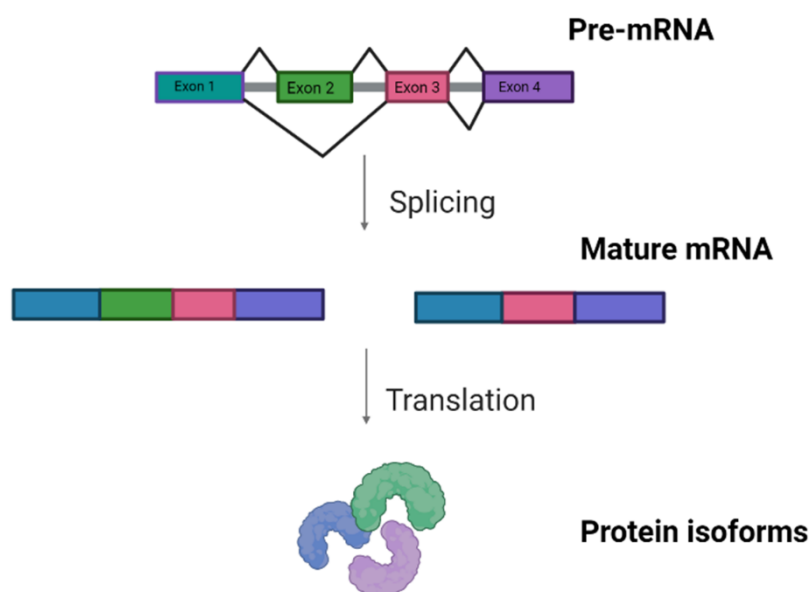


Figure 8. Splicing: Pre-mRNA is the first form of RNA generated through the transcription process. To generate mature RNA, all introns have to be removed from the transcribed RNA through the splicing process. Illustration generated using Biorender.com.

A mature mRNA molecule consists of a 5' untranslated region (UTR) that functions as a regulatory sequence to control gene expression and a start codon that instructs the ribosome to begin translation of the coding region. A stop codon (followed by a 3' UTR and a polyadenylated tail) instructs the ribosome to stop translation. After the mRNA is transported into the cytoplasm, it is translated into protein by the ribosomes in the endoplasmic reticulum, as shown in Figure 9 [105].

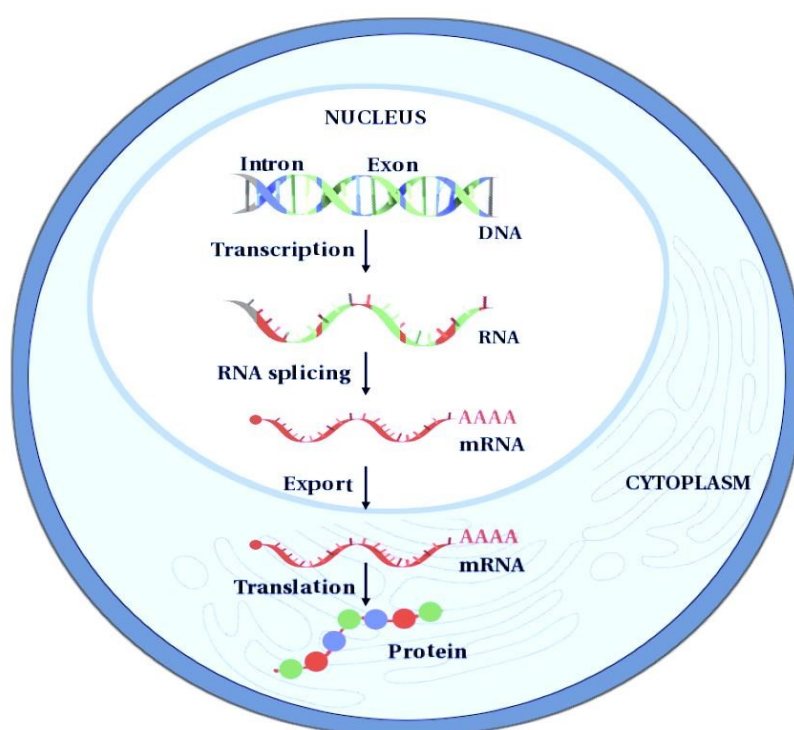


Figure 9. An overview of the eukaryotic cell from Nature Education [106] showing the flow of information from the transcription of DNA to RNA, splicing into mature RNA and translation into a protein. Illustration redrawn using Biorender.

The expression levels of a specific gene can be quantified by measuring the abundance of the final product (protein or functional RNA species) or its precursor (mRNA) [106]. Measuring the amount of mRNA can allow us to evaluate cellular activity. When measuring gene expression levels peripheral blood is considered the first choice because it is easy to access, and most importantly, peripheral blood cells and other organs (heart, brain, lung, kidney, colon, spleen, stomach, prostate, liver) can share more than 80% of the transcriptome, thus reflecting the immunological state of the organism [61]. A study from *Herberg et al.* [91] examined whether the pattern of host genes activated or suppressed in blood in response to infection could distinguish bacterial infection from other causes of fever in children. In Herberg cross-sectional study that included 370 febrile children, those with bacterial infection were distinguished from those with viral infection, using a 2-transcript signature.

In the next two chapters the two specific genes *IFI44L* and *EMR1* are identified and analysed as the basis for a diagnostic test. The gene *IFI44L* stands for Interferon-induced protein 44-like, composed of 452 amino acids it is located on chromosome 1, area p31.1. It represents a protein coding gene that belongs to the IFI44 family and functions as a type I interferon-stimulated gene. As a result of the type I interferon response, cells are protected from invading viral pathogens. This defence is mediated by cellular factors produced by interferon-stimulated genes (ISGs). Among the ISGs, *IFI44L* appears to exhibit a more targeted antiviral specificity [107]. As reported by Schoggins J *et al*, *IFI44L*

appears to be involved in a variety of innate immune processes and can be stimulated by a variety of viruses, including influenza and HIV-1 [107]. It has also been observed that IFI44L is upregulated after a wide range of viral infections and may therefore be used as a promising biomarker of viral infection [108], [109]. The *EMR1* gene, also known as *ADGRE1*, encodes a seven-transmembrane G protein-coupled receptor with a domain containing repeated Epidermal Growth Factor (EGF)-like calcium binding regions [110]. According to some studies, the F4/80 antigen, encoded by the *Adgre1* locus, has been widely used as a monocyte-macrophage marker in mice, but its utility as a macrophage marker in other species remains unclear [109]. In response to infection or injury, classical monocytes proliferate in the bone marrow and are released into the bloodstream. During an infection with bacteria, these monocytes travel to the infection site and phagocytose pathogens. They release chemokines that lead to the recruitment of other immune cells, and present antigen through class II major histocompatibility complex. Recent studies [111] have supported the hypothesis that *ADGRE1* has evolved in response to selection by pathogens and participates in the host defense. However, due to lack of knowledge, it has been difficult to evaluate the function of this gene [111].

The next section gives an overview of the state-of-the-art amplification technologies employed in this chapter to quantify gene expression: RT-qPCR, RT-qLAMP and microarrays.

Reverse Transcriptase – quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR is considered the gold standard for accurate, sensitive and fast measurement of gene expression. To quantify mRNA levels, two steps are usually performed: reverse transcription (RT) and real-time quantitative PCR [112].

The RT step uses mRNA as a template to synthesise a more stable complementary DNA (cDNA) molecule in a reaction catalysed by the enzyme reverse transcriptase (RT). The RT enzyme requires a primer of poly-T that is complementary to the poly-A tail at the 3' end of the mRNA molecule. RT then copies the RNA to make a double-stranded DNA/RNA hybrid. The RNA strand is degraded, and the new single-stranded DNA is copied by DNA polymerase to form a double-stranded cDNA copy of the original RNA. Therefore, cDNA is the starting material for qPCR amplification and is much more biologically stable than using RNA. In a standard PCR reaction, two primers (forward and reverse) of ~18-25 nucleotides in length anneal to strands of the DNA duplex, and due to the availability of the 3' hydroxyl group the polymerase can extend. The quantitative PCR consists of three stages: 1) denaturation of the double-stranded DNA at approximately 95°C, 2) annealing with the new forming chain at 55°C, and 3) elongation at 72°C. These three steps make up a cycle, and repeated cycles (typically 35 cycles) lead to exponential amplification of genetic material, generating thousands of millions of copies of the targeted sequence within a few hours [113].

When using real-time qPCR, the amount of DNA product can be monitored at every cycle of the PCR reaction. The detection method is based on fluorescence intensity, measured at each cycle of amplification through a spectrophotometer incorporated in the machine. The amount of DNA synthesised in the PCR reaction is measured at each cycle, using, for example, probes labelled with fluorescent tags that are only capable of fluorescing when annealed to their target sequence. The rate of increase of fluorescence is directly proportional to the number of molecules of the specific RNA present at the start of the reaction [114][115]. The cycle number at which the fluorescence first exceeds the threshold line is known as the quantification cycle, C_q (often referred to as the threshold cycle, C_t - Figure 10). The C_q value will be large if the DNA molecules present at the start are small and vice versa. By using the C_q value, it can either use relative quantification to analyse changes in the target DNA molecule when compared to the reference sample or use C_q and the standard curve generated from a known quantity sample to determine the absolute quantity of the target DNA molecule. For a positive reaction, the amplification curve shows four phases: ground, exponential, linear and plateau (Figure 10). In the ground phase, the reactions have not yet taken place. At the exponential stage, the PCR efficiency achieves 100%, the copies of DNA samples double in each cycle, and there are still plenty of all reaction components. In the linear stage, the amplification continues but slows down. In the plateau phase, PCR reactions are saturated.

Even though RT-qPCR is a sensitive and accurate method, it is still expensive, requiring high-level expertise and a laboratory setting. Nevertheless, it can be a highly reproducible technique that allows the detection of extremely low amounts of RNA, making it one of the most-used technologies in research and diagnostics laboratories [116].

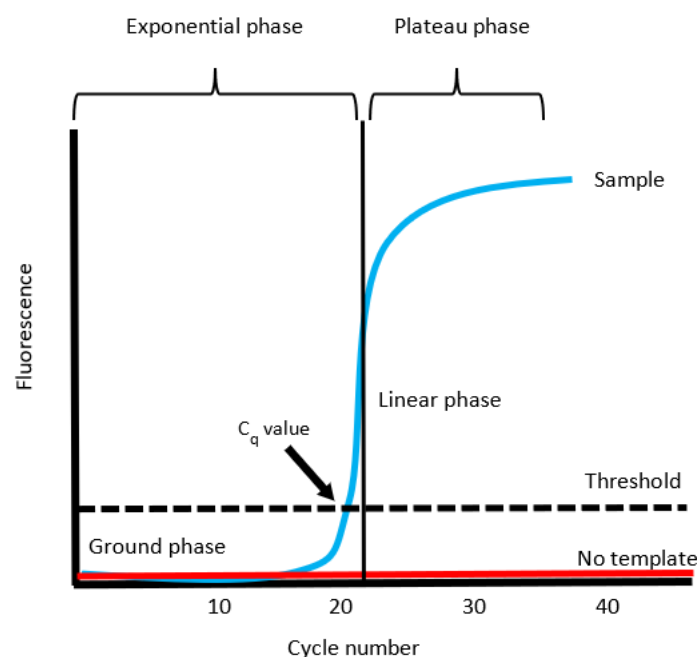


Figure 10. Readout signal of a typical PCR reaction for samples in which the template is either absent or present, known as a positive or negative reaction.

Loop-mediated isothermal amplification (LAMP)

Isothermal amplification methods have recently begun to be used in the diagnostic field, especially during the COVID-19 pandemic [117]. This is due to their capability to provide rapid and accurate DNA and RNA detection using isothermal temperature. Although PCR-based detection approaches are the most used technologies for NA amplification, due to their need for thermocycling, they have several disadvantages compared to isothermal amplification, such as lack of portability, cost-effectiveness, and non-suitability in low-resource diagnostic fields [118].

One among the most efficient isothermal amplification technologies is the LAMP. This technology was developed by *Notomi et al.* [119] and requires the use of Bst DNA polymerase, an enzyme with a strand-displacement activity that performs at the optimal temperature of 60°C to 65°C [120]. A common LAMP reaction contains four to six primers targeting six to eight regions in the targeted sequence, which helps increase the specificity of the amplification. Two inner primers (FIP and BIP) are key for the formation of a dumbbell structure, two outer primers (F3 and B3) and two loop primers (LF and LB) that accelerate the DNA amplification by hybridising and extending from the hairpin loops.

The forward (FIP) and backward (BIP) inner primers consist of two sequences (F1c-F2 and B1c-B2) targeting two different regions on the target. The F2 region is at the 3' end and the F1c region at the 5' end. The F2 region is complementary to the F2c region of the template sequence. The F1c region is identical to the F1c region of the template sequence. The BIP consists of a B2 region at the 3' end and a B1c region at the 5' end. The B2 region is complementary to the B2c region of the template sequence. The B1c region is identical to the B1c region of the template sequence [120].

The LAMP mechanism depicted in detail in Figure 11 is initiated by strand invasion of the DNA template by hairpin-forming LAMP primers that anneal and extend. The annealed primers are displaced by displacement primers, F3 and B3, leading to the formation of a dumbbell structure that has multiple sites for initiation of synthesis. The final products obtained are a mixture of stem-loop DNA with various stem lengths and various cauliflower-like structures with multiple loops (Figure 11). The structures are formed by annealing between alternately inverted repeats of the target sequence in the same strand. This results in less time for exponential amplification and detection.

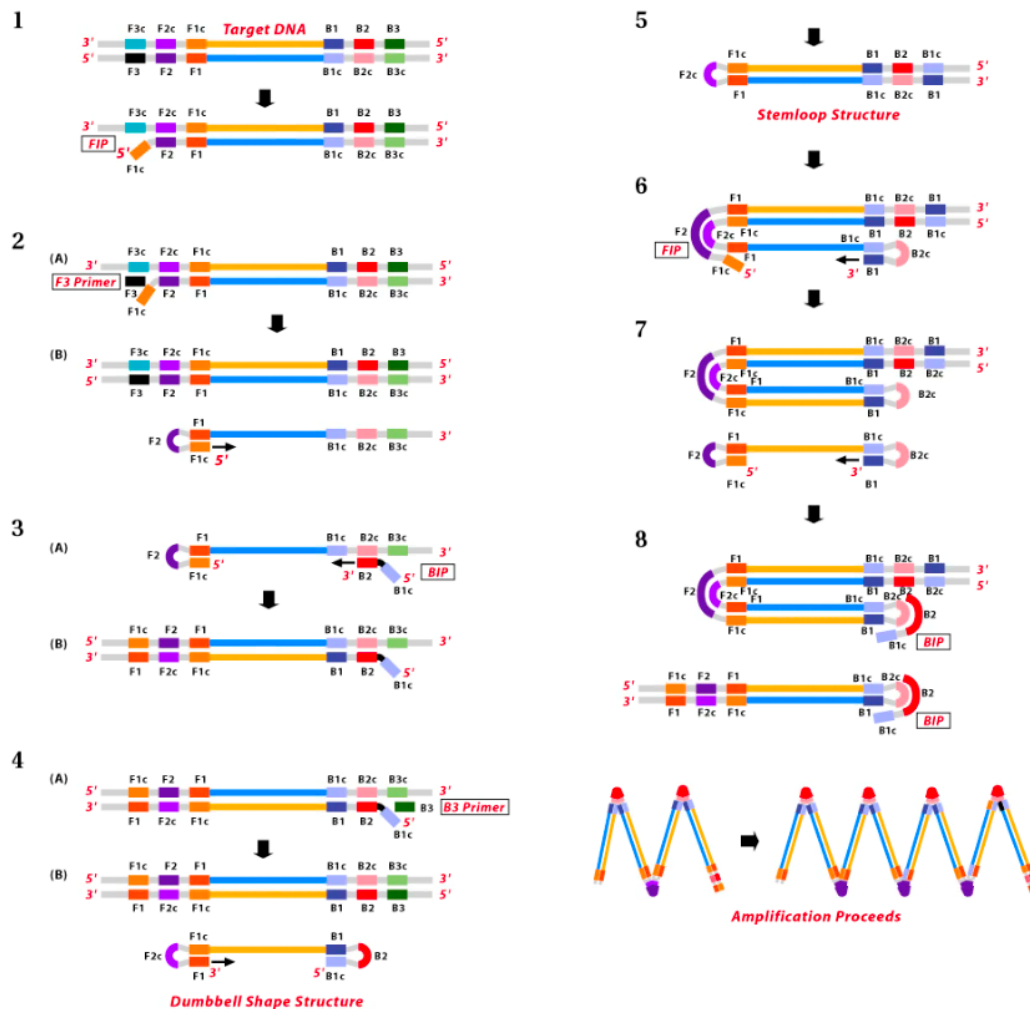


Figure 11. LAMP amplification initiation, cycling and elongation.
 Readapted from premierbiosoft (http://www.premierbiosoft.com/tech_notes/Loop-Mediated-Isothermal-Amplification.html).

LAMP has been extensively validated for the detection of viral, bacterial and parasitic diseases, [121] demonstrating several advantages over the years, some of which are as follows:

1. DNA amplification takes place at an isothermal condition (63-65°C) with higher efficiency, generating DNA even up to a billion copies over less than an hour, compared to a million copies yielded by the PCR [122].
2. The Bst DNA polymerase most used in LAMP shows a high strand displacement activity, which eliminates the thermal DNA denaturation stage.
3. LAMP enhances specific amplification due to the use of two to three pairs of primers (internal, external, and loop), which recognise up to eight specific locations on the DNA or RNA targets.
4. LAMP is cost-effective as it does not require special reagents or sophisticated equipment.
5. This technology can be used for the amplification of RNA templates in one step by the inclusion of the reverse transcriptase enzyme in the reaction mix.

6. The products of LAMP can be detected much faster than in standard techniques, sometimes only requiring analysis with the naked eye [123], [124].

Microarrays

Microarrays use the array technology introduced in the 1950s, when the attachment of biomolecules to a solid surface was used for the first time to generate immunoassays for antibody binding and later for NAs (Southern blot) [125]. The principle behind the DNA microarray is that complementary DNA sequences will bind to each other and emit fluorescence proportional to the amount of NA that is bound. Thousands of distinct strands of DNA oligonucleotides are immobilised onto a solid glass surface in discrete spots with high density ($\sim 500\text{spots}/\text{cm}^2$). Each array contains over 30,000 spots, typically around 100-300um in size, and each spot contains multiple identical oligonucleotides. The spots are organised in a grid of rows and columns with known locations. Due to the micro-scale of the experiment, the fabrication of the array involves robotic machinery of high precision and speed, which prints the oligonucleotide probes on the surface [103]. The arrayed probes are complementary to the transcript of interest and therefore suitable for hybridisation. The steps involved in cDNA-based microarray are illustrated in Figure 12.

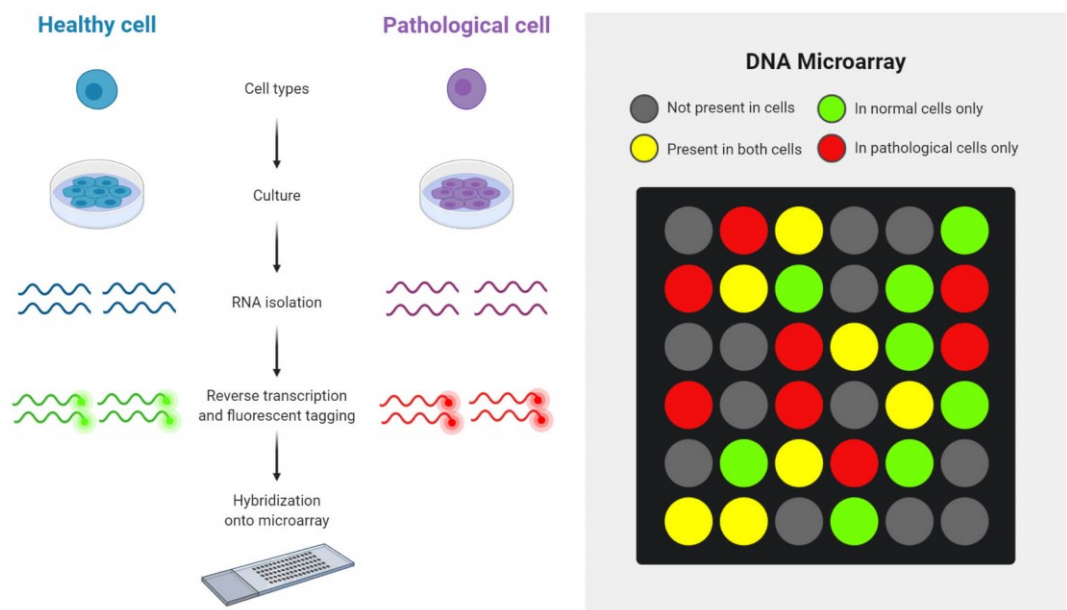


Figure 12. cDNA-based two-colour microarray steps.
Illustration reprinted from http://www.premierbiosoft.com/tech_notes/microarray.html. Image by Sagar Aryal.

The steps to measure gene expression are as follows [126]:

- (1) RNA is extracted from the sample of interest.
- (2) Purified mRNA is reverse transcribed to cDNA. The latter is then amplified and tagged with a fluorescent dye.

- (3) Subsequently, the tagged cDNA is specifically hybridised to the array and bound to complementary sequences. To eliminate non-specific bindings, the array is washed and inserted into a scanner that excites the fluorescent dyes recording the emitted intensity. CDNA's abundance for a specific probe is correlated to the fluorescence emission.
- (4) The data are collected by a scanner that detects the signals, and an imaging algorithm process assigns intensity measurements to each probe.

Measurements made are transformed into numerical values that are then related to the abundance of the corresponding RNA transcript in the sample. The output is a highly dimensional dataset that requires bioinformatic analysis to process and understand. The microarray uses fluorescent dyes of different wavelength emissions, in multicolour arrays usually red (670nm) and green (570nm), as it allows the comparative analysis between different diseases and cell types. mRNA from the two different conditions can be made into cDNA with different fluorescent dyes resulting in probes emitting shades of red, green and yellow fluorescence relative to the amount of starting cDNA in each sample [126].

Although microarray enables high throughput transcriptional profiles of large sets of samples using advanced technologies, it also presents some limitations due to the large number of probe designs based on sequences of low specificity, the high sensitivity of the experimental setup to variations in hybridisation temperature alongside the high cost of performing a single experiment, the short shelf life of the chips, and the complicated bioinformatics data analysis [125][126].

Materials and Methods

Patients recruited and diagnostic process of the IRIS study

A host gene expression signature to distinguish febrile children with bacterial infections from those with viral infections was discovered by the Paediatrics Infectious Disease Team studying children recruited in different hospitals. Details about the cohort and research ethics are described in *Herberg et al* and are briefly summarised here to provide context. The prospective study was part of a UK National Institute of Health Research-supported study (NIHR ID 8209), the Immunopathology of Respiratory, Inflammatory and Infectious Disease Study (IRIS). Patients were also recruited in Spain (GENDRES network, Santiago de Compostela), and the United States (Rady Children's Hospital, San Diego) [127].

Using local approval of the research ethics committee permissions (St Mary's Research Ethics Committee (REC 09/H0712/58 and EC3263), and the Ethical Committee of Clinical Investigation of Galicia (CEIC ref 2010/015), informed consent was obtained from parents or guardians. In addition, the Academic Medical Centre, University of Amsterdam (NL41846.018.12 and NL34230.018.10) is included in 140220 [91].

The study included children under the age of 17 with fever and illness of sufficient severity to request a blood test. Patients with conditions that might affect blood gene expression (bone marrow transplant, immunodeficiency, or immunosuppressive treatment) were not included in the study [108]. For RNA analysis, blood samples were collected, together with clinical blood tests at presentation to the hospital, irrespective of antibiotic use at the time of collection. Patients recruited were screened with complete blood testing and culture and urine culture and cerebrospinal fluid analysis. C-reactive protein levels were also recorded. Multiplex PCR was used to screen for common respiratory viruses. Clinically indicated chest radiographs were taken.

Patients were divided into two diagnostic groups: the definite viral infection group included only patients who had viral infections confirmed by using immunofluorescent, molecular or culture tests, and the definite bacterial infection group included only children with infections confirmed by the cultural method. Children with indeterminate infections were categorised into probable or unknown bacterial or viral infections based on the level of suspicion. The definite bacterial infection group included children in whom pathogenic bacteria were detected in normally sterile sites or with sepsis and shock syndrome. The definite viral infection group included children with the viral clinical syndrome, not presenting any bacterial feature. Children categorised in the unknown bacterial or viral group had inconclusive clinical features. The healthy control group comprised healthy children without

recent or recurrent infection to compare the expression level of the biomarker genes and better understand the direction of differential expression (up or down).

From the study reported by Herberg and colleagues [91] a set of 39 whole blood clinical samples stored in PAXgene Blood RNA Tubes together with gene expression data from Illumina microarray (Table S1, S2 in the Appendix) were made available to study how the development of RT-LAMP assays can be used as an alternative to the state-of-the-art technologies (RT-qPCR and microarray), while still demonstrating good analytical sensitivity and specificity.

Clinical Samples

A set of 39 whole blood samples stored in PAXgene Blood RNA Tubes to preserve RNA stability were provided: definite bacteria ($n=16$), definite viral ($n=16$), and healthy control ($n=7$). In terms of RNA amount and quality of tested clinical samples, all RNA exactions ranged from 30ng/ μ l to 381ng/ μ l with an average of 160.2ng μ l (SD=110ng/ μ l).

Synthetic DNA

Synthetic DNA gBlocks containing the region of interest for each of the genes screened *IFI44L*, *FAM89A*, *GAPDH*, *PPIB* and *EMR1* were purchased from Integrated DNA Technologies, resuspended in TE buffer to 10 ng/ μ L stock solution and stored at -20°C until required for further use.

To characterise the reaction performance (dynamic range of quantification and analytical sensitivity) for the developed RT-LAMP assays, serial dilutions of synthetic RNA were prepared in molecular grade water ranging from 1×10^6 copies/reaction to 1×10^1 copies/reaction. For these experiments, synthetic DNA (gblocks, IDT) containing a T7 promotor (Figure 13) were initially transcribed into RNA by T7 RNA polymerase using the HiScribe T7 Quick High Yield RNA Synthesis Kit from NEB.

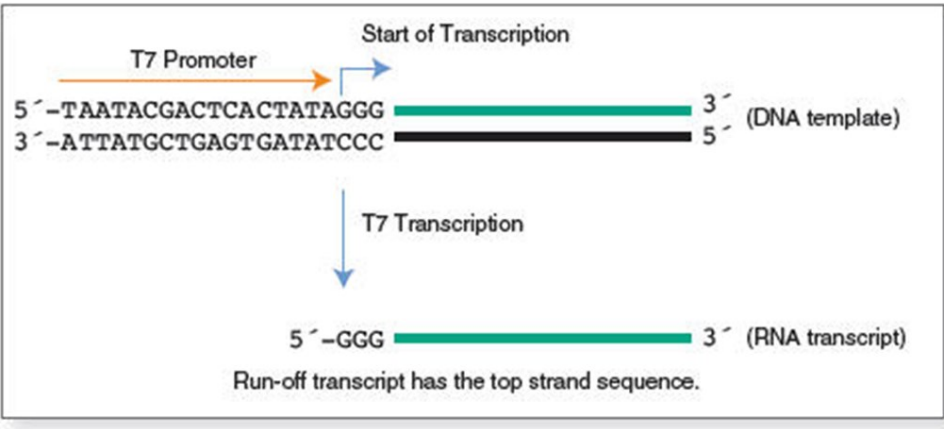


Figure 13. HiScribe T7 Quick High Yield RNA Synthesis Kit from NEB.

A reaction was assembled at room temperature following the order in Table 2. The reaction was mixed and incubated at 37°C for six hours. A DNase treatment to remove the DNA template was conducted. To remove template DNA, 2 µl of DNase I (RNase-free) were added to each of the 20 µl reaction mixes and incubated for 15 minutes at 37°C and 15 minutes at 80°C to inactivate the enzyme activity. All RNA sequences produced were purified following the NEB protocol and appropriately diluted in nuclease-free water. To verify the RNA concentrations, the control RNAs were quantified using Qubit™ RNA High Sensitivity from Thermo Fisher.

Nuclease-free water	0 µl
NTP Buffer Mix	10 µl (10 mM each NTP final)
Template DNA	8 µl (1 µg)
T7 RNA Polymerase Mix	2 µl
Total reaction volume	20 µl

Table 2. Reaction mix to synthesise high yield RNA.

RNA extraction and processing

Whole blood (2.5mL) was collected into PAXgene Blood RNA Tubes containing 5mL of RNA stabilisation reagent (PreAnalytiX, Germany), incubated for two hours, frozen at -20°C, and then stored at -80°C for later extraction. RNA was extracted using PAXgene Blood RNA kits (PreAnalytiX, Germany), following the manufacturer’s instructions. The samples were removed from -80°C and incubated at room temperature overnight to ensure complete lysis. Following lysis, the tubes were centrifuged for ten minutes at 5,000 × g, the supernatant was decanted, and 500 µL of RNase-free water was added to the pellet. The tube was vortexed to thoroughly resuspend the pellet and centrifuged for a further ten minutes at 5000 × g, and the entire supernatant was discarded. The remaining pelleted lysate was resuspended in 360 µL of buffer BR1 by vortexing. Details of the extraction protocol are illustrated in Figure 14.

Freshly extracted RNA was quantified in NanoDrop® ND-1000 spectrophotometer (from ThermoFisher Scientific) to ensure the integrity and purity of RNA with a 260:280 ratio between 1.95 and 2.3. Absorbance and values are summarised in Table S1, S2 in the Appendix.

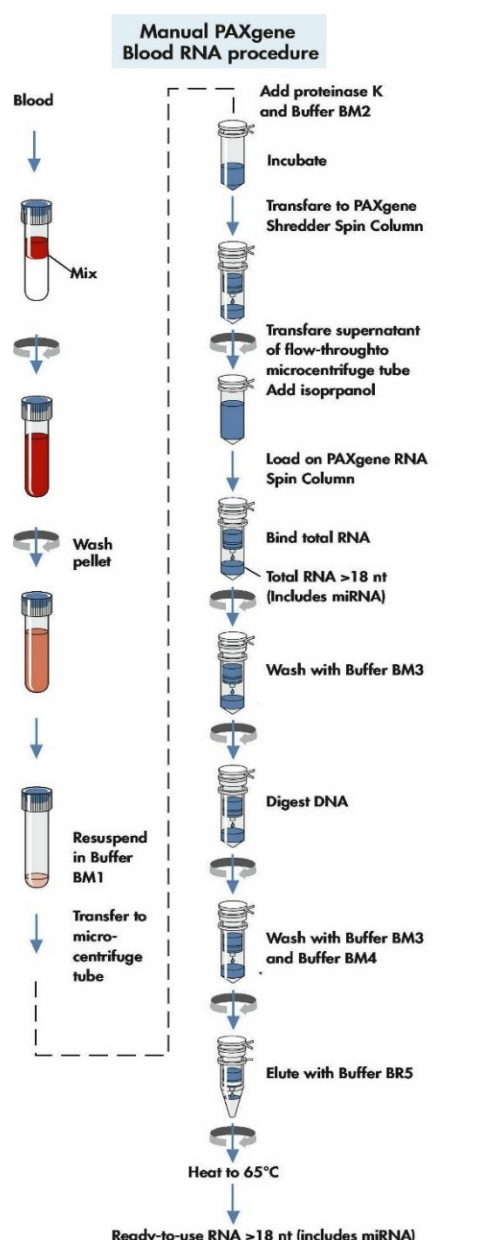


Figure 14. mRNA extraction protocol from Paxgene tubes by PreAnalytiX GmbH. The PAXgene Blood RNA procedure begins with a centrifugation step to pellet NAs in the PAXgene Blood RNA Tube. The pellet is washed, resuspended, and incubated in optimised buffers together with proteinase K to bring about protein digestion. Additional centrifugation through the PAXgene Shredder spin column is carried out to homogenise the cell lysate and remove residual cell debris, and the supernatant of the flow-through fraction is transferred to a fresh microcentrifuge tube. Ethanol is added to adjust binding conditions, and the lysate is applied to a PAXgene RNA spin column. During brief centrifugation, RNA is selectively bound to the PAXgene silica membrane as contaminants pass through. The remaining contaminants are removed in several efficient wash steps. Between the first and second wash steps, the membrane is treated with DNase I to remove trace amounts of bound DNA. After the wash steps, RNA is eluted in elution buffer and heat-denatured at 65°C.

Splice variant analysis

Using a publicly available blood RNA-seq data set comprising 255 children with bacterial and viral infections (GSE69529), gene expression abundance was measured at the level of splice junction to identify the best regions to design the primers for each gene that is part of the signature. This process allows to optimise the translation of the signatures to RT-qPCR because PCR primer sets are designed to specifically target splice junctions, preventing the amplification of genomic DNA. Investigation of splice events through RNA-seq was performed for the final signatures (*IFI44L*, *EMR1-ADGRE1*), aligned for each subject group of RNA-seq reads (bacterial, viral and healthy control) by Dominic Habgood-Coote.

LAMP primers were designed to detect the same transcript as the mature RNA. Gene counts were analysed for the bacterial and viral gene candidates to assess the sum of the reads associated with each of the exons that belong to that gene. Figure 15 shows the area under the curve (AUC) between bacterial and viral group for each region as well as the abundance of RNA in each genomic position (depth normalised exon counts) for each of the two transcripts, respectively *IFI44L* (Figure 15a) and *EMR1-ADGRE1* (Figure 15b), where primers span an exon-exon boundary between highlighted exons.

The height of the bar indicates the abundance of the gene, and this allows us to select the exact location of the RNA transcript where the gene is highly expressed and can be clearly differentiated among different patient groups. The small purple beads indicate the location where the primers sit for each of the genes of interest. In Figures 15a and 15b, primers span to the exon and therefore only capture mature RNA rather than genomic DNA. The exons and primers for both *IFI44L* and *EMR1* show a high correlation of 1. Therefore, the primers span an exon-exon boundary between the exons, confirming they are mRNA specific. The correlation between exon counts for each gene is depicted in the heat maps in Figure S1 in the Appendix.

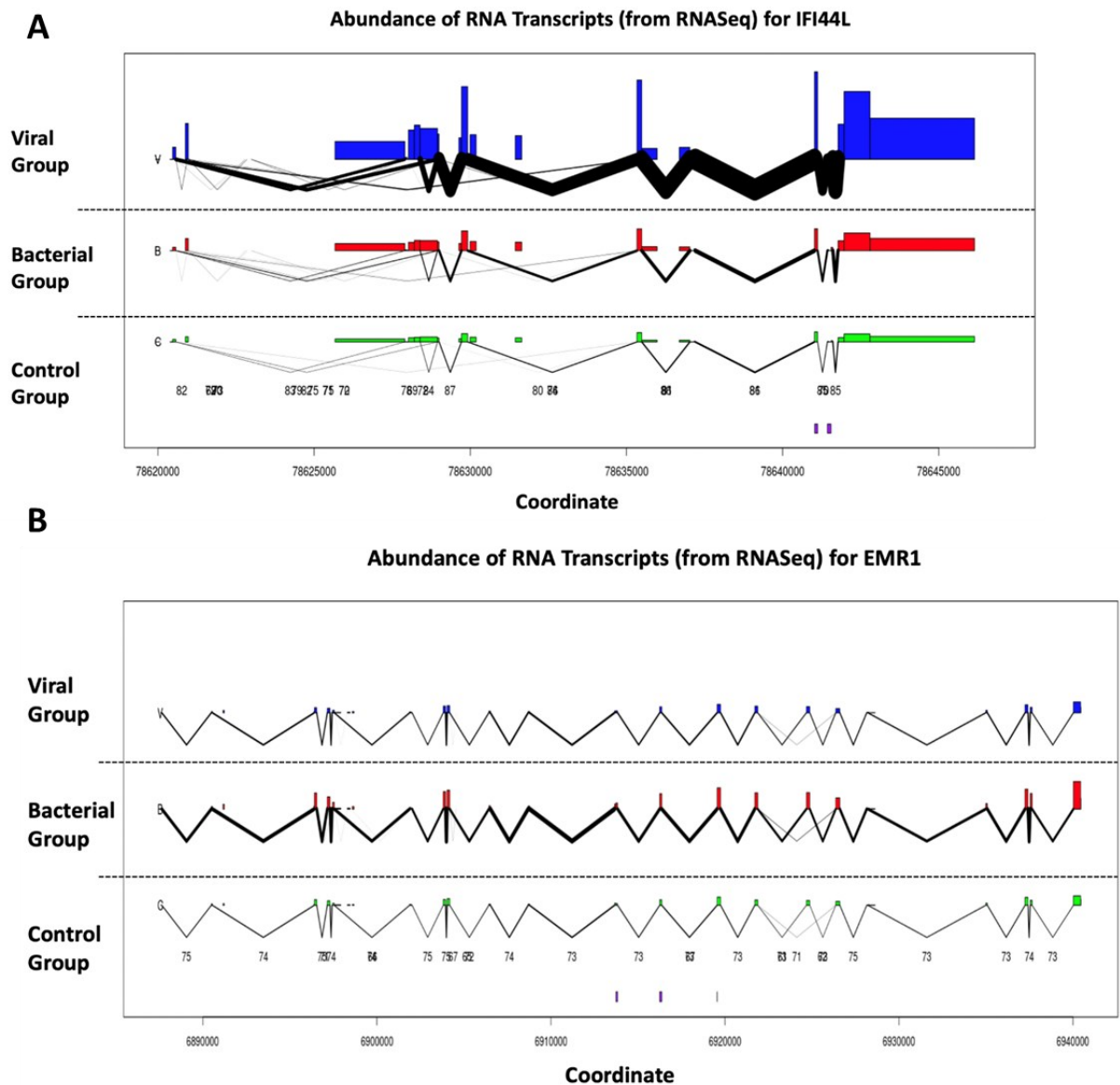


Figure 15. Abundance of RNA Transcripts from RNA-Seq for IFI44L and EMR1. Represent RNA-seq reads for three patient groups (viral group in blue, bacterial group in red, and healthy control group in green) aligned with the splice events of the viral gene (A) and the bacterial gene (B). The abundance of the transcript is estimated by the height of the coloured bar. The overlapping of the primers (purple bits) to the high abundant transcripts indicates that they span to the coding part of the gene (exons). Therefore, they can be considered good candidates as they are capturing mature RNA.

Selection of the gene candidates

A list of five candidate genes, *IFI44L*, *EMR1-ADGRE1*, *FAM89A*, *GAPDH* and *PPIB*, were made available to me together with the microarray expression levels (Table S3 in Appendix). Specifically, the *IFI44L* gene as discussed in Herberg and colleagues was found to be up-regulated in antiviral responses mediated by type I interferons and is therefore considered a viral gene. *EMR1* (egf-like module containing, mucin-like, hormone receptor-like 1) and *FAM89A* (Family with sequence similarity 89,

member A) genes were found to be up-regulated in children with septic shock and are considered bacterial genes. *PPIB* (peptidylprolyl isomerase B) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) genes were found to show the same level of expression in bacterial and viral infections and therefore used as reference genes.

In the analysis, the criteria used to down-select only the most efficient gene targets consist of different parameters for each of the genes studied, such as:

Gene counts: Candidates with sufficient average gene counts were considered.

LAMP primers set: Priority was given to LAMP primers showing complete sets of primers, including loops primers to enhance the speed of the amplification reaction.

Specificity: LAMP primer sets were specifically designed to pick up the targeted gene without amplifying other DNA sequences.

Sensitivity: LAMP primers are specifically designed to achieve an LoD of ten copies per reaction.

Reaction speed: LAMP primers were optimised to show amplification of the product within 20 minutes for the lowest LoD (ten copies per reaction).

A total of 20 sets of LAMP primers (Table S4 in Appendix) were designed and tested for the initial five gene candidates, each of them being assessed according to the listed criteria.

To minimise the number of candidate genes and design an assay compatible for future POC application, one combination of bacterial and viral genes (*IFI44L* and *FAM89A*) and one reference gene (*PPIB*) from the initial list of genes reported in Herberg et al. [127] down-selected and then replaced *FAM89A* with *EMR1-ADGRE1* (RefSeq ID: [NM_001974.5](#)).

LAMP primer design

The LAMP primer sets were designed and screened to evaluate their performance in detecting each of the gene candidates: *IFI44L*, *PPIB* and *EMR1-ADGRE1*. The screening process included the use of genomic DNA to check the specificity of the LAMP primers and synthetic RNA previous transcription by T7 RNA polymerase. LAMP assays were designed using Primer Explorer V5 (Eiken Chemical Co. Ltd., Tokyo, Japan) and sequences listed in Table 3. Sequences were aligned using the MUSCLE algorithm in Geneious Prime® 10.0.5 (<https://www.geneious.com>) software. Primers were manually optimised through the IDT OligoAnalyzer software, considering as key factors melting temperature (T_m), stability at the 3' end of each primer, GC% content, and possible secondary structures such as primer dimer formation checked by using NUPACK37 software (<http://www.nupack.org/>). The T_m of the amplification product of each primer set was determined by the Melting Curve Predictions Software (uMELT) package (<https://dna-utah.org/umelt/umelt.html>). Primers were purchased from Integrated

DNA Technologies and resuspended in Tris EDTA to 100 μ M stock solutions. The stocks were stored at -20°C.

The performance of the assays was evaluated based on their analytical sensitivity to achieve a lower LoD of ten copies per reaction. The best candidate's primers were selected according to the efficiency of the amplification reaction (LoD and reaction speed).

Gene	Primer	Sequence (5' – 3')
<i>IFI44L</i>	F3	TGTGGTATAGCATATGTGGC
	B3	GAGAGGATGAGAATATCCTTCA
	FIP	CGGCTTTGAGAAGTCATAGATCTACCTTGCTTACTAAAGTGGATGATTG
	BIP	ATGAATGTCCATAAAATGCTAGGCAGTCCAGTTCCAAATCTGAAG
	LF	AGTTGTCTTGAAGAACCTCACTG
	LB	CCAATATTTTGATGGTTGGAACTATG
<i>PPIB</i>	F3	GGAGATGGCACAGGAGGA
	B3	CCACATGCTTGCCATCTAGC
	FIP	GAAGTTCTCATCGGGGAAGCGCGGAGACTTCACCAGGGGA
	BIP	CTGGGTGAGCATGGCCAACGGCTGTCTTGACTGTCGTGAT
	LF	GCTCTTTCCTCCTGTGTGCCATC
	LB	CAGGCAAAGACACCAACGG
<i>EMR1</i>	F3	CCACAGTCTTCCTGGAGAGT
	B3	CCAGTGGTCTCTGTGGATTC
	FIP	CGTCCGAACAGCCGGAGTGATATGGAAAGCATGACACTGGC
	BIP	TGTGACGTTGGACTTGGTAGCCCCCTCAATTGTGGAACACCCG
	LF	TGCTGAGGGTTTCAAAAAGAT
	LB	AAGGGGGATAAGATGAAGAT

Table 3. List of designed primers for *IFI44L*, *PPIB* and *EMR1-ADGRE1*.

RT-LAMP reaction conditions

Each LAMP reaction was prepared in a total 10 μ L reaction: 0.625 μ L of Bst 2.0 DNA Polymerase (8000 U/mL stock), with 1 μ L of template, 1 μ L of 10X isothermal buffer (New England Biolabs), 0.6 μ L of MgSO₄ (100mM stock), 1.4 μ L of dNTPs (10mM stock), 0.25 μ L of BSA (20mg/mL stock), 1.6 μ L of Betaine (5 M stock), 0.25 μ L of AMV reverse transcriptase (Promega) (25,000 U/mL stock), 0.25 μ L of SYTO 9Green (20 μ M stock), 1 μ L of 10X LAMP primer mixture, 1.925 μ L of Nuclease-free water. Reactions were performed at 63°C for 30-40 minutes. One melting cycle was performed at 0.1°C/s from 65°C to 97°C for validation of the specificity of the products. Experiments were carried out in triplicate, loading the reactions into LightCycler Multiwell Plates 96 (Roche Diagnostics), utilising a LightCycler 96 Real-Time PCR System (Roche Diagnostics). Furthermore, validation of the specificity of

the products was performed running one melting cycle in the LC96 at 0.1°C/s from 65°C to 97°C for validation of the specificity of the products.

RT-PCR reaction conditions

RT-qPCR is considered the gold standard for RNA quantification. Each reaction mixture contained the following reagents: 0.2 µL of Superscript III RT/Platinum Taq Mix, 5 µL of 2X reaction mix, 0.5 µL of 20X Primer mix, 0.2 µL of ROX Reference Dye [50 nM], 0.2 µL of RNaseOUT, 2 µL of a template (1pg to 1ug total RNA) and 1.9 µL of DEPC-treated water. Each condition was run in duplicate (10 µL/reaction). Samples were loaded into the LightCycler 480 Multiwell Plates 96 (Roche Diagnostics) using a LightCycler 96 Real-Time PCR System (Roche Diagnostics). Reactions were performed following an initial step of 15 minutes at 95°C for the cDNA synthesis, followed by a denaturation step of 95°C for two minutes and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Each infection group's Ct values were compared in the presence of each of the target genes (where Ct is the cycle threshold, defined as the number of cycles required for the fluorescence signal to exceed the background level, and is inversely correlated with the amount of target NA in the sample).

Analytical sensitivity of the primer sets screened for each gene

The sensitivity of the LAMP primer set for each of the five gene candidates was evaluated using tenfold serial dilutions in molecular grade water of synthetic DNA ranging from 1×10^6 to 1×10^1 copies per reaction using RT-LAMP and RT-pH-LAMP. For each method, a standard curve was obtained by plotting the time-to-positive (TTP) against copies per reaction, including standard deviation. A total of 20 sets of primers were screened for each of the five genes. Only one set of LAMP primers was selected for each of the five genes based on their LAMP performance. Only three genes were selected as final biomarkers to develop the RT-LAMP assay.

Data analysis

Expression levels from Illumina microarrays were provided by Kaforou and colleagues. A trained support vector machine classifier was used to identify clinical samples exhibiting differential transcript expression between definite viral and bacterial groups with $|\log_2 \text{FC}| > 1$ and a p-value of 0.05. These thresholds were chosen to ensure that differential expression for selected variables could be distinguished using the resolution of other validation techniques (i.e., RT-qPCR, RT-qLAMP).

Normalisation of the cycle threshold or TTP data for RT-qLAMP, RT-qPCR was presented as mean $\pm$ standard deviation.

Results and Discussion

The choice of the signatures

The importance of choosing the best transcripts for discriminating bacterial infection from viral infection is crucial to avoid compromising the transferability across platforms in the next stage, therefore facilitating the translation into a POC system. Herberg and colleagues [91] reported a two-transcript signature (*IFI44L* and *FAM89A*) discovered using gene expression microarrays in a set of 455 children with fever and bacterial and viral infections (IRIS) [127]. From this cohort, 39 RNA samples were used to study the performance in RT-qPCR and RT-qLAMP. Samples from patients in the IRIS study with confirmed bacterial and viral infections were matched for severity and were collected between September 2009 and May 2017.

To choose transcripts suitable for translation to the RT-LAMP assay, counts of *IFI44L* and *FAM89A* were assessed in a publicly available blood RNA-seq dataset comprising 255 children with bacterial and viral infections (GSE69529) [128]. The AUC of the Herberg 2016 JAMA signature in that cohort, which comprises patients with diarrhoea and of Mexican origin, was 80.0% (72.6–87.4). The *IFI44L* – *EMR1* signature showed an AUC of 82.1% (75.7–88.6), showing consistency and slight improvement [127]. The average gene counts for *IFI44L* were sufficient (2281.9) but low for *FAM89A* (20.2). The low count for *FAM89A* could represent a potential risk of compromise transferability across platforms. Therefore, using the list of previously identified 38 highly correlated transcripts, [127] *FAM89A* was replaced with *EMR1-ADGRE1* (RefSeq ID: NM_001974.5), which had sufficient average gene counts (511.3) [82], and in combination with *IFI44L* (RefSeq ID: NM_006820) had a similar performance to the original two-transcript signature (area under the curve [AUC] in the training, test, and validation data sets: 93.4%, 97.4%, and 97.2%, respectively). (Table S5 in Appendix).

Microarray expression in three gene candidates

Microarray data were provided by Kaforou's team. Figure 16 shows the expression level in depth normalised counts for each targeted gene in the bacterial, viral, and healthy control groups of the IRIS cohort. Looking at the three significant genes, *ADGRE1-EMR1* (Figure 16a) shows a significant difference in each of the other groups, with a higher level of expression (in red) compared to the viral and healthy groups, and when put in a logistic regression model against the viral phenotype it shows an AUC of 97.2 %. Looking at the normalised counts for *IFI44L* (viral marker), the blue boxplot in Figure 16b shows higher expression levels in the definite viral group compared to the other phenotypes.

The reference gene, *PPIB* (Figure 16c), shows a constant expression level independently of whether the patients are bacterially or virally infected. Horizontal lines in boxes indicate medians; lower and upper edges of boxes, interquartile ranges; whiskers, ≤ 1 times the interquartile range. Expression data were provided by Dominic Habgood-Coote. This analysis provides evidence that the two transcripts proposed, EMR-1 and IFI44L, are significantly differentially expressed in patients with bacterial or viral infections.

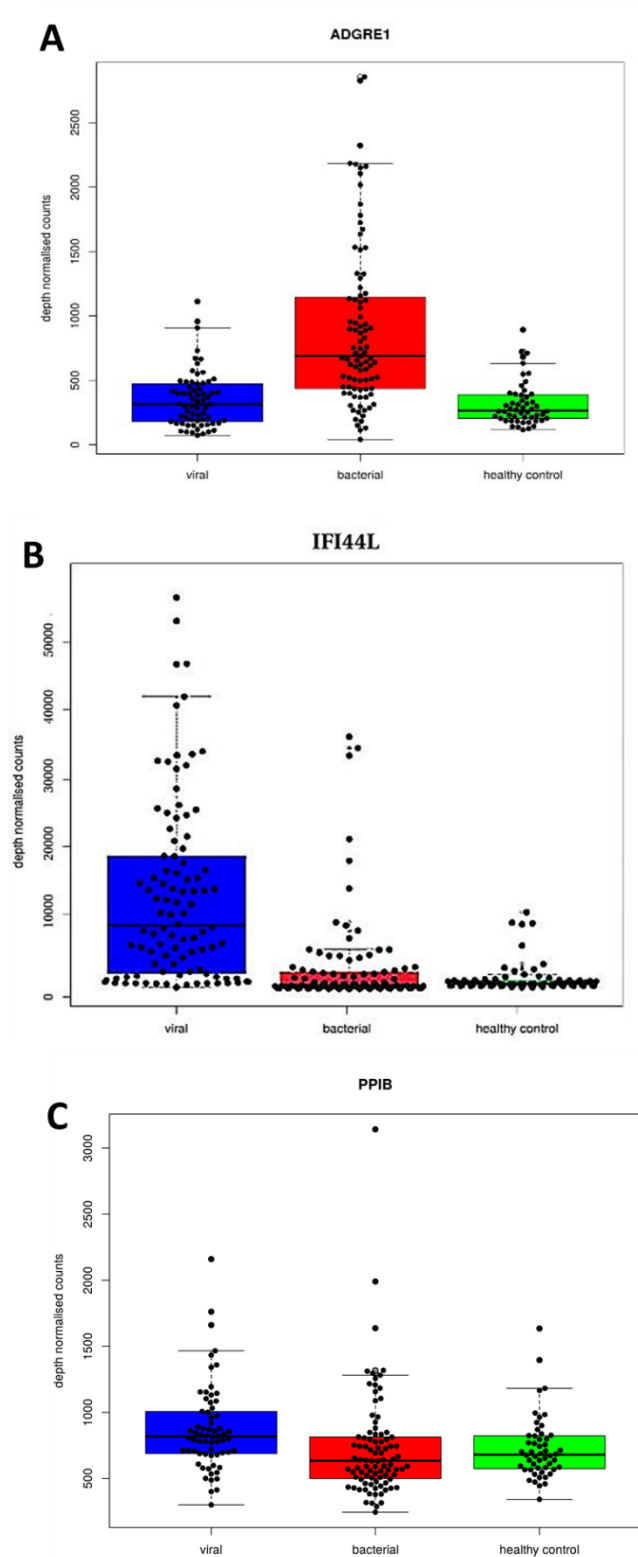


Figure 16. Microarray classification of three genes in the IRIS cohort. Classification of the bacterial, viral and healthy control groups based on microarray expression level of EMR1-ADGRE1(A) IFI44L (B), and PPIB (C) signature.

RT-LAMP assay for detection of *EMR1-ADGRE1* transcript

LAMP assays targeting the gene *EMR1-ADGRE1* were designed. The selected sequence shown in Figure 17a contains eight different LAMP primers designed and their location in the selected amplicon. The T7 promoter at the beginning of the sequence regulates the transcription process through the RNA polymerase.

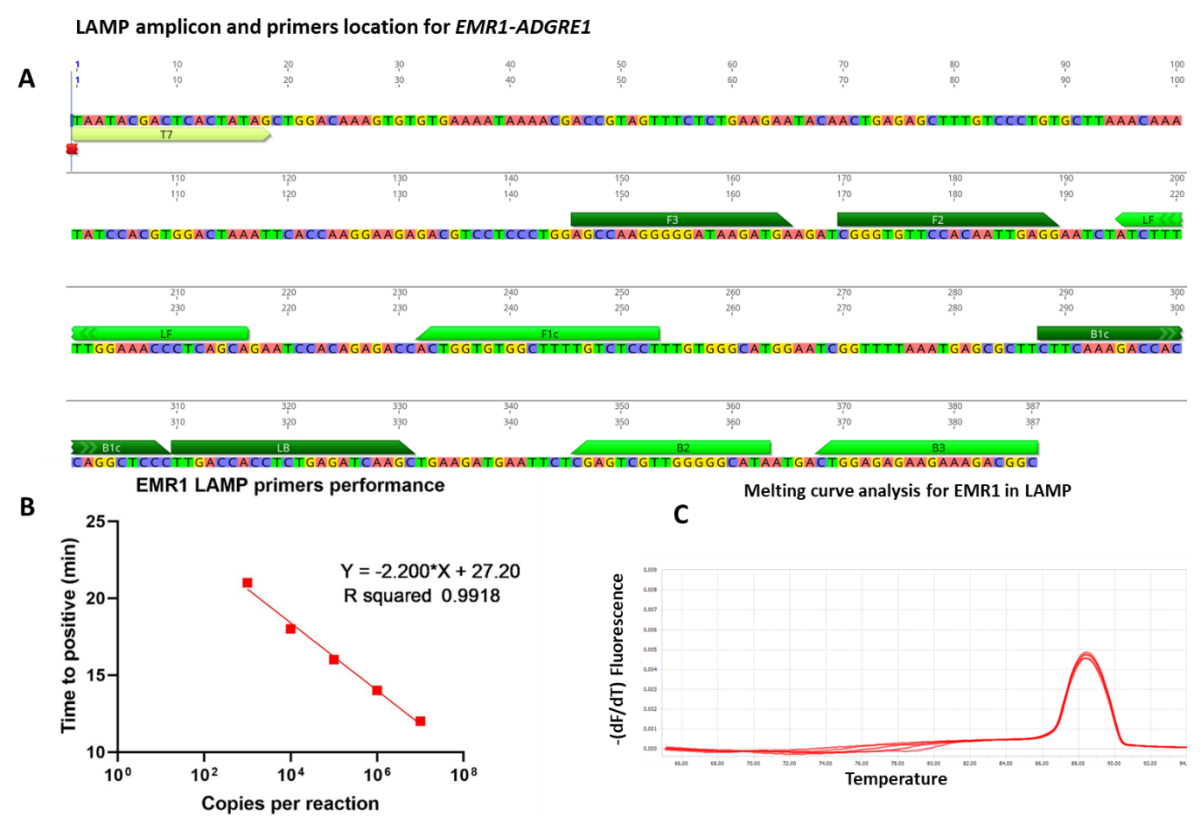


Figure 17. Amplicon of the *EMR1-ADGRE1* LAMP assay, selected LAMP primers and analytical sensitivity and specificity. (A) Illustration using Geneious version 2021.2 of the LAMP amplicon (5' to 3' direction) for *EMR1-ADGRE1*, showing in green the designed LAMP primers and their respective binding sites. (B) RT-LAMP standard curve. (C) Melting curve analysis.

Analytical sensitivity of the LAMP assay was evaluated using synthetic RNA at concentrations ranging from 1×10^7 to ten copies per reaction. A standard curve was obtained by plotting the Ct values derived from the amplification curves of the dilution series against the logarithm of the standard quantification with a linear correlation $R^2=0.988$, and as shown in Figure 17b, the LoD was equal to 1×10^3 copies per reaction with a TTP within 20 minutes, and the propagation of a single product in the LAMP reaction was confirmed by a melting curve analysis of the reaction. A single sharp dissociation

peak at 95°C was observed in the LAMP reaction in Figure 17c; no amplification was observed in the no-template control reactions.

LAMP assay for detection of *IFI44L* transcript

LAMP assays targeting the gene *IFI44L* were designed. The selected sequence shown in Figure 18a contains the amplicon with a T7 promotor and eight different designed LAMP primers indicated in blue on their binding sites. To study the dynamic range of quantification, tenfold serial dilutions using synthetic RNA at concentrations ranging from 1x10⁶ to ten copies per reaction were performed. In these reactions, amplification was confirmed after three minutes, detecting 1x10⁶ copies per reaction, with the reaction reaching its endpoint within eight minutes.

The LAMP assay targeting the *IFI44L* gene, showed a LoD of ten copies per reaction within eight minutes (TTP) and linearity of the reaction ($R^2 = 0.96$) as shown in Figure 18b. A melting curve analysis showed the specificity of the LAMP product (melting peak observed at 85°C), and no amplification was observed in the template control (Figure 18c).

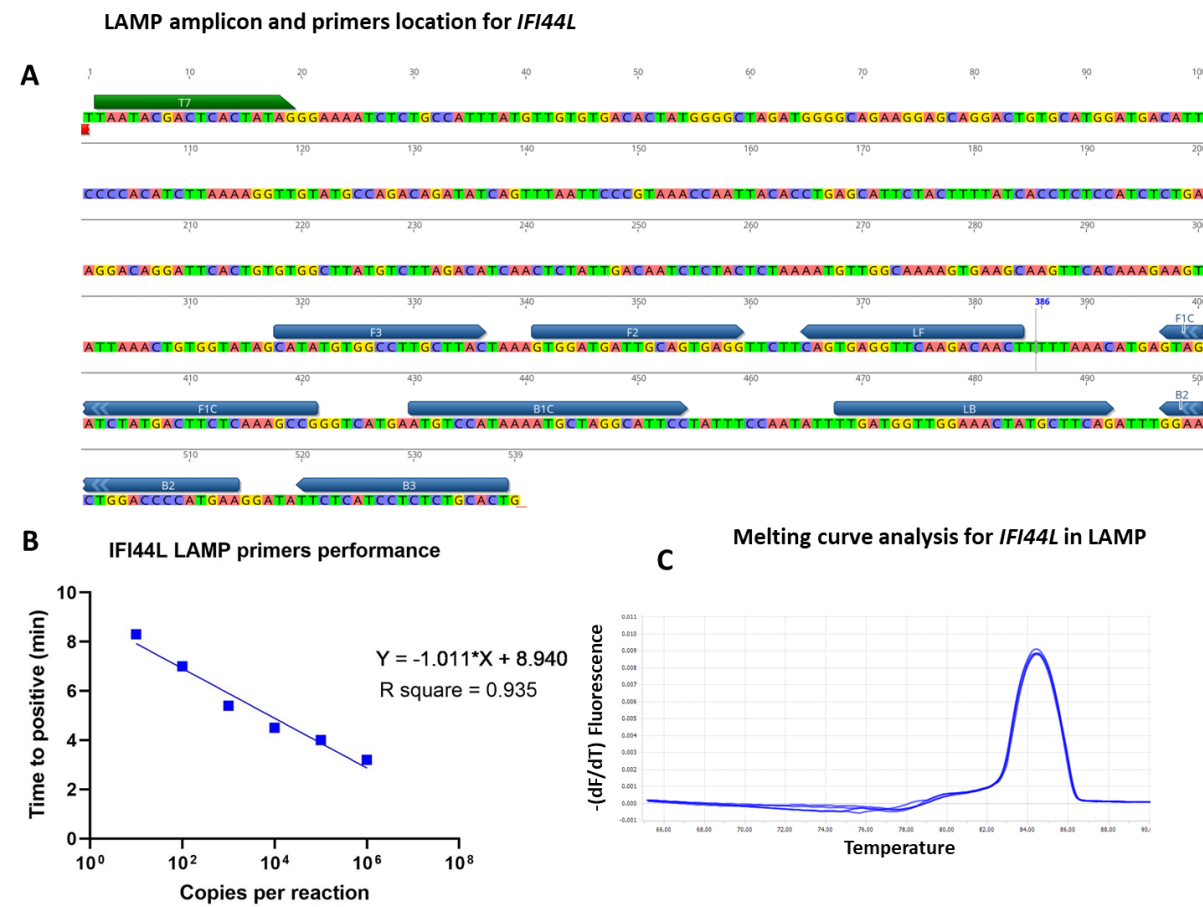


Figure 18. Amplicon of the *IFI44L* LAMP assay, selected LAMP primers and analytical sensitivity and specificity. (A) Illustration using Geneious version 2021.2 of the LAMP amplicon (5' to 3' direction) for *IFI44L*, showing in blue the designed LAMP primers and their respective binding sites. (B) RT-LAMP standard curve. (C) Melting curve analysis to confirm product specificity.

RT-LAMP assay for the detection of *PPIB* transcript

LAMP assays targeting the gene *PPIB* were designed. The selected sequence shown in Figure 19a contains the amplicon with a T7 promotor in blue and eight designed LAMP primers indicated in green on their respective binding sites.

Tenfold serial dilutions using synthetic RNA at concentrations ranging from 1×10^5 to ten copies per reaction were performed. *PPIB* LAMP assay showed a LoD of ten copies per reaction within seven minutes (TTP) and linearity of the reaction ($R^2 = 0.95$) as shown in Figure 19b. A melting curve analysis (Figure 19c) showed the specificity of the LAMP product, and no amplification was observed in the template control.

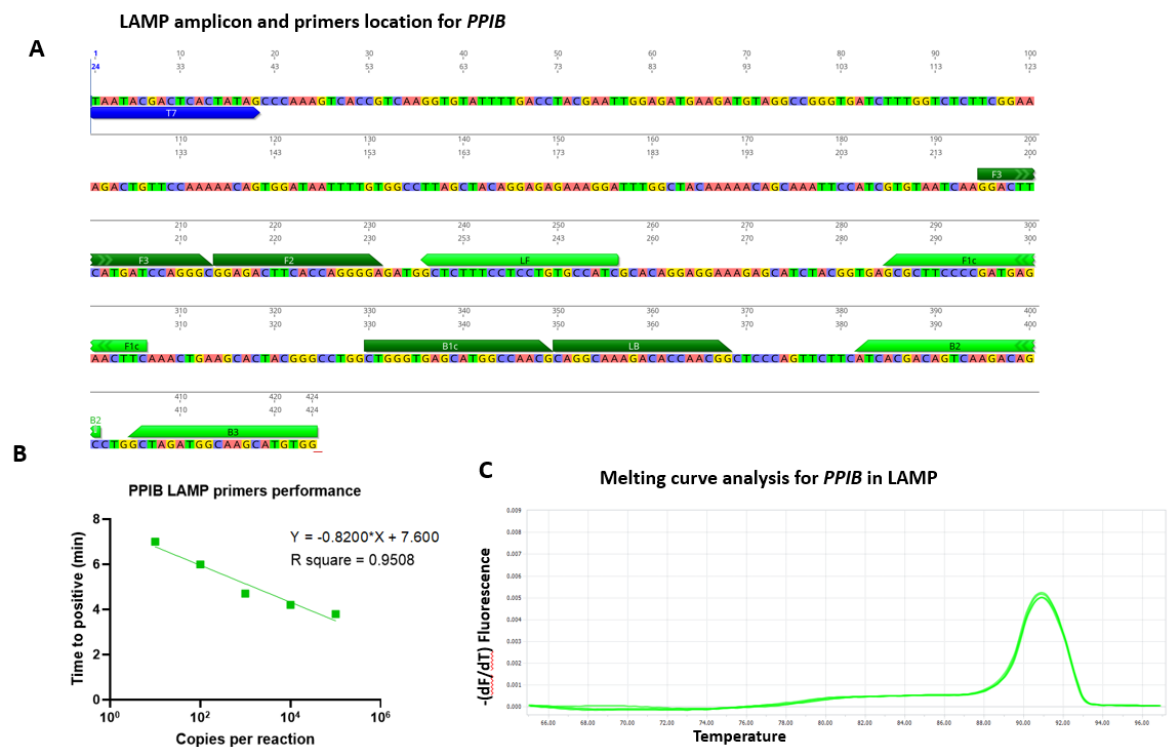


Figure 19. Amplicon of the *PPIB* LAMP assay, selected LAMP primers, and analytical sensitivity and specificity. (A) Illustration using Geneious version 2021.2 of the LAMP amplicon (5' to 3' direction) for *PPIB*, showing in green the designed LAMP primers and their respective binding sites. (B) RT-LAMP standard curve. (C) Melting curve analysis to confirm product specificity.

Taken together, these preliminary data showed the analytical specificity of the target, absence of secondary peaks and false positives, speed of reaction appropriate for POC applications and detection down to ten copies per reaction for the *PPIB* and *IFI44L* assay, while *EMR1* showed a LOD of 1×10^3 copies per reaction, suggesting further optimisation was needed.

Clinical samples performance in microarray

The microarray data (Figure 20) from a subset of clinical samples made available to me from the IRIS study were analysed, to test and evaluate the classification of the clinical samples (definite bacterial and definite viral patients) before proceeding with the classification process across the other techniques (RT-qPCR and RT-qLAMP).

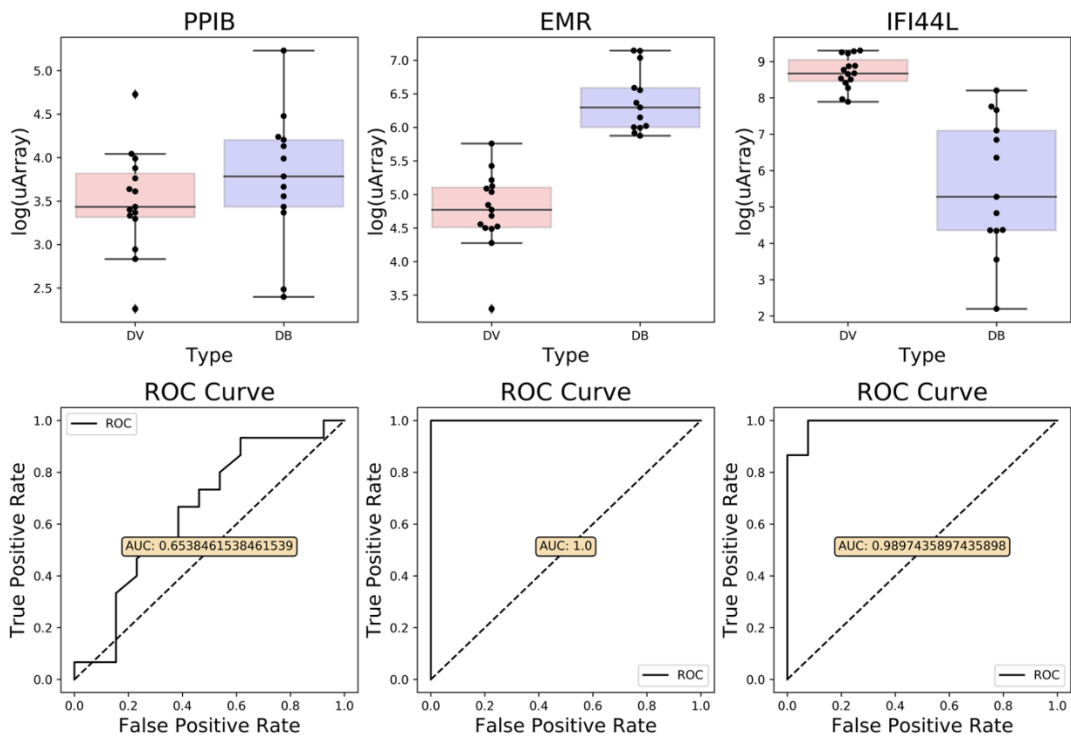


Figure 20. Microarray analysis of bacterial and viral samples targeting three transcripts. The classification was performed using logistic regression.

PPIB showed similar expression levels for bacterial and viral patients with an AUC of 0.653. A higher expression level was achieved in definite bacterial patients when targeting the *EMR1* gene showing good separation between the two groups with an AUC of 1.0. Similarly, good separation between bacterial and viral patients was obtained when targeting the *IFI44L* gene with an AUC of 0.98. Microarray results were considered as the reference methodology to evaluate the translation process across techniques.

Clinical samples performance on RT-LAMP

Clinical samples were analysed using the developed RT-LAMP assays to study their classification performance (Figure 21). Extracted RNA was run on commercial real-time instruments

(LC96) and quantified based on the normalisation of the mean values of TTP. The more target RNA is in the sample, the faster the amplification will happen. This means fewer cycles will be required before the fluorescence signal crosses the background threshold (lower TTP). Conversely, if there are low amounts of target RNA, the gene will undergo lower expression in that sample, and it will require more cycles before the fluorescence can cross the background threshold (higher TTP).

PPIB showed a different expression level in bacterial and viral patients, with an AUC of 0.72. *EMR1* and *IFI44L* were found to have a lower average Ct difference ($\Delta C_T = C_{T[\text{bacteria}]} - C_{T[\text{viral}]}$) compared to the microarray, suggesting poorer discrimination between the two infection categories. *EMR* showed an AUC of 0.789 and *IFI44L* showed a better classification with an AUC of 0.871.

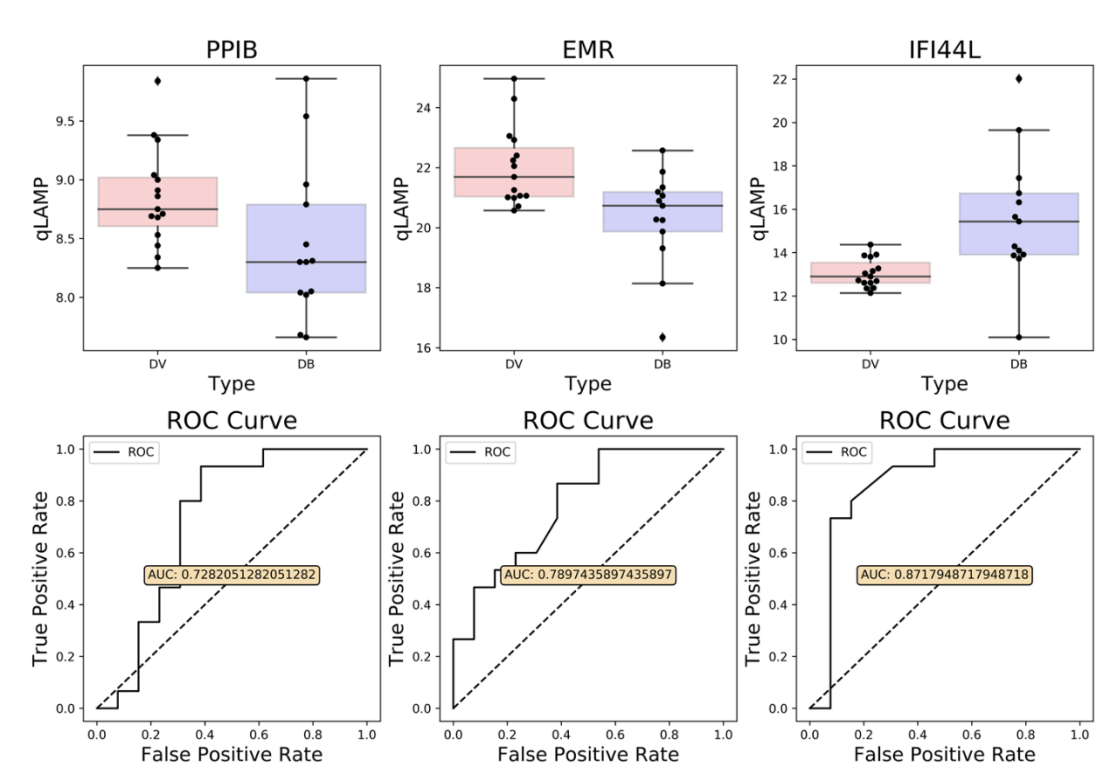


Figure 21. qLAMP analysis of bacterial and viral samples targeting three transcripts. The classification was performed using logistic regression.

To visualise the correlation between microarray and RT-qLAMP, the log (microarray) and TTP from RT-qLAMP were plotted for the *IFI44L* and *EMR1* genes and are shown in Figure 22. When targeting the *EMR1* gene, microarray and qLAMP show a correlation of -0.59 (in grey the 95% CI of the best-fit line). When targeting the *IFI44L*, gene microarray and RT-qLAMP show a higher correlation of -0.91 with a CI of 95%. Interestingly, targeting the bacterial gene *EMR1* within the two infectious groups does not seem to show a linear correlation between the two techniques, suggesting optimisation for the bacterial LAMP assay might be needed. *IFI44L* shows a much better correlation

between the two techniques, probably also due to the abundance of the gene being much more expressed than *EMR1*, as shown in the splice variant study.

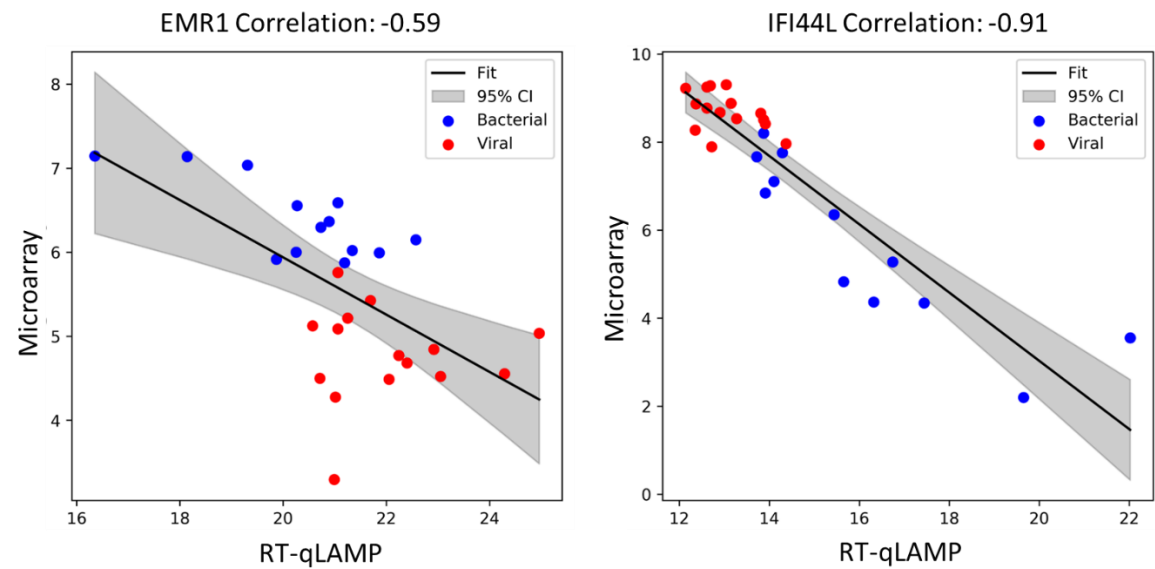


Figure 22. Correlation between RT-qLAMP and microarray for each of the target genes: *EMR1* and *IFI44L*. The blue dots indicate the definite bacterial patients, and the red dots indicate the definite viral patients.

RT-qPCR as gold-standard amplification methodology

To obtain a further comparison across techniques, clinical samples were tested on the RT-qPCR as the gold standard technique. Commercial probes and primers targeting the three genes were used to perform RT-qPCR. Extracted RNA was quantified based on the normalisation of the mean values (Ct). Similarly to qLAMP, the more target RNA is in the sample (higher expression levels = lower Ct and vice versa), and the faster the amplification will happen.

As shown in Figure 23, in RT-qPCR lower values for the bacterial infection group are shown when targeting the *EMR1* gene with an AUC of 0.91 (95% CI, 79.8%-100%) corresponding to a higher *EMR1* expression level in microarray, and higher values for the control and viral group, as expected. Lower values for the viral infection group were observed when targeting the *IFI44L* gene with an AUC of 0.84 (95% CI, 69.9%-97.6%) corresponding to a higher *IFI44L* expression level in microarray, while higher values were shown for the control and bacterial groups.

Similar Ct values were observed in all three infection groups when the reference gene (*PPIB*) was targeted with an AUC of 0.77 (95% CI, 60.6%-93.6%). PCR and LAMP products were absent in all the negative controls.

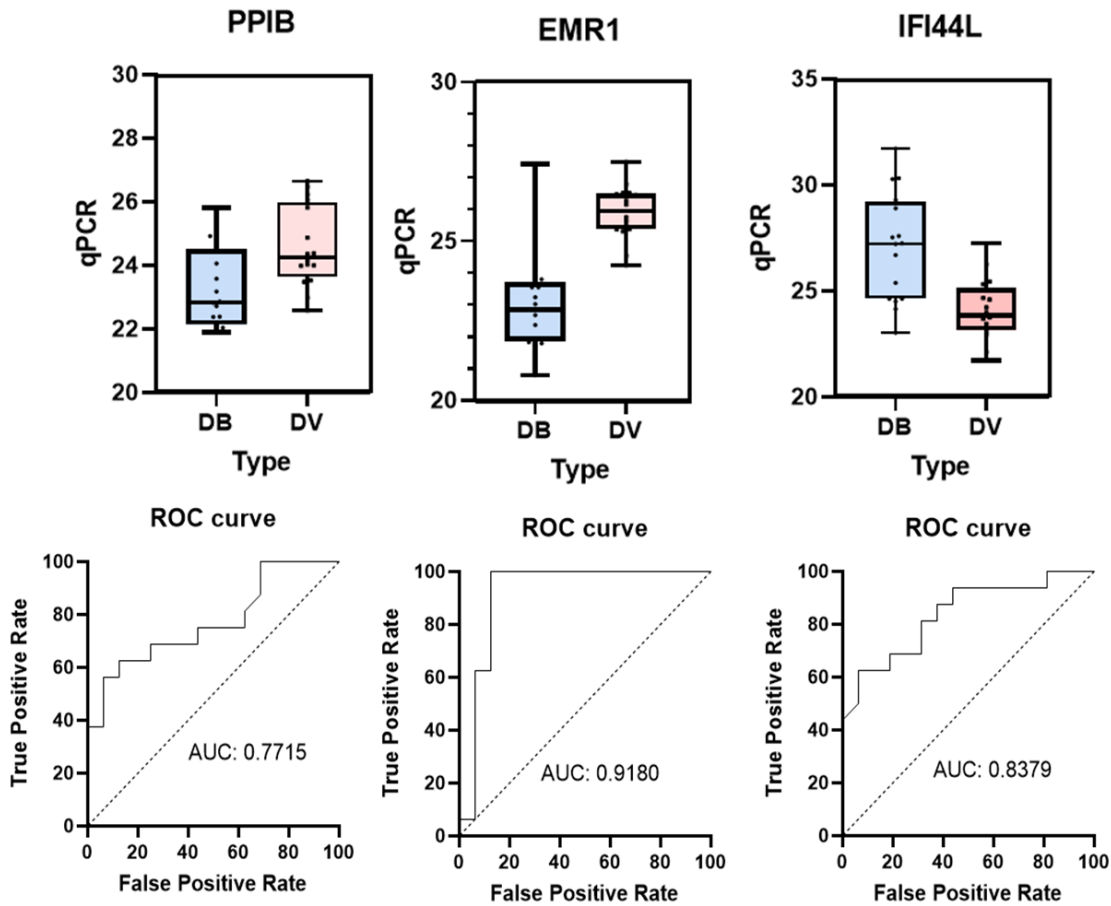


Figure 23. RT-qPCR analysis of bacterial and viral samples targeting three transcripts.

RT-qPCR and RT-qLAMP results were plotted for the IFI44L and EMR1 genes and are shown in Figure 24. When targeting the *EMR1* gene, RT-qPCR and qLAMP show a correlation of -0.84 (95% CI, 66.7%-91.1%). When targeting the *IFI44L* gene, RT-qPCR and qLAMP show a correlation of -0.91 (95% CI, 79.5%-94.8%). The two techniques show linear correlation. These data confirmed that the LAMP assay, comparably to the gold standard qPCR, could be used to distinguish and classify patients with a bacterial diagnosis from those with a viral diagnosis. However, to get better classification accuracy on-chip and analytical sensitivity when targeting bacterial patients, the EMR1 LAMP assay was further optimised, and data are shown in the next chapter. The results provided sufficient confidence to proceed toward the translation of the LAMP assay into RT-eLAMP (electronic LAMP) to develop a POC platform.

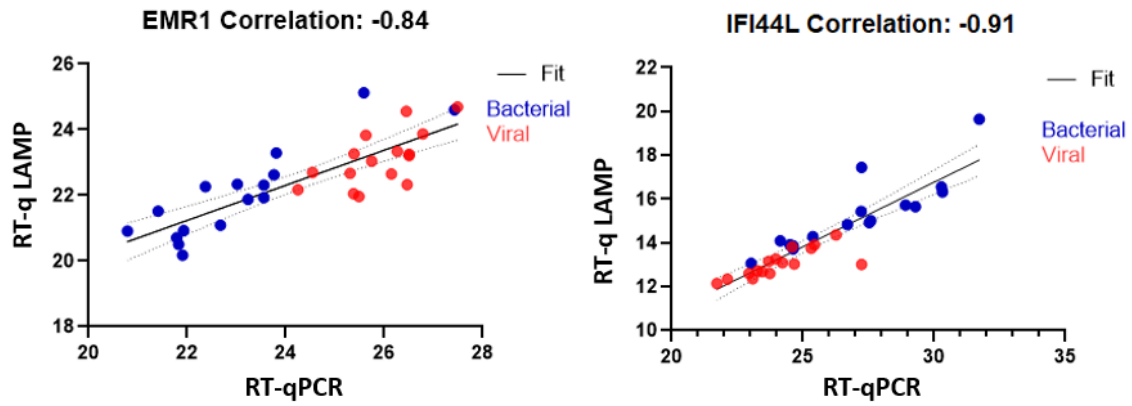


Figure 24. Correlation of RT-qPCR and RT-qLAMP for each of the target genes EMR1 and IFI44L. The blue dots indicate the definite bacterial patients, and the red dots indicate the definite viral patients. Dotted lines show the 95% CI of the best-fit line.

Conclusion

Discrimination of viral from bacterial infections in children remains challenging. Studies previously published report that children with bacterial and viral infections can be distinguished by their blood host RNA signature. In this chapter, RT-LAMP assays were developed for the *PPIB*, *EMR1* and *IFI44L* genes to measure gene expression levels in children with definite bacterial and definite viral infections and healthy controls. LAMP assays were developed and compared to different techniques to investigate their potential to be translated into a microchip platform suitable for development as a POC test. The study demonstrated the capability of amplifying RNA under isothermal conditions with high efficiency, sufficient analytical sensitivity and rapid TTP (<25 min). Using a conventional benchtop qPCR platform, the calculated LoD for the LAMP assays was ten copies per reaction for *IFI44L* and *PPIB* and 1000 RNA copies per reaction for *EMR1*. The LoD of the POC platform depends upon the chemistry of choice. To achieve a low LoD, optimisation of the LAMP assay was conducted to enhance the capability of this approach to be considered as a molecular technique for the future development of the diagnostic platform. Further discussion on the topic is reported in Chapter 7.

Part I

Chapter 3

Translation of Gene Expression Signatures into a Point-of-Care Test

Chapter 3 concludes Part I of the thesis and focuses on how the developed RT-LAMP assays targeting host transcripts can be translated into a microchip-based technology for POC applications. To the best of our knowledge, this is the first work demonstrating the use of host-gene expression detection on electrochemical sensors. I have contributed to conceptualising and performing all the laboratory work. The data analysis was undertaken by me and Dr. Moniri. On-chip experiments were kindly assisted by Dr. Miscourides and Dr. Moser. This study was conducted within my two research groups: the section of Paediatrics and EEE departments and provides proof of concept that the two-gene RNA signature can be detected within 25 minutes, discriminating bacterial from viral infection in a format suitable for development as a POC diagnostic test.

The concepts in this chapter resulted in the following journal articles and patent:

- **I. Pennisi**, J. Rodriguez-Manzano, A. Moniri, M. Kaforou, J.A. Herberg, M. Levin and P. Georgiou, "Translation of a host blood RNA Signature distinguishing bacterial from viral infection into a platform suitable for development as a Point-of-Care Test", JAMA Paediatrics, Jan 2021.
- **I. Pennisi**, J. Rodriguez-Manzano, N. Miscourides, A. Moniri, N. Moser, D. Habgood- Coote, M. Kaforou, J. A. Herberg, M. Levin and P. Georgiou, "A method for determining a diagnostic outcome", (Assignee: Imperial Innovations Limited). Filed 24-08-2020.
- **I. Pennisi**, A. Moniri, N. Miscourides, L. Miglietta, N. Moser, D. Habgood-Coote, J. A. Herberg, M. Levin, M. Kaforou, J. Rodriguez-Manzano, P. Georgiou "Discrimination of bacterial and viral infection using host-RNA signatures integrated in a lab-on-a-chip technology" (accepted in Bioelectronic and Biosensors).

Introduction

Biosensors and biomarkers

The successful design of diagnostic tests requires the identification of suitable biomarkers for the targeted disease and high-performing biosensors to detect them [129]. A biomarker is defined as any biologically derived signal that indicates the presence or absence of a pathogen or disease state [130]. Biosensors are usually biological sensors or chemical biosensors and are composed of a bioreceptor, such as an antibody, NA or enzyme, and some form of transducer, which gives a signal when the analyte is present. The resulting signal can be in the form of an electrical, optical, magnetic, or chemical signal [94]. In recent years great progress has been made in applying silicon-based biosensors in the field of bioanalytical applications due to their favourable characteristics. Ion-sensitive field effect transistors (ISFET) have been employed as biosensors to provide chemical inputs to electronic circuits, allowing the symbiosis of electronics and biology [131]. Due to the economy of scale of silicon, ISFET-based sensing systems have the advantage of being miniaturised and mass-fabricated at a low cost.

In this chapter, ISFET in complementary metal-oxide semiconductor technology (CMOS) is used as the main electrical biosensing methodology to detect NA using an LoC system [132].

Electrochemical detection on ISFET

The ISFET, conventionally referred to as a pH sensor, was used to measure ion concentrations (H^+ or OH^-) in a solution, causing an interface potential on the gate insulator [133]. When using microchip-based semiconductor technologies that integrate sensitive surfaces (ISFET), it is possible to detect NAs using isothermal amplification (LAMP) at low buffering conditions (pH-LAMP), [93] enabling the detection of H^+ , or equivalently a pH change as a result of the amplification process. pH-LAMP represents the chemistry of choice because it enables the use of pH-based sensing devices avoiding the need for expensive, optical instrumentation [134].

One of the applications of the DNA-based ISFET in the diagnostic field is the Ion Torrent technology for DNA sequencing. In this system, the pH sensing capabilities of ISFET sensors are employed to detect single nucleotide polymorphisms and DNA sequencing with 99.6% accuracy, 600 nucleotides per read, and 100 Mb per run [135]. Georgiou's and Rodriguez-Manzano's group in EEE has successfully applied DNA-based ISFET for pathogen detection, expanding the applicability of this technology to the detection of different pathogens [38], [50], [82], [83].

When the NA polymerisation reaction happens, hydrogen ions (H^+) are released, and the consequent chemical signal (H^+) is detected using ISFETs as pH sensors. The ISFET is a chemically sensitive transistor that can be tailored to different chemicals by depositing an ion-selective membrane on the gate. Insulators such as Aluminium Oxide (Al_2O_3), Silicon Nitride (Si_3N_4) and Silicon Dioxide (SiO_2) make the transistor sensitive to pH, which makes the ISFET useful for DNA detection using LAMP. Figure 25 shows a visual representation of the ISFET. Given that the gate of the ISFET is made of the chemical solution (and reference electrode), the properties of the ISFET (threshold voltage) will be sensitive to pH fluctuations such as protons released. The exchange of hydrogen ions between the electrolyte and the reactive sites at the insulator surface causes the change in potential at the electrolyte insulator interface. As the pH changes, the electrolyte oxide interface potential also changes, due to which the threshold voltage of the ISFET changes. This variation of threshold voltage causes a change in drain current [136].

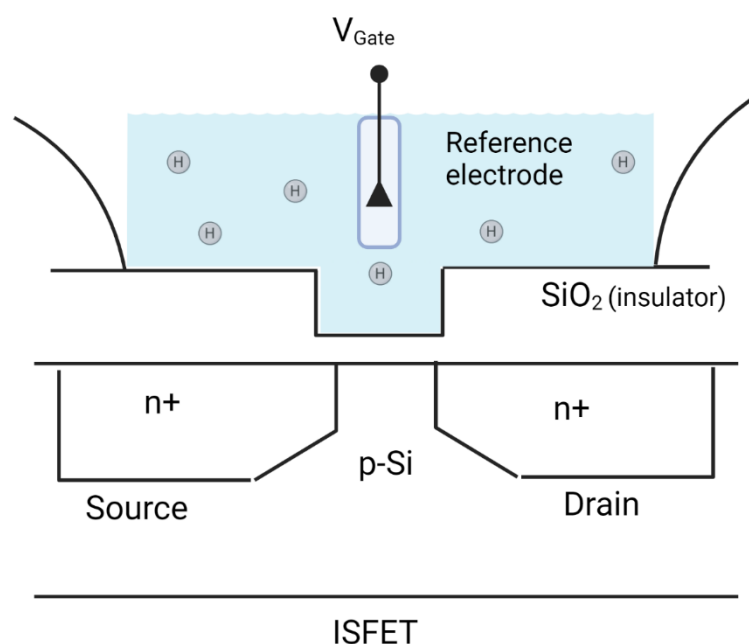


Figure 25. Bergveld schematic representation of the ISFET as electrochemical biosensor during DNA amplification. In general, a field-effect transistor (FET) consists of three terminals: the source, drain, and gate. The voltage between the source and drain of the FET regulates the current flow in the gate voltage. Specifically, the current-control mechanism is based on an electric field generated by the voltage applied to the gate. When a voltage is applied between the drain and source with the created channel, a current flows through this p-channel via the mobile electrons. Illustration generated with Biorender.com

DNA-based ISFET: from DNA to protons

Traditionally, amplification methods are performed in a buffered background where the pH is constant to maintain an optimal environment for the enzyme activity. When using pH-LAMP this restriction is removed, allowing unbuffered background and induction of proton generation with a

consequent pH variation when an amplification reaction occurs [134]. During NA amplification, when a dNTP is incorporated into the growing DNA strand, a proton (H^+) and a pyrophosphate (PP_i) are released as described in the reaction represented in Figure 26. Due to the ISFET capability for pH sensing when fabricated in unmodified CMOS, the sensors are capable of detecting H^+ accumulation. A pH change in solution (correlating to the DNA yield produced) is transduced by ISFETs into an electric output (correlating to voltage change). The translation of the amplification signal from nucleotides to protons allows the use of this technology, which is inherently scalable, low-cost, and portable, for quantitative detection of the target of interest [93].

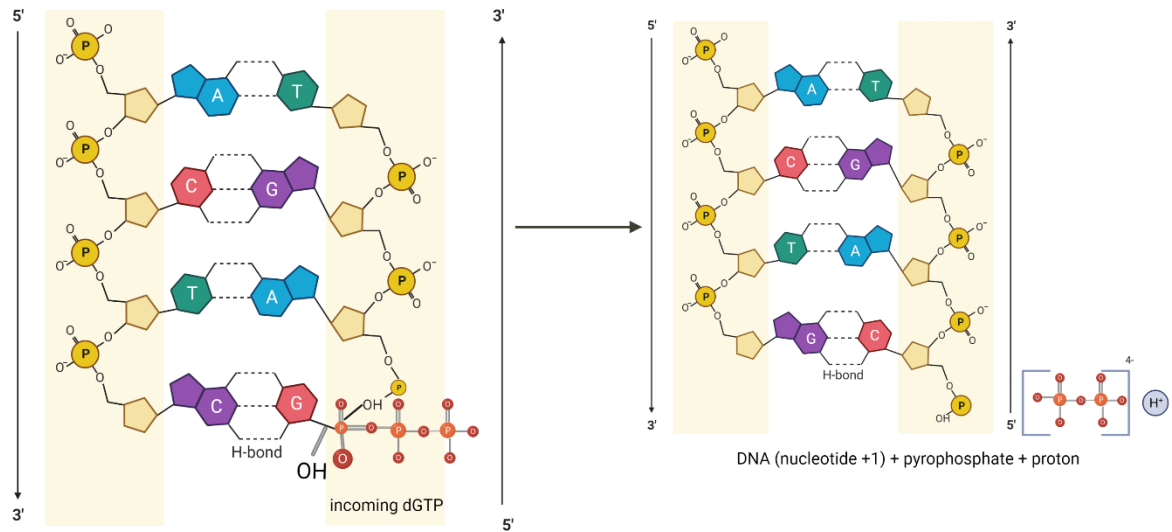


Figure 26. Representation of DNA hybridisation (single-strand (ss) DNA to double-strand (ds) DNA) and release of pyrophosphate, magnesium, and hydrogen ions because of nucleotide insertion [137]. DNA represents the target strand DNA and dNTP represents any of the four triphosphate bases (dATP, dCTP, dTTP, dGTP). Hydrolysis and incorporation of the nucleotide results in an extended strand represented as DNA+1, pyrophosphate, magnesium and hydrogen ion.

Materials and Methods

Clinical samples

The translation process focused on a new subset drawn from a larger group of samples (IRIS study). A total of 24 clinical samples categorised as definite bacteria and definite viral patients were selected with the main proviso being availability of remaining RNA sample for this analysis. Healthy controls samples were excluded from the study as the biomarker genes are being used to discriminate between different causes of fever and not between healthy and sick individuals. While this small pilot study is limited in sample size to fully address the applicability of the results obtained to the full range of infectious disease presentations, Table 4 summarises the diversity of the patients included. Data shown include the age, gender and ethnicity of the patients, the causative organism and the main presenting syndrome. The first column ('Type') indicates whether the patient category was bacterial (B) or viral (V).

Type	Age (months)	Gender	Ethnicity	Organism	Syndrome
B	22	M	Black (African)	Pneumococcus	Pneumonia with pleural effusion
B	24	F	White (British)	Gp A streptococcus	Pneumonia with bacteraemia
B	7	M	White (British)	Pneumococcus	Meningitis and bacteraemia
B	46	F	White (British)	Gp A streptococcus	Pneumonia with pleural effusion and bacteraemia
B	87	F	Black (Other)	Gp A streptococcus	Purpura fulminans with bacteraemia
B	10	M	White (British)	Gp A streptococcus	Pneumonia
B	10	M	Black (African)	<i>S. aureus</i>	Empyema
B	36	F	White (British)	Pneumococcus	Pneumonia
B	84	M	Not Stated	<i>Klebsiella oxytoca</i>	Abdominal sepsis
B	15	F	Other	Pneumococcus	Meningitis
B	58	F	Mixed (Other)	Gp A streptococcus	Osteomyelitis
B	21	M	White (European)	Meningococcus	Bacteraemia
V	16	M	Other (Middle East)	RSV	Bronchiolitis
V	49	M	White (British)	Influenza	Upper respiratory tract infection
V	24	M	Other (Middle East)	Influenza	Pneumonia
V	63	M	White (British)	Influenza	Pneumonia and asthma exacerbation
V	10	M	White (British)	RSV	Bronchiolitis
V	7	M	Black (African)	RSV	Bronchiolitis

V	102	F	Other (Middle East)	Influenza	Gastroenteritis
V	162	M	White (British)	Adenovirus	Tonsilitis
V	16	M	Other (Middle East)	RSV	Bronchiolitis
V	29	F	White (British)	Influenza	Upper respiratory tract infection
V	6	F	Other	Influenza	Pneumonia
V	59	F	White (European)	HSV1	Encephalitis

Table 4. Demographics of the patients utilised for this study. The first column ('Type') indicates the patient category, bacterial (B) or viral (V). The second column indicates the age in months. The third column shows the gender and the fourth column the ethnicity of the patients. Causative organism and main presenting syndrome at hospital presentation are shown in the last two columns.

The demographic features show a broad spread: median age was 24 months (IQR 11-59); gender balance was 14 males to ten females; the ethnicity mix included 12 with white ethnicity out of the 23 with data given. The range of infections was broad. The bacterial group included gram-negative and gram-positive bacteria causing a range of presentations, including respiratory infection, abdominal sepsis, bacteraemia, meningitis, and osteomyelitis. The viral group included RNA and DNA viruses causing respiratory, gastrointestinal, and neurological presentations.

LAMP assays

The two developed LAMP assays targeting the *IFI44L* and *EMR1* gene discriminating bacterial from viral infections in febrile children were transferred on the microchip-based platform: electronic amplification (eLAMP). *EMR1* was used as a candidate gene over-expressed in children with bacterial infections, and *IFI44L* was the candidate gene over-expressed in children with viral infections. LAMP primers conditions were increased in a final 10µL reaction from 0.8µM to 1.5µM for FIP and BIP and from 0.4µM to 1µM for LoopF and LoopB; 0.2µM for F3 and B3 were maintained. HPLC purified primers were purchased from IDT and suspended in molecular grade water to 100uM stock solutions and primer mixes were prepared for each LAMP primer set, ensuring no buffers were affecting the pH conditions. Aliquots were stored at -20°C for further analysis. Primers are listed in Table 5.

Gene	Primer	Sequence (5' – 3')
<i>IFI44L</i>	F3	TGTGGTATAGCATATGTGGC
	B3	GAGAGGATGAGAATATCCTTCA
	FIP	CGGCTTTGAGAAGTCATAGATCTACCTTGCTTACTAAAGTGGATGATTG
	BIP	ATGAATGTCCATAAAATGCTAGGCAGTCCAGTTCCAAATCTGAAG
	LF	AGTTGTCTTGAAGAACCTCACTG
	LB	CCAATATTTTGATGGTTGGAACTATG

<i>EMR1</i>	F3	CCACAGTCTTCCTGGAGAGT
	B3	CCAGTGGTCTCTGTGGATTC
	FIP	CGTCCGAACAGCCGGAGTGATATGGAAAGCATGACACTGGC
	BIP	TGTGACGTTGGACTTGGTAGCCCCTCAATTGTGGAACACCCG
	LF	TGCTGAGGGTTTCCAAAAAGAT
	LB	AAGGGGGATAAGATGAAGAT

Table 5. LAMP primer sequences developed for IFI44L (NM_006820) and EMR1 (NM_001974.5) targets.

RT-pH-LAMP reaction conditions

RT-LAMP reaction conditions were modified to enable the measurement of pH change. This allows for compatibility of LAMP with the on-chip amplification (eLAMP). RT-pH-LAMP reaction mix contained the same reagents used in the common RT-LAMP, except for the isothermal buffer, which was replaced with a customised isothermal buffer solution without Tris-HCl to lower the buffer capacity of the solution. NaOH was added to increase the starting pH of the solution (~8.5 pH) and enzymes were increased in concentration to reduce their volume in the reaction. The RT-pH-LAMP reaction mix comprised of 1 µL of 10X customised isothermal buffer (pH 8.5 - 9), 0.6 µL of MgSO₄ (100mM stock), 0.56 µL of dNTPs (25mM stock), 0.6 µL of BSA (20mg/mL stock), 1.6 µL of Betaine (5M stock), 0.25 µL of NaOH (0.2 M stock), 0.25 of AMV reverse transcriptase by Promega (25,000 U/mL stock), 0.10 µL of RNase inhibitor (20,000 U/mL stock), 0.25 µL of SYTO 9 Green (20µMstock), 0.042 µL of Bst 2.0 DNA Polymerase (120,000 U/mL stock), 1 µL of 10X LAMP primer mixture, 1 µL of template and enough nuclease-free water (Thermo Fisher Scientific) when necessary, to bring the volume to 10 µL reactions.

All reagents except the isothermal buffer (in-house) and AMV reverse transcriptase (Promega) were purchased from New England Biolabs. To validate the performance of the LoC technology pH-LAMP reactions were carried out at 63°C for 30 minutes and loaded in parallel on the chip and into a LightCycler® 96 System (Roche Diagnostics) for real-time experiments. Furthermore, one melting cycle was run in the LC96 at 0.1°C/s from 65°C up to 97°C for validation of the specificity of the amplification products. Each experimental condition was run in triplicate. For RT-eLAMP, the reaction mixture contained the same LAMP reagents except for the SYTO 9 Green.

RT-pH-LAMP reaction conditions

Electronic LAMP (eLAMP)

The illustration in Figure 27 shows a disposable cartridge containing the microchip surrounded by an acrylic manifold that allows approximately 10 µL solution to be transferred to the reaction

chamber on the top of the chip using a common micropipette. To carry out DNA amplification reactions on a chip, the microfluidic chamber was filled with 10 μL of pH-LAMP reaction. The well was successfully sealed with PCR tape to avoid evaporation and contamination of amplicons while running on the platform. The disposable cartridge was inserted into a portable device with an embedded thermal controller and battery. During the isothermal amplification the release of hydrogen ions is directly proportional to the nucleotides incorporated into the double-stranded chain. Therefore, pH change in solution was measured before and after amplification, using a pH probe to verify the success of the amplification.

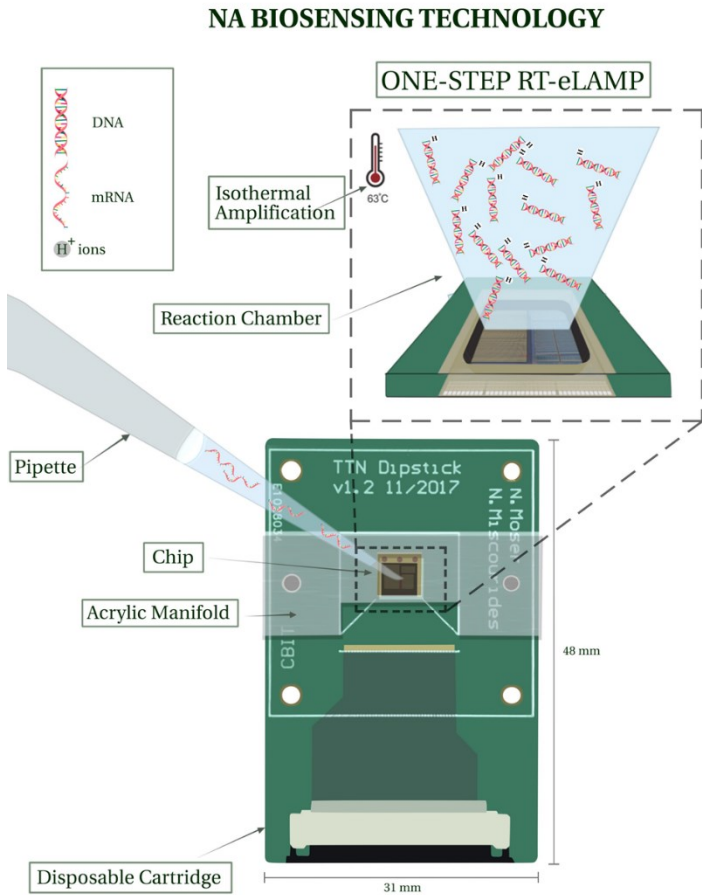


Figure 27. NA biosensing technology illustrated on a disposable cartridge. Following RNA extraction from whole blood, the mRNA target is mixed with the LAMP reagents and a total of 10 μL reaction is loaded on top of the ISFET sensors to allow the electrochemical reaction to happen.

Chip assembly

The chip contained 4368 ISFET pixels fabricated in a commercial CMOS process using silicon nitride (Si_3N_4) as a sensing layer. Each pixel consisted of an ISFET configured for pixel quantisation, improving robustness and better sensitivity to pH change [138]. The ISFET sensing arrays are encapsulated on a ground panel (gold) of a disposable PCB cartridge 4.84 x 3.12 cm and prepared for

contact with the wet reaction mix solution. To prevent short circuits in bond pads, the sides of the chip and any pad openings are covered by biocompatible epoxy.

A 3D-printed or laser-cut acrylic manifold is screwed down on the cartridge and taped using Tessa adhesive gasket to contain the solution that will be in contact with the microchip. The forming reaction chamber of 3x2 mm allows approximately 15 μ L solution to be contained (Figure28).

A chlorinated in 1M KCl, Ag/AgCl reference electrode (0.03mm diameter) is placed on the chip surface in contact with the solution and connected to a cartridge contact to allow voltage reading.

The chip also contains temperature sensors spread through the array, and an external heater or a Peltier module added at the bottom provides temperature regulation, which is needed for the LAMP assay. Signal processing and control circuitry are all contained within the same portable chip platform, making it an optimal candidate as a portable diagnostic device.

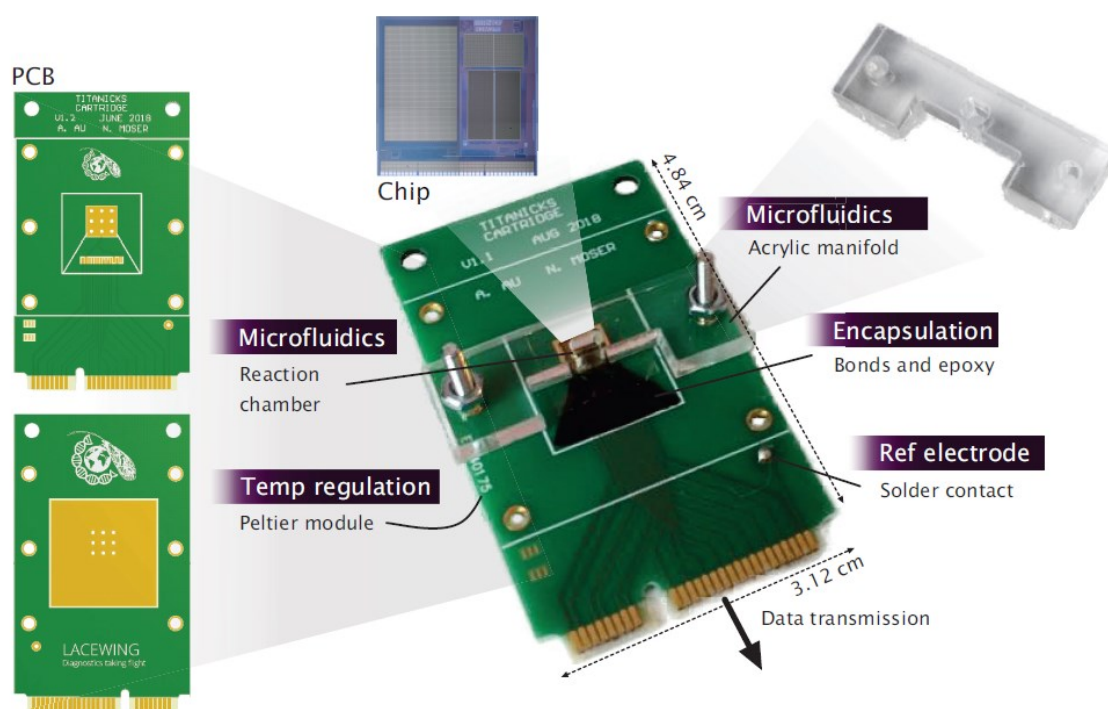


Figure 28. Cartridge components for portable diagnostics. Illustration of a disposable cartridge before and after the assembly step. The chip is mounted on the PCB and encapsulated using epoxy. The headers are connected by wire-bonding to the cartridge for data transmission. An acrylic manifold seals the reaction chamber and the reference electrode, soldered to a contact. A Peltier module is added at the bottom to regulate temperature. Illustration kindly provided by N.Moser.

Benchtop instrument experimental setup

To demonstrate the performance of the on-chip amplification reaction compared to the gold standard real-time amplification, both reactions were run in parallel using a conventional real-time PCR machine. The benchtop setup shown in Figure 29 was used to conduct amplification reactions on-chip in a controlled environment. To perform on-chip amplification while providing a stable

temperature condition of 63°C, the acrylic manifold shown in Figure 28 was loaded with 10 µL of pH-LAMP reaction and the cartridge PCB was inserted in a flat thermal block within the PCR machine (Veriti Thermal Cycler, Applied Biosystems) for temperature monitoring with a ribbon cable used to isolate the cartridge inside the thermal chamber. From the chip, the ISFET array was continuously monitored using MATLAB software and the average output of the active pixels was used as an indicator of pH changes in the solution. The same reaction mix was run in parallel in the LC96 to compare amplification results. The experimental setup of the benchtop approach (Figure 29) was conducted as follows:

1. The end of a 0.03mm diameter silver wire was chlorinated to obtain an Ag/AgCl reference electrode and inserted on the top of the microchip to provide biasing. The voltage was controlled externally.
2. The cartridge PCB containing the microchip was assembled by placing an acrylic manifold containing the reaction chamber on the top of the chip and the wire, to contain the reaction solution and prevent leakage.
3. The response of the chip and the sensors was evaluated by inserting deionised water in the chamber and tuning the voltage of the reference electrode until the point with the maximum number of active pixels was reached.
4. The benchtop instrument (Veriti Thermal Cycler) was set to provide a stable and controlled temperature of 63°C for the cartridge to allow the amplification reaction to happen on top of the microchip.
5. Once the isothermal condition was reached, the deionised water was removed and replaced with the reaction mix containing the target and sample of interest to allow amplification to occur. The chip was constantly monitored in real-time by reading data from the array of sensors through the motherboard PCB connected to a PC via USB. The reaction was stopped at 30 minutes for further analysis.

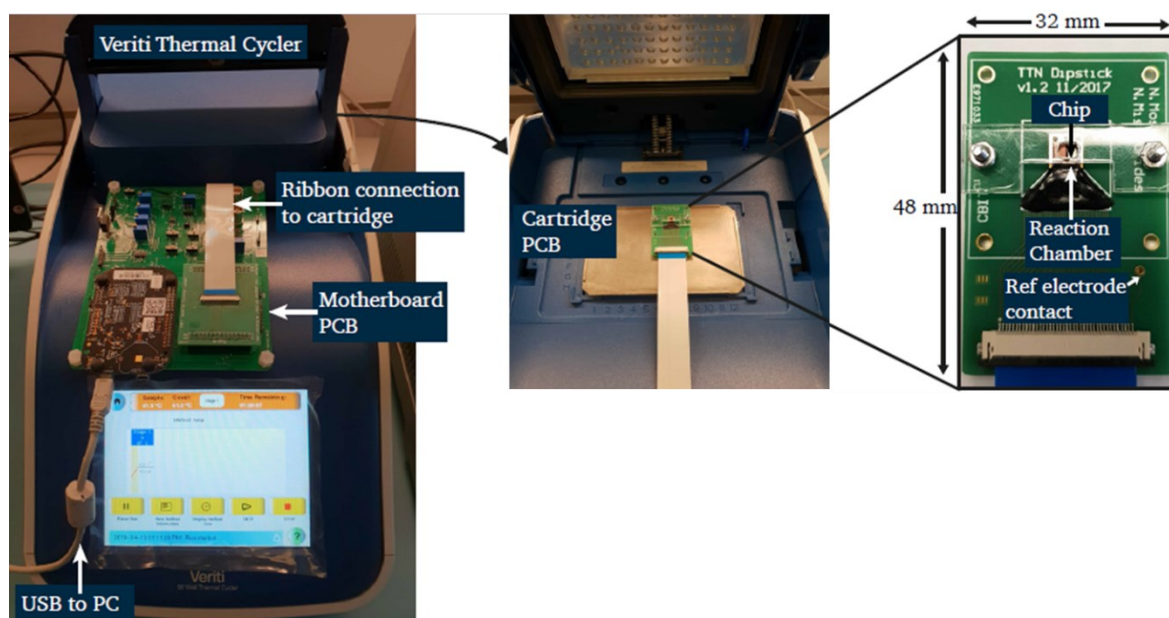


Figure 29. On-chip amplification using a benchtop thermal cycler to maintain a constant temperature.

Portable lab-on-a-chip platform

The previous setup was used to conduct amplification reactions on-chip in a controlled environment by keeping the cartridge at a stable temperature of 63°C. As a transition from benchtop diagnostics to portable diagnostics, a handheld platform developed by Nicolas Moser and members of the CBIT called Lacewing (LW) was utilised. Lacewing stands for location-aware counter-epidemics wireless ISFET networking. Lacewing is the first portable CMOS sensing platform (Moser et al.) [139] to be handheld and to work without the direct use of electricity. The prototype integrates the microchip described in a motherboard (Figure 30) with integrated circuits and analogue components including a Peltier module for thermal supply controlled by a microcontroller, a power supply using a rechargeable battery, a micro-USB for battery charging, a holder for the disposable cartridge and a PIC microcontroller for digital algorithms and data acquisition.

A companion application named Firefly is interfaced with the system, allowing real-time monitoring of amplification reaction on-chip and controlling the motherboard and cartridge via Bluetooth connectivity. The app also enabled geotagging and cloud storage of the test results.

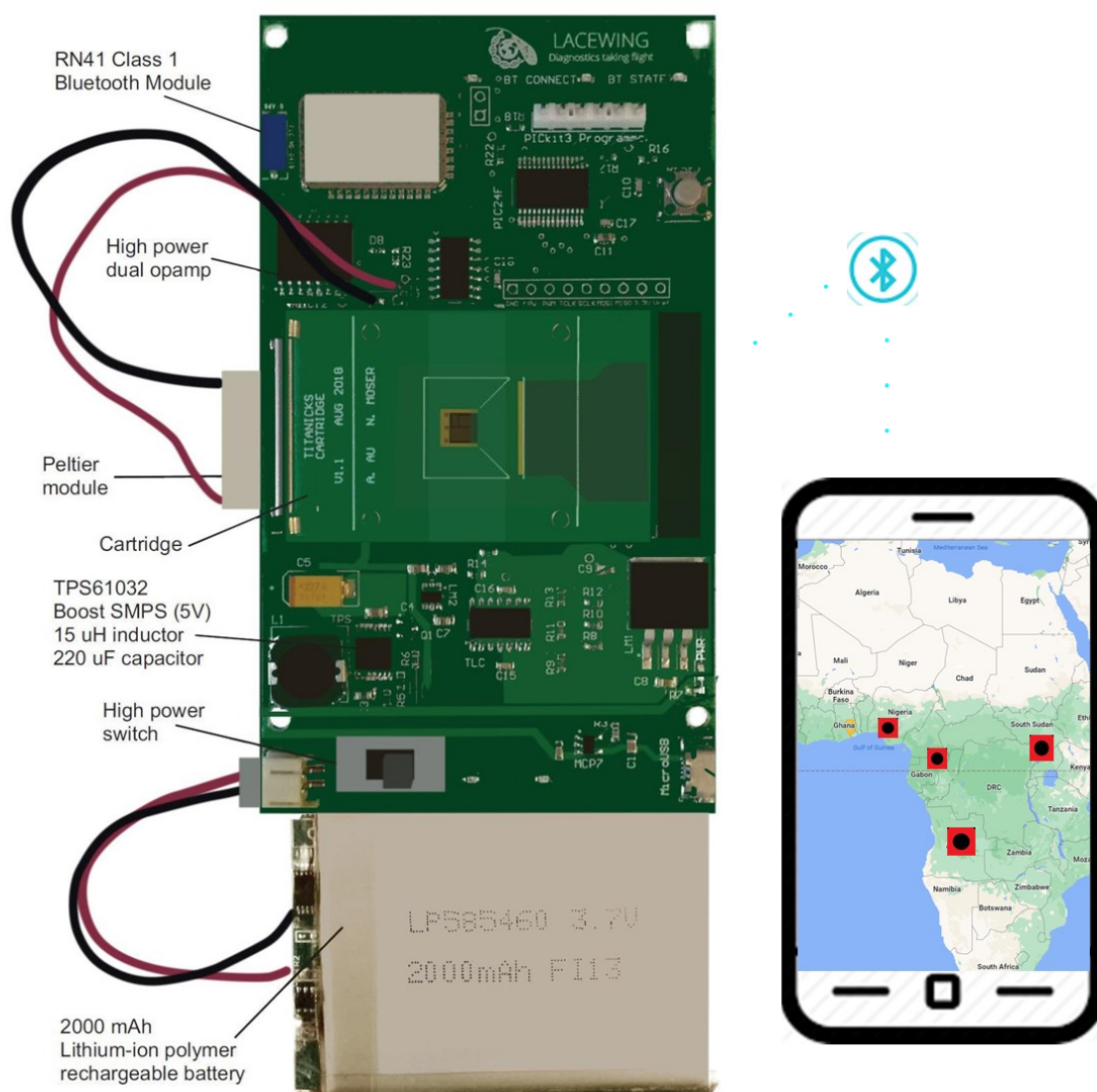


Figure 30. N. Moser portable LoC platform.
 Illustration of Lacewing device with disposable cartridge integrated on the motherboard. A Bluetooth module provides real-time monitoring of the reaction and data can be visualised through a companion app designed to interface and control the cartridge and the motherboard. Geolocation and cloud storage are also enabled for epidemiological surveillance.

The clinical samples were tested on the Lacewing platform to demonstrate the capability of a LoC device to detect and discriminate bacterial and viral infections with similar results compared to conventional instruments. To perform amplification reaction on LW, 10 μ L of reaction mix were loaded on the microfluidic well and sealed with tape to avoid evaporation, and the assembled cartridge was inserted onto LW.

The LW platform is controlled by the Firefly application and consists of seven steps that are illustrated on the app step by step. The sequence of the operations is summarised below:

- The initialisation step takes less than one second and resets the chip’s RAM to 0.
- The calibration step takes one second and calibrates the temperature of the array.

- The third and the fourth steps find the optimal calibration by looking at the best reference electrode voltage and take approximately one minute.
- The fifth step loads the calibrated values for the temperature array and compares them to the expected value at 63°C.
- The sixth step regulates the Peltier voltage from the output of the temperature array. Once the regulation is ready, the array readout starts, and regulation happens at every frame.
- The last step allows the readout to begin.

The whole sequence of steps lasts less than two minutes and is required before each LW run. Before starting a measurement, the sensors are exposed to deionised water for several hours. This allows the sensor drift to settle and reach linear behaviour. Data were gathered regarding the time of the test, typically 25 minutes, with results being saved on the cloud server and geotagged to enable visualisation on the infection map. A controller maintains the temperature at around 63°C during the reaction. A slight variation around 63°C justifies the choice of LAMP as a DNA amplification method, demonstrating that the reaction does not require temperature cycles like with PCR, but also that it offers tolerance around the absolute temperature.

Data analysis of ISFETs with the conventional method

The data analysis of ISFET arrays consisted of averaging the output of every sensor to give a single time series, removing drift and subsequently processing the data (ATP method) so that the output is similar to conventional PCR instruments. By using the conventional method, amplification data were analysed from all ISFETs in contact with the chemical solution. The resulting signal was normalised by removing the chemical drift, and the signal from all the sensors was analysed from the same distribution. The TTP was extracted via the Cy0 method (Guescini et al.,[140]).

Data analysis of ISFETs with the proposed method

The proposed method (Figure 31) consisted of five steps and was performed using MATLAB. Raw data were collected from the ISFET sensors (A). The pre-processing removed the background of each signal by subtracting the average of the first five voltage measurements (B). Then, the drift was removed using an adaptive signal processing approach where the learnt parameter was updated at each time step and the overall behaviour was captured over time (C). Sensors with similar behaviours were clustered together in the unsupervised clustering (D). Spatially close sensors were expected to behave similarly; therefore spatial validation was performed to confirm non-random clustering (E). The signals in each cluster were averaged to remove noise, and a peak detection algorithm was used to extract a TTP value (F).

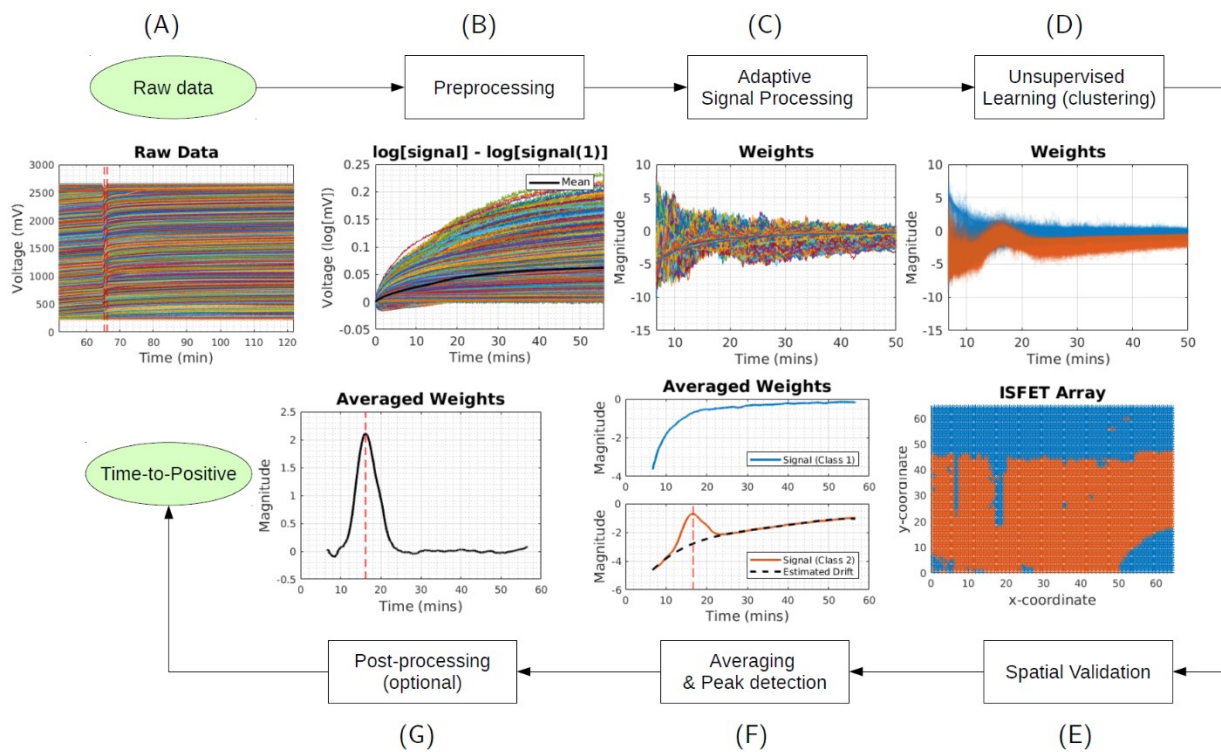


Figure 31. A proposed method for extracting TTP values from ISFET arrays. Developed by Dr Moniri.

Statistical analysis for discriminating infectious groups

To define a decision boundary between the viral and bacterial infection groups, a support vector classifier from the scikit-learn package in Python was used. The linear combination of TTP values was used as a score in the regression model. To avoid over-fitting, tenfold cross-validation was used to select the values. The confidence interval for the classification accuracy was determined using the Pearson correlation and a p-value using the F-statistic.

Microarray analysis

Values for gene expression data, analysed on the Illumina microarray platform, were provided by Herberg and colleagues.

Using the data provided, the clinical samples were identified and selected that had differentially expressed transcripts between the definite viral and bacterial groups with an $\log_2\text{FCI}$ above 1 and an adjusted p-value less than 0.05, using a trained support vector machine classifier. These thresholds were chosen on purpose to ensure that differential expression for selected variables could be distinguished using the resolution of other validation techniques, such as qPCR.

Results and Discussion

RT-eLAMP assays performance

LoD data was generated on a benchtop instrument by detecting synthetic RNA in a series of tenfold dilutions, in a range from 1×10^6 to 1×10^1 copies per reaction, using five replicates at each concentration. Figure 32 shows the standard curves for *IFI44L* and *EMR1* assays, developed for eLAMP. Results demonstrate highly sensitive assays, with an LoD down to ten copies per reaction, high specificity alongside appropriate speed for POC applications (TTP reaction within 25 minutes). The calculated LoD for the two assays is shown in Figure 32.

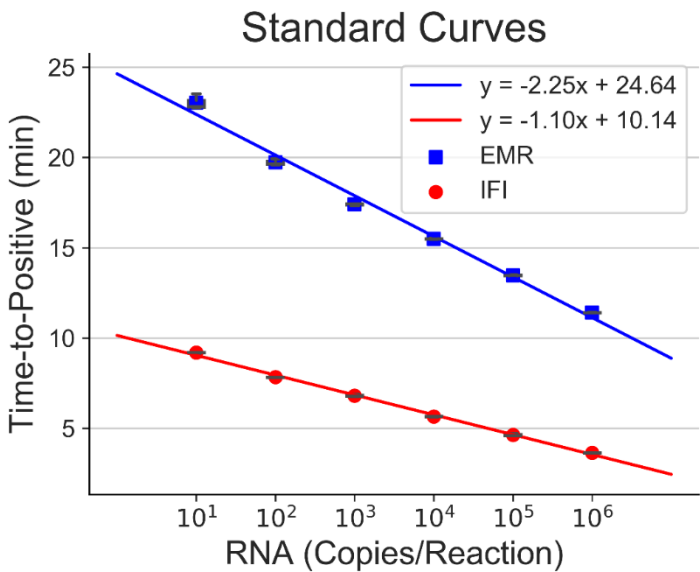


Figure 32. RT-pH-LAMP assays *IFI44L* and *EMR1* performance in eLAMP.

Microarray analysis using definite bacterial and viral samples

Illumina microarray values targeting the two-gene signature (*IFI44L* and *EMR1*) using definite bacterial and definite viral samples were analysed and are shown in Figure 33. The scatterplot of the log microarray expression values shows the decision boundary for a trained support vector machine classifier, indicated by a black dashed line (Figure 33a). The classification accuracy, as determined by cross-validation, was 100:0% (95% CI, 95.7%-100:0%) [Sensitivity of 100% (95% CI, 95.7%-100%), specificity of 100% (95% CI, 95.7%-100%) and AUC of 100% (95% CI, 91.6%-100%)]. (Figure 33b) shows boxplots of the classification with scores that correspond to the Euclidean distance between each data

point and the decision boundary. The log RNA expression values can be found in the Figure 33c and show the values for each isolate and their true class. The success column indicates whether the experiment gave a valid output for each of the two-gene signatures indicated in the success column by (Y). If one of the genes failed to give an output, the isolate would be considered unsuccessful and would be indicated by (N). In microarray, all the isolates were considered successful. This analysis demonstrated that the two-gene signature has high power in classifying children with definite bacterial infections and children with definite viral infections and can be considered a successful method in microarray analysis. However, detection with gene expression microarrays needs sophisticated lab equipment, which is expensive and cannot be used for routine diagnostics. Therefore, clinical samples were assessed on the benchtop platform using the RT-pH-LAMP assay.

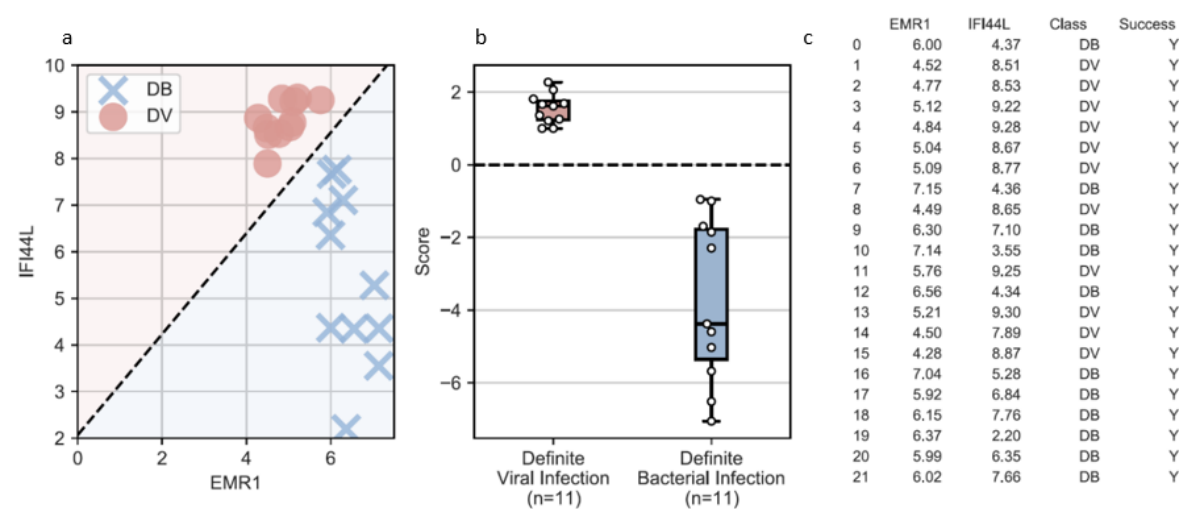


Figure 33. Viral versus bacteria classification using Illumina microarray analysis. A scatter plot of the log microarray expression values is shown in (A). The boxplot of the classification score is shown in (B). A Table with log expression values for each patient sample and their rate of success is shown in (C).

RT-pH-LAMP on benchtop platform

The same assays and clinical isolates were tested using RT-pH-LAMP using the benchtop instrument. The results in Figure 34a show the scatterplot of the TTP values obtained for the two genes using RT-pH-LAMP. The assay provided a 100% (CI, 95.7-100%) accuracy when separating the two infection groups, with a sensitivity of 100% (95% CI, 95.7%-100%), specificity of 100% (95% CI, 95.7%-100%) and AUC of 100% (95% CI, 91.6%-100%) when compared to the reference standard (RTqLAMP) confirming the successful translation of the pH-LAMP assay developed. In the boxplot in Figure 34b. it is possible to observe a clear separation of the two infection groups. The TTP values for each isolate are reported in Figure 34c and are all considered valid. An aspect to ease the visualisation of the data across the two platforms (microarray and RT-pH-LAMP) is that microarray measures the RNA expression level (abundance of the target) and LAMP measures the TTP (in minutes), so it is expected that TTP values from LAMP are inversely correlated to the expression values of microarray

(the higher the expression level, the smaller the TTP). This can be easily visualised by observing that the positions of DB and DV isolates in the scatterplot results in Figures 33a and 34a are swapped in 2D space. For example, in the microarray classification in Figure 33a, DB patients are in the lower right and DV patients are in the upper left part of the scatter plot, while in the RT-pH-LAMP classification in Figure 34a, the DV patients are in the lower right and DB patients are in the upper left part of the scatter plot.

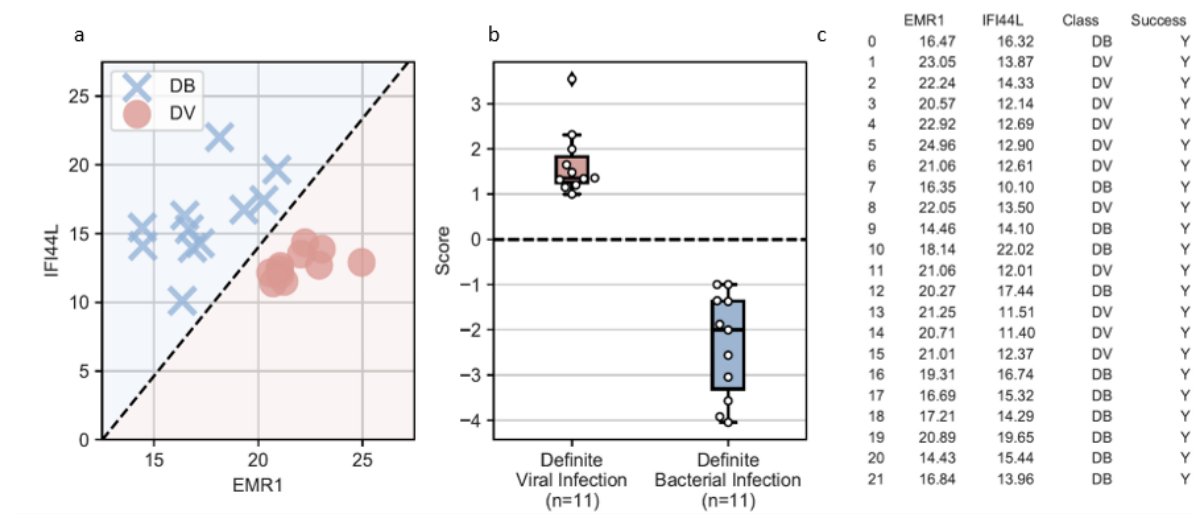


Figure 34. Viral versus bacterial classification using RT-pH-LAMP on benchtop instrument. The scatterplot of the TTP values for definite bacterial and definite viral patients is shown in (a). A boxplot of the classification score, which corresponds to the decision boundary is shown in (b). A Table with the TTP values for each patient sample and their rate of success is shown in (c).

RT-pH-LAMP on CMOS platform

To evaluate the classification performance on the microchip platform, the same clinical isolates were tested on the ISFET-based LoC platform using the conventional method based on the ATP algorithm. The results in Figure 35a show the scatterplot of the TTP values for the two-gene using RT-eLAMP, and a non-ideal classification can be observed among the two infection groups, with clinical isolates overlapping and suggesting poor classificatory performance in distinguishing bacterial from viral infection. A total of 36.4% of the experiments were considered invalid (N) (Figure 35c), failing in giving a valid output for each of the two-gene signatures, resulting in a loss of 50% of the isolates when generating a 2D signature. Furthermore, it can be observed that due to the lack of data, the decision boundary has a negative slope coefficient (Figure 35b), which is not correct.

This behaviour is due to the limitations caused by ISFET noise: trapped charge (an offset in the signal), drift (electronic or chemical that changes the output of the sensors over time), and temperature effects (uncontrolled temperature regulation that changes the output of the sensors). When ISFET presents the mentioned behaviours, it becomes challenging when analysing the data using the ATP method. The ATP method is sufficient in the scenario where most of the sensors have

similar behaviour and are not impacted by noise and trapped charge effects. When these effects are present an alternative method should be considered when analysing data.

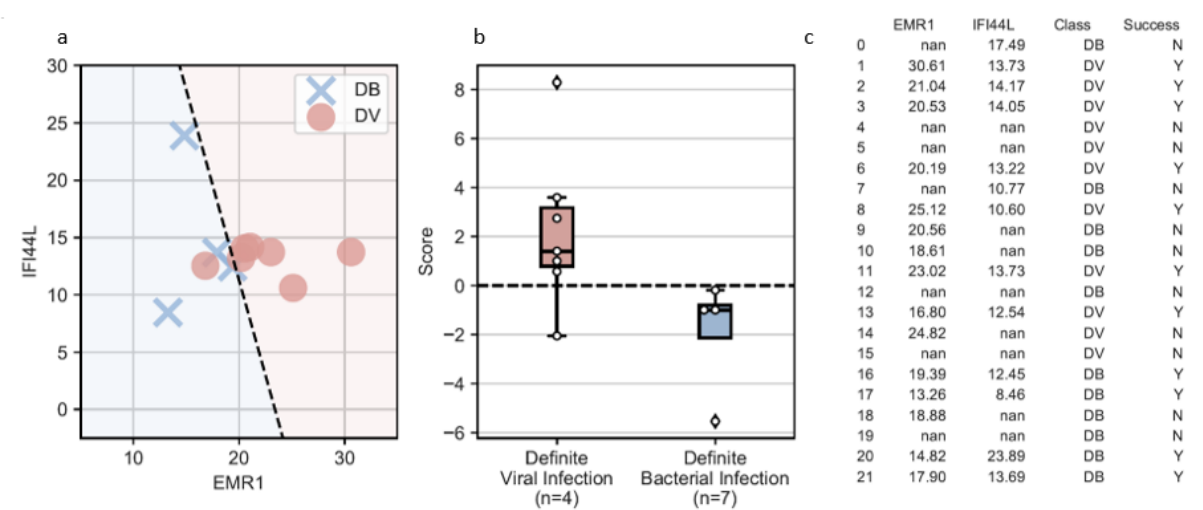


Figure 35. Viral versus bacterial classification applying the ATP method on RT-eLAMP. The scatterplot of the TTP values for definite bacterial and definite viral patients is shown in (a). A boxplot of the classification score, which corresponds to the decision boundary, is shown in (b). A Table with the TTP values for each patient sample and their rate of success is shown in (c).

RT-eLAMP using the proposed method

To directly compare and evaluate the differences in the classification performance when using the ATP and the proposed method, RT-eLAMP results were analysed following the proposed method as described in the section on materials and methods. In contrast to the results shown in Figure 35, where the ATP method was applied, the proposed method showed the correct classification of definite bacterial and definite viral groups Figure 36a. The classification accuracy results are 100% (CI, 95.7-100.0%) showing a positive decision boundary slope and sensitivity of 100% (95% CI, 95.7%-100%) specificity 100% (95% CI, 95.7%-100%) and AUC of 100% (95% CI, 91.6%-100%). Although the two infection groups are not distinctively separated, as shown in microarray and RT-pH-LAMP classification, they still show a 100% rate of success (Y) for each isolate value as in Figure 36c. The boxplot in Figure 36b shows a clear separation of the two infection groups, and taken together, these results confirm that the proposed method might be considered a good alternative to the conventional method to overcome ISFET non-idealities and correctly classify patient groups when using the CMOS-ISFET platform.

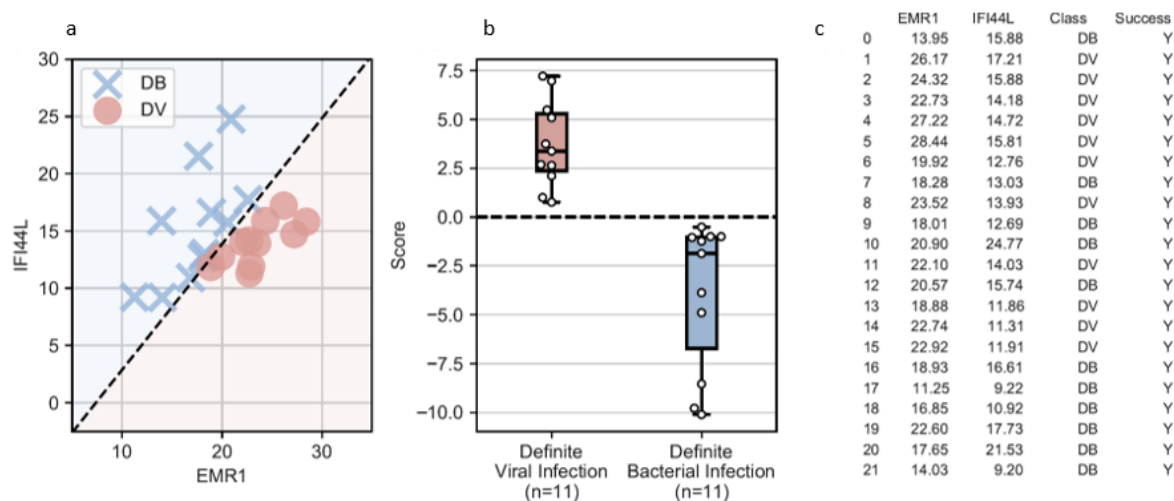


Figure 36. Viral versus bacterial classification using RT-eLAMP applying the proposed method. The scatterplot of the TTP values for definite bacterial and definite viral patients is shown in (a). A boxplot of the classification score, which corresponds to the decision boundary is shown in (b). A Table with the TTP values for each patient sample and their rate of success is shown in (c).

Evaluating the translation process

Figure 37 shows the correlation analysis of the predictive score in the RT-eLAMP and traditional RT-qLAMP that has been evaluated and assessed for each bacterial and viral patient using the two signatures. The predictive scores were tightly correlated between the two techniques, the Pearson’s correlation coefficient was 0.90, R^2 0.82 and the F-statistic p-value 1.36e-09. This transfer learning approach provided the confidence that the eLAMP methodology could be reliably used in the translation process, given the strong one-to-one correlation when using the gold standard method.

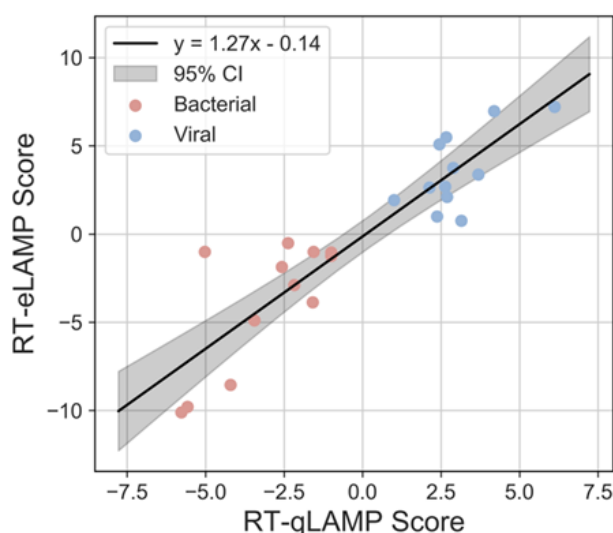


Figure 37. Correlation analysis between RT-eLAMP and RT-qLAMP. The performance shown in the correlation when comparing the LAMP assay on different platforms confirms the capability of the CMOS-based platform to detect and correctly classify samples with the same accuracy provided when using the gold standard instrument qPCR. The bottleneck in the translation process might instead be represented by the limitations due to the complexity associated with the LAMP assays design.

Although it has been shown that the RT-qLAMP and RT-eLAMP can be used to classify different infection groups reliably and correctly, representing a potential diagnostic solution, especially for limited resource settings, microarray methods remain key to discovering and identifying gene expression signatures. To study the translation process from microarray to RT-eLAMP, normalised gene expression values (log2 fluorescence) and TTP values have been compared using support vector classifiers, with tenfold cross-validation, and each patient was represented by a score reflecting the risk of bacterial or viral disease. In Figures 38a and 38b, the two-transcript signature showed a sensitivity of 100% (95% CI, 95.7%-100%), and 100% (95% CI, 95.7%-100%), with AUC 100% (95% CI, 91.5%-100%). Translating the two-gene signature to RT-eLAMP showed similar results: sensitivity and specificity were 100% (95% CI, 96.0%-100%), and 100% (95% CI, 96.0%-100%), with AUC 100% (95% CI, 92.2%-100%) (Figure 38c,d). To visualise the differences and similarities between microarrays and the RT-eLAMP, the per-gene expression values and TTP values correlations of the two platforms were evaluated and can be visualised in Figures 38e and 38f. Plot E shows the *EMR1* gene with a correlation between microarray and eLAMP of -0.54, R^2 : 0.29, F Statistic p-value: 0.01. Plot F shows the *IFI44L* gene with a correlation between microarray and eLAMP of -0.47, R^2 : 0.22, F Statistic p-value: 0.0274. Overall, the translation process performed in this proof-of-principle study demonstrated that differentiating bacterial from viral infections using host-transcriptomics and the semiconductor-based platform is a feasible reality and can be moved closer to clinical application, providing faster results than microarray.

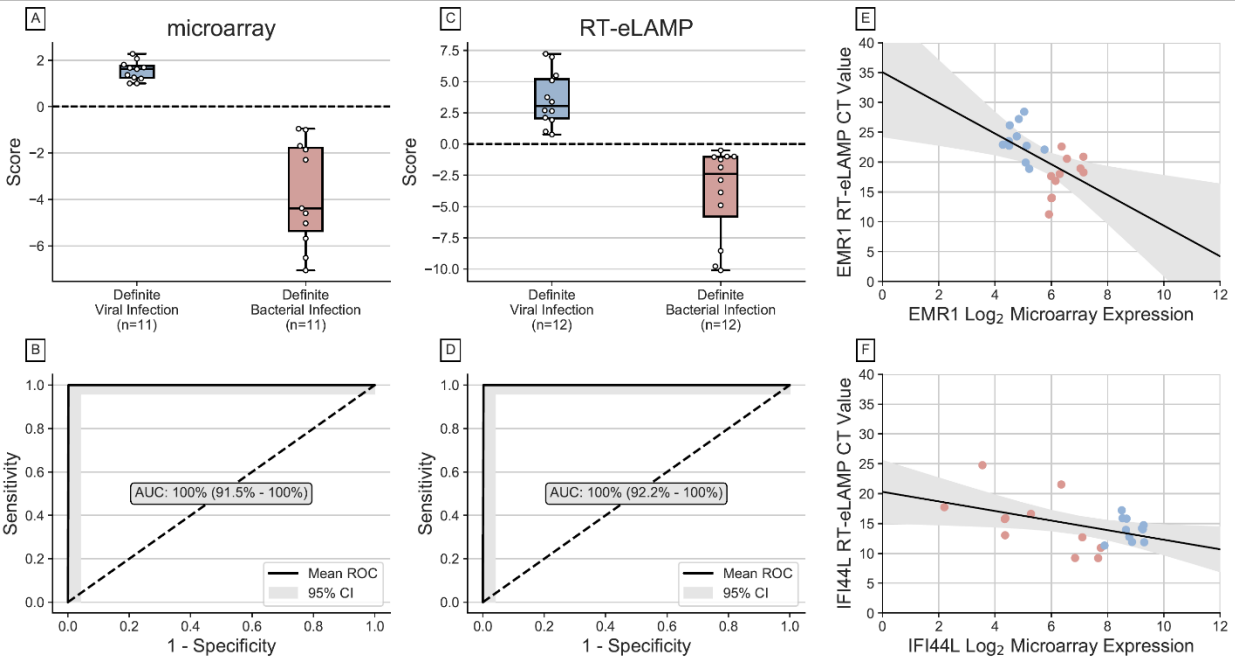


Figure 38. Microarray vs RT-eLAMP. Predictive score, receiver operating characteristic (ROC) curves, expression values and correlation analysis based on the two-transcript signature for distinguishing definite viral (dark blue) and definite bacterial (light red) samples. Boxes indicate the

interquartile ranges and the median (middle line). Whiskers indicate 1.5 times the interquartile range. A and B show performance using expression values obtained from the microarray platform. C and D show performance using the RT-eLAMP platform. AUC indicates the area under the curve. E and F show the correlation between techniques.

Conclusion

This chapter concludes Part I of the thesis. RNA signatures reported in the literature that differentiate bacterial from viral infections use inconvenient and bulky transcriptomics technologies. In this proof-of-principle study, the promising transcript signatures were moved closer to clinical application, highlighting the potential of a two-gene RNA signature to be translated into a rapid and portable LoC test suitable for POC applications. To translate the findings from microarray gene expression discovery to LoC diagnostics, sensitive LAMP assays were developed to detect host transcripts on chips. The algorithm developed by Ahmad Moniri was implemented in this study and thoroughly discussed in his dissertation as method to extract valuable information from an array of electrochemical sensors, providing better separation between the two infection groups on-chip.

The sample-to-result workflow (Figure 39) can be summarised in three main steps:

1. NA extraction from a patient sample
2. eLAMP detection on ISFET sensors using the proposed algorithm
3. Diagnostic outcome

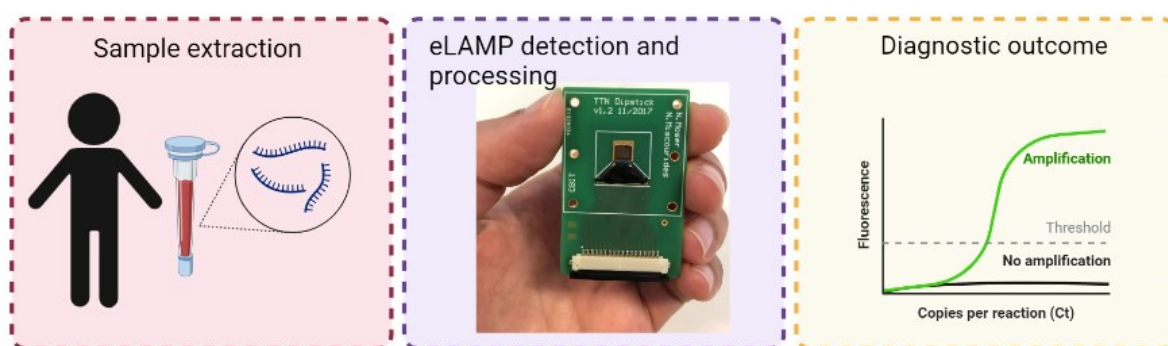


Figure 39. The LoC sample-to-result workflow.

The absence of fluorescent technologies and the economy of the microchip employed make this method suitable for implementation at a low cost. Furthermore, the capability of the electrochemical sensors to translate NA detection into the release of hydrogen ions during isothermal amplification enables faster detection of any target, increasing the level of quantification of the final product.

Overall, the technology is building towards a fully integrated platform with sample-to-result diagnosis.

This study raises several interesting questions for future directions:

- How would the microchip-based method described here work on a broad spectrum of illnesses?

It would be interesting to assess the performance of the method in a wider population. As this is a proof-of-concept study, the focus was only on the patients with single-pathogen infections. However, in this study, patients with a range of severity, some with moderate and some with severe illness, were included. Eight of 22 patients required paediatric intensive care unit admission for their treatment suggesting the results presented confirm that the discrimination among bacterial and viral infection was independent of the severity of the illness.

- Where would this approach be more beneficial?

I believe a key advantage of a POC diagnostic would be its use in primary care settings, where the spectrum of paediatric infection would include those with a milder phenotype, and I believe further work will be required to carefully validate its use in this setting.

- What kind of outcome would provide this method?

The diagnostic outcome may be a differentiation between a biological sample from a subject that has an infection and a biological sample from a subject that does not have an infection, or differentiation between a biological sample from a subject that is predisposed to an infection a subject that is not predisposed to an infection. Or the diagnostic outcome may be a differentiation between an active form of an infection and a latent form of an infection.

- Could the number of transcripts included in this approach be increased so that patients with co-infections could be detected?

Further work will address whether using a higher number of genes could identify patients with bacterial and viral co-infections. The platform used had a limited number of wells per microchip. However, a new microchip with higher capacity in terms of microfluidic wells might allow higher detection capabilities. Several methods can be used on-chip to achieve detection of multiple gene signatures. For example, in the case of a 6-gene signature six separate reactions plus controls may be run on the new chip (with enough space for twelve reaction wells), or reactions may be combined depending on the regulation direction of the biomarker (up-down).

- How can mRNA extraction from whole blood be obtained at the POC?

The technique for RNA purification used in this study is labour-intensive and can only be performed in a laboratory, as shown in Figure 14. The sample preparation step is far from being suited to POC or limited resource settings. Indeed, available technologies that detect transcript abundance are only used in diagnostic laboratories, where accurate rather than rapid diagnostic testing is needed. LAMP has been extensively validated for whole blood applications and quality of the NA extraction (inhibitors carry over) is normally not a problem [141]–[143]; This opens the door to the design of an assay using finger-prick sampling (<100 µL blood). I am developing a challenging microfluidic device that will allow a simple RNA purification from whole blood with no input from laboratory instrumentation.

Despite its limitations, this study provides proof of concept that combining the advantageous sensing properties of microchips with innovative molecular biology approaches such as LAMP and host RNA gene transcripts could enable the origin of an innovative class of sensitive, reliable and resourceful diagnostic tests.

Part II

Chapter 4

SARS CoV-2 detection on lab-on-a-chip technology

Part II of the thesis expands to direct-pathogen detection. The previous chapter demonstrates the feasibility of the microchip-based platform to detect and discriminate bacterial from viral infections in children using a host-transcriptomics approach. This chapter focuses on SARS-CoV-2 detection from extracted RNA and how the microchip-based platform was rapidly adapted during the pandemic to detect the presence of SARS-CoV-2 in under 20 minutes. This retrospective study using a set of 183 clinical samples was conducted with the CBIT team to evaluate the performance of the developed RT-LAMP assay on a real-time benchtop instrument (RT-qLAMP). A subset of 40 samples was tested to validate the translation of the RT-LAMP assay onto the POC platform (RT-eLAMP). Results demonstrated comparable performance to the gold-standard benchtop commercial instrument and provided confidence that the LoC platform can reliably detect the presence of SARS-CoV-2 with high analytical sensitivity in as little as 20 minutes, surely a more portable, affordable, and scalable solution than existing POC methods in healthcare systems. As part of a team effort, I contributed to the conceptualisation and laboratory work, whereas the statistical analysis was conducted by my colleagues in the CBIT.

The concepts in this chapter resulted in the following journal article:

J. Rodriguez-Manzano, K. Malpartida-Cardenas, N. Moser, **I. Pennisi**, M. Cavuto, L. Miglietta, A. Moniri, R. Penn, G. Satta, P. Randell, F. Davies, F. Bolt, W. Barclay, A. Holmes and P. Georgiou, "A handheld point-of-care system for rapid detection of SARS-CoV-2 in under 20 minutes", ACS Central Sciences, vol. 7, no. 2, pp. 307-317, Jan 2021.

Introduction

SARS-CoV-2 and the pandemic scenario

The novel coronavirus identified in Wuhan, China Wuhan for the first time in December 2019 developed into a dramatic scenario that obliged the WHO to declare a pandemic status [144]. Two years ago, it was not clear whether the new coronavirus would represent a historic threat to global health, with a large and prolonged impact on every aspect of society. In the current Coronavirus Disease 2019 (COVID-19) pandemic, the virus responsible is SARS-CoV-2, which is an enveloped, positive-sense, single-stranded RNA betacoronavirus belonging to the Coronaviridae family [145].

The genome of SARS-CoV-2 measures more than 30 kb in length and contains 14 open reading frames (ORFs) encoding 27 proteins. A polyprotein is encoded at the 5' end of the SARS-CoV-2 genome by ORF1a/b, which is post-translationally cleaved into 16 nonstructural proteins (nsp1–16), which form the replicase/transcriptase complex (RTC). At its 3' end, the viral genome contains four major structural proteins, spikes (S), envelopes (E), membranes (M), and nucleocapsids (N), as well as nine other proteins [146], [147]. Known as the matrix protein, the M glycoprotein traverses the viral envelope and is responsible for attaching the membrane of the virion to the NA within it. Viral lifecycles require the N protein during the morphogenesis phase. Virus attachment and fusion to host membranes are essential functions of the S protein [148].

SARS-CoV-2 enters the host cell by binding to the angiotensin-converting enzyme 2 (ACE2) receptor protein, which is present on the surface of the cell membrane. When the virus binds to ACE2, its exposure facilitates virus fusion with the host membrane [149]. It has been demonstrated that the pathogenesis of SARS is associated with delayed interferon (IFN)-1 signaling and immune toxicity [150]. Acute respiratory distress syndrome is thought to be caused by the immunopathology associated with these infection responses [145].

Once in the host cytoplasm, Coronavirus RNA transcription is initiated by RdRp, a catalytic subunit. In the process of viral transcription, a large viral mRNA transcript is generated containing ORFs, 1a and 1ab, as well as other subgenomic mRNAs (sgmRNAs) [151]. Generally, all mRNAs encode polyproteins, except for a few exceptions. Expression of the mRNA gene is copied directly to the leader TRS strand and the 3' end of the viral genome by a direct base pair. RNA polymerase, capping, and proofreading activities are integrated into a replication system orchestrated by a replicase which consists of a polymerase and several other proteins [152]. The discontinuous transcription mechanism is quite complex, and it is likely responsible for the extraordinarily high rate of recombination observed within Coronaviruses, which is as high as 24%. RNA viruses, and in particular positive-sense RNA viruses, exhibit extremely low levels of recombination. Therefore, the unique feature of the large and

complex genomic RNA of coronaviruses could have evolved in order to maintain such a large genome [145].

Stringent measures such as quarantine and transportation bans were developed and put into effect to prevent the spreading. This, however, was not enough to prevent it, and it was hard to believe that its impact would be felt for years to come [153]. Since then, the world has adjusted dramatically to the challenge. As countries have plunged in and out of lockdown, mask-wearing and self-testing have become regular parts of daily life [154]. The ability to diagnose SARS-CoV-2, the cause of the COVID-19 disease, is important to contain the pandemic and avoid the transmission of new variants accurately and reliably [155].

COVID-19 is still characterised by the high rate of transmission, especially since the new Omicron variant became dominant, and the ongoing increase of cases with consequent pressure on hospitals is a global concern [155]. Rapid POC diagnostics to reliably detect the causative virus, SARS-CoV-2, are still widely used to identify and isolate patients, preventing the spread of the virus and more importantly providing prompt treatment [79] [117]. The most common health problem caused by SARS-CoV-2 is lower respiratory tract infection, including symptoms such as fever, coughing, fatigue, and pneumonia, which can severely develop into acute respiratory distress syndrome and hyperinflammation, especially in immune-compromised subjects [156]. SARS-CoV-2 is characterised by a high transmissibility rate (mainly through aerosolised droplets from saliva or nose), high infectivity and a variable incubation period (from 0 to 14 days) [157]. In particular, the high number of subjects who are asymptomatic or present mild symptoms (mainly children and young adults) might be one of the major contributions to the rapid spread of this disease [155].

Even if the high levels of immunity built up through vaccination programmes and previous waves of infection appear to have improved the world-leading epidemic trend by reducing deaths rate and hospitalisations [24]. Therefore, improving our understanding of the virus with modelling, major trials of treatments, more adequate vaccine technology, and faster and more reliable diagnostic methods are still crucial for the scientific community to achieve its goal to bring an end to the COVID-19 pandemic [24].

Gold standard molecular methods to detect SARS-CoV-2

During the COVID-19 pandemic, various diagnostic methods have been employed for the detection of SARS-CoV-2, including rapid tests such as antigen and antibody detection, serologic tests, computerised tomography (CT) imaging, and molecular amplification tests (PCR, NAAT) [117]. Common diagnostic methods used during the pandemic to detect the presence of SARS-CoV-2 are illustrated in Figure 40. While rapid antibody and antigen tests have the benefit of providing rapid results (within 30 minutes) using a simple and affordable method, they suffer from low sensitivity and

specificity (<89%) compared to molecular methods and consequently leading to false-negative and false-positive rates [69]. Serum-specific IgG and IgM antibody detection methods are limited because of the late appearance of antibodies, thus making early diagnosis difficult [117]. In contrast, molecular methods such as RT-qPCR are still used as the gold standard diagnostic test to assess the presence of viral RNA achieving diagnostic sensitivity and specificity above 98%, critical for correctly identifying populations of individuals with the disease [29],[56]. However, it remains an expensive technique that requires highly skilled personnel and is relatively time-consuming and difficult to adopt in rural areas [117]. The proposed method analysed in this chapter combines the accuracy obtained by molecular detection together with the speed of detection (<20 minutes) and is an affordable methodology.

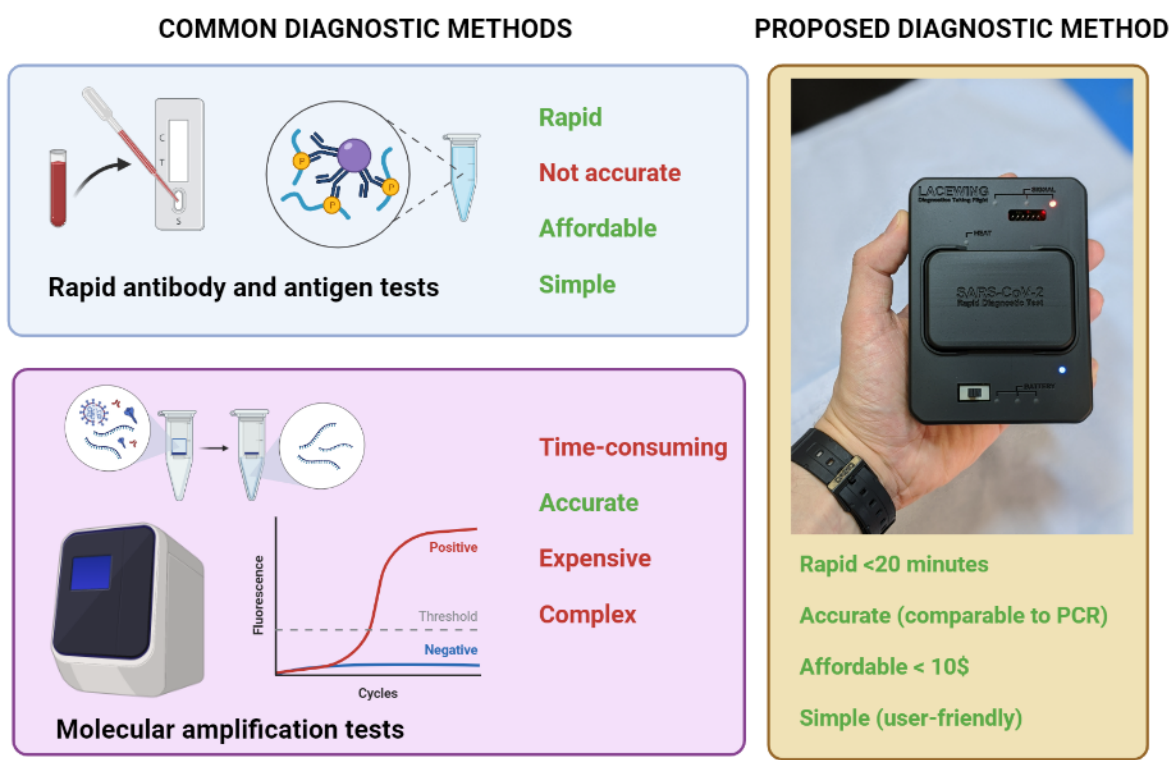


Figure 40. Overview of the approved clinical diagnostic methods for the effective detection of COVID-19 infection and the method proposed. The proposed method combines the optimal characteristics (in green) of the existing methods. Illustration created using Biorender.com.

Diagnosis of SARS-CoV-2 using RT-LAMP

Due to the high volume of diagnostic tests and the need for accelerating time to result, isothermal amplification techniques became part of the molecular tests employed for the detection of SARS-CoV-2. They have posed a good alternative to the PCR-based NA detection, which was limited by delays and increases in prices and was restricted to use in health care centres only [118]. These methods are ideal for POC applications because they have a fast turnaround time and can be carried out in areas with basic resources. This is due to the reduced hardware complexity of these methods,

as they do not require fluorescence-based apparatus. Furthermore, their results have comparable sensitivity and specificity to the PCR techniques [117]. To date, reported isothermal assay LAMP merged with reverse transcription (RT-LAMP) has been successfully used for several respiratory RNA viruses, including SARS-CoV-2 detection, but its use is still limited by the need for trained personnel to perform the analysis and by the laboratory equipment to perform the sample preparation step before amplification [158].

Diagnosis of SARS-CoV-2 using the LoC platform

As an essential worker during the pandemic, I have focused on the detection of SARS-CoV-2 using an optimised version of the proposed label-free electrochemical-based detection platform reported in the previous chapter. Compared to the previous LoC platform, the platform employed contained integrated enhanced thermal management and a new microfluidic system made capable of running two reactions in parallel due to the new two-well 3D printed manifold design. NA detection occurred by monitoring pH changes during NA amplification in parallel for the sample and control reactions, making it suitable for POC applications. The platform was adapted for the viral RNA detection from nasopharyngeal, oral and nasal clinical specimens, its compatibility with real-time RT-LAMP, and sample types when coupled with a sample preparation module [79].

Materials and Methods

Study design

This study demonstrates that the ISFET-based LoC platform could be used to rapidly and efficiently detect SARS-CoV-2 from clinical RNA extracted. LAMP assay targeting the conserved region of the *nucleocapsid* (N) gene of SARS-CoV-2 based on reported sequences was designed by Rodriguez et al. The assay used was validated on a real-time benchtop instrument (RT-qLAMP) and 183 clinical samples from, oral, nasal and nasopharyngeal swabs of symptomatic patients were collected at the microbiology laboratory at West London Hospital Laboratory (UK) and have been screened using the LoC technology. The results were compared with the gold-standard RT-qPCR [33]. LAMP assay was implemented on the ISFET-based LoC platform to assess its performance for rapid POC detection. A subset of 40 samples was screened on the LoC platform, including positive (n=34) and negative (n=6) samples. The study was conducted in agreement with the ethical approval (20/HRA/1561; DOCUMAS 20SM5875).

The number of samples selected to be screened on the LoC platform was determined using the following formula, where p is the suspected sensitivity and x is the desired margin of error:

$$n \geq \frac{1.96^2 p(1-p)}{x^2}$$

Sensitivity, defined as the true-positive rate, is the proportion of SARS-CoV-2 positives correctly identified by the LoC platform compared to the benchtop instrument. Sensitivity and specificity were considered 90%, similarly to those obtained with RT-qLAMP, with a desired 10% margin of error. Following these conditions, the number of required samples is 34.57 (rounded up to 35); therefore, a total of 40 samples (34 positives and six negatives) were tested on the platform.

LAMP Primers

NCBI was used to obtain genomic viral sequences using the algorithm nucleotide BLAST, with the amplicon of the proposed RT-LAMP assay containing 4506 nucleotides. The GISAID EpiCov database was used to obtain complete genomic sequences specific to SARS-CoV-2. The alignment of the sequence was performed using the MUSCLE algorithm in Genious Prime Software v2019.04 and the sequence NC_045512 as a reference. Primers were optimised based on the Zhang et al assay and analysed in silico for the presence of hairpins and dimers using NUPACK, IDT OligoAnalyzer software

and the Thermo Fisher Multiple Primer analyser tool. All primers were synthesised by IDT, and sequences are listed in Table 6.

SARS-CoV-2 LAMP primers	Sequence 5' – 3'
F3	ACCAATAGCAGTCCAGATGA
B3	GCAGCATTGTTAGCAGGAT
FIP	TCTGGCCCAGTTCCTAGGTAGT-CCAGACAAATTCGTGGTGG
BIP	GGACTTCCCTATGGTGCTAACAAA-CGGGTGCCAATGTGATCT
LF	GGACTGAGATCTTTCATTTTACCGT
LB	ACTGAGGGAGCCTTGAATACA

Table 6. Sequences of primers of LAMPcov assay developed in Rodriguez-Manzano et al [40]. LAMP assay targeting the N gene. One mismatch highlighted in red was introduced in the F2 primer to avoid hairpin formation during LAMP.

Clinical samples and controls

Archived clinical specimens including nasopharyngeal, oral and nasal swabs were made available for this study with no clinical information associated to ensure the privacy of the data. The stored clinical samples were collected within the microbiology laboratory at West London Hospital Laboratory (UK). A total of 183 positive and negative samples already extracted were provided with an elution volume ranging from 50 µL to 200 µL. Quantified RNA from the viral culture was used as a control as reported in detail in Rodriguez-Manzano et al.

RT-qPCR reaction conditions

RT-qPCR experiments were conducted in duplicate using the CDC-recommended assays targeting the viral N1, N2 regions, and for validation of the RNA extraction procedures, the RNase P (IDT) target was included as quality control for the extraction method and amplified using the GoTaq® 1-Step RT-qPCR kit (Promega). Each reaction mix contained 10 µL of 2× GoTaq® qPCR master mix, 0.4 µL of 50× GoScript™ RT mix for 1-Step RT-qPCR, 1.2 µL of 16.6× N1/RNase P assay primer mix by the CDC (Forward Primer (20 µM), Reverse Primer (20 µM), Probe (5 µM)), 4 µL of extracted RNA and enough nuclease-free water (GoTaq® 1-Step RT-qPCR kit, Promega) to bring the volume to 20 µL.

The reaction mix was plated into 96-well plates, loaded into a LightCycler 96 Real-Time PCR System (LC96) (Roche Diagnostics) and run following the manufacturer's recommendations: 15 minutes at 63°C for cDNA conversion, two minutes at 95°C for AMV deactivation, and 45 cycles at 95°C for 15 seconds and finally 55°C for 45 seconds.

RT-qLAMP reaction conditions

RT-qLAMP experiments were conducted in duplicate, and each reaction mix contained 2 μL of 10 $\times$ isothermal buffer (New England Biolabs), 1.20 μL of MgSO_4 (100 mM stock), 2.80 μL of dNTPs (10 mM stock), 0.50 μL of BSA (20 mg/mL stock), 3.20 μL of Betaine (5M stock), 0.50 μL of SYTO 9 Green (20 μM stock), 1.25 μL of Bst 2.0 DNA polymerase (8,000 U/mL stock), 0.64 μL of AMV (25 U/ μL stock, Promega), 0.20 μL of RNase inhibitor (20 U/ μL stock, ThermoFisher Scientific), 4 μL of extracted RNA, 1 μL of 10 $\times$ LAMP primer mixture (20 μM of BIP/FIP, 10 μM of LF/LB, and 2.5 μM B3/F3) and enough nuclease-free water (ThermoFisher Scientific) to bring the volume to 20 μL .

The reaction mix was plated into 96-well plates and loaded into a LightCycler 96 Real-Time PCR System (LC96) (Roche Diagnostics) and the run was performed at 63°C for 30 minutes. One melting cycle was performed at 0.1°C/s from 65°C up to 97°C for validation of the specificity of the products.

RT-eLAMP reaction conditions

RT-eLAMP experiments were conducted on the LoC platform at 63°C for 30 minutes. Each reaction mix contained the following: 0.50 μL of 10 $\times$ isothermal pH-based buffer (pH 8.5–9), 0.30 μL of MgSO_4 (100 mM stock), 0.28 μL of dNTPs (25 mM stock), 0.30 μL of BSA (20 mg/mL stock), 0.13 μL of NaOH (200 mM stock), 0.80 μL of Betaine (5M stock), 0.02 μL of Bst 2.0 DNA polymerase (120,000 U/mL stock), 0.13 μL of AMV (25 U/ μL stock, Promega), 0.05 μL of RNase inhibitor (20 U/ μL stock, ThermoFisher Scientific), 2 μL of extracted RNA and 0.50 μL of 10 $\times$ LAMP primer mixture (20 μM of BIP/FIP, 10 μM of LF/LB, and 2.5 μM B3/F3). A 5 μL reaction was loaded into the sample well and control well onto the disposable cartridge. Data were analysed in MATLAB by extracting the signal associated with the pH change.

Data analysis and statistics

MATLAB (R2018b) and OriginPro 2019 were used to perform the data analysis. TTP values are reported as the mean $\pm$ std using built-in functions in MATLAB. To verify that the data were normally distributed, the One-sample Kolmogorov-Smirnoff Test was employed. The two-sample Student's t-test was performed when comparing groups that presented normal distribution with equal mean and variance. As a threshold for statistical significance, a p-value ≤ 0.05 was considered.

Results and Discussion

Development of a LAMP assay for SARS-CoV-2

The LAMP assay was designed and optimised by Rodriguez-Manzano et al [79]. The gene targeted was the nucleocapsid (N) gene of SARS-CoV-2 because as previously discussed it is one of the conserved gene across the available sequences. LAMP primers sequence and location in the N gene are illustrated in Figure 41a. In the analytical sensitivity of the assay shown in Figure 41c, TTP values ranged between nine and 24 minutes, with an LoD of ten copies per reaction. A standard was built using a serial dilution of a control RNA showing a high correlation of $R^2=0.98$. The LAMP assay developed by Zhang et al. is included as a reference in Figure 41b and is comparable to the one developed in this study.

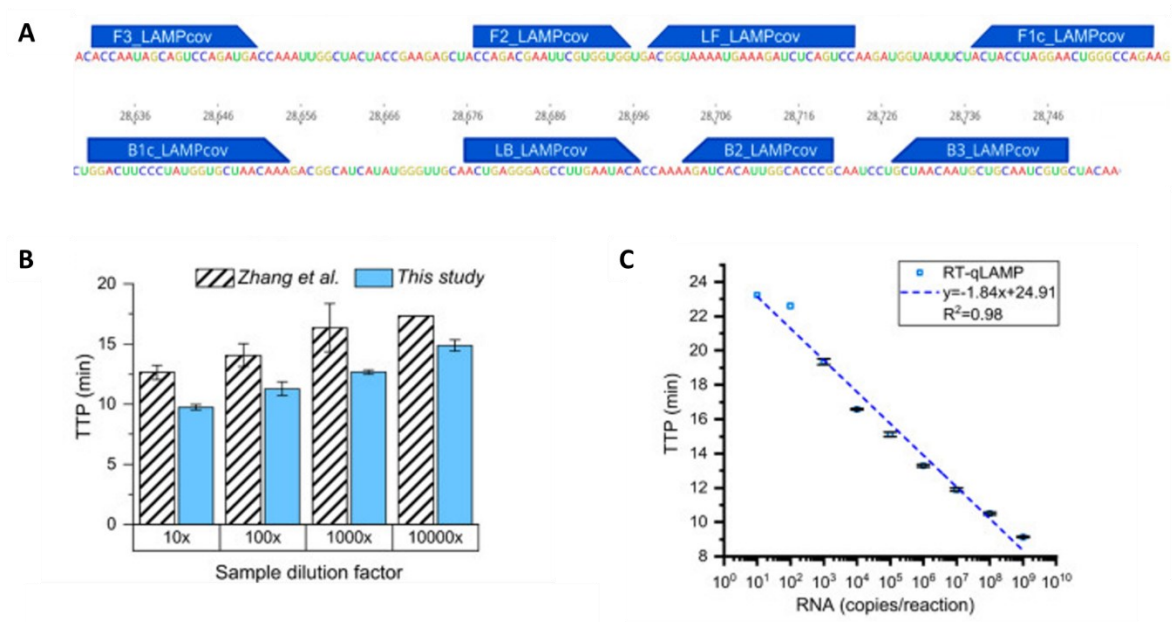


Figure 41. Analytical sensitivity and specificity of the LAMP assay. (A) Reference sequence NC_45512 SARS-CoV-2 showing LAMP primers location. (B) TTP comparison between LAMP assay and reference assay (Zhang et al.). (C) Standard curve for RT-qLAMP assay using a control RNA ranging from 10^1 to 10^9 copies per reaction. Results published [79].

Performance comparison of RT-qPCR and RT-qLAMP using clinical samples

A total of 183 nasopharyngeal swab samples collected and stored in viral transport medium (VTM) were provided already extracted and purified. Samples were tested with two of the PCR-based assays recommended by the CDC, detecting two targets in N gene (N1+N2) and the RNase P human gene as quality control for the extraction method. RT-qPCR results obtained targeting the N assay

were considered as a reference to cluster the clinical samples into five infection categories based on their Ct values (high, upper-medium, lower-medium, low and negative).

Ct was determined by the cycle-threshold method using 0.2 as the fluorescence cut-off value. This classification considered a six-cycle difference per category, corresponding to approximately 100-fold dilution. The “high” concentration group values were 20.20 ± 1.82 cycles and 10.08 ± 0.83 minutes for RT-qPCR and RT-qLAMP, respectively. The “upper-medium” concentration group values were 26.51 ± 1.54 cycles and 12.55 ± 1.41 minutes; the “lower-medium” concentration group values were 31.78 ± 1.69 cycles and 16.47 ± 3.97 minutes; and the average TTP values of the samples within the “low” concentration group were 36.51 ± 0.88 cycles and 21.64 ± 4.17 minutes for RT-qPCR and RT-qLAMP, respectively. Results obtained with RT-qLAMP and RT-qPCR are summarised in Table 7.

Categories	RT-qPCR Ct range (cycles)	RNA conc. (copies/ μ L)	Samples (n)	RT-qPCR Ct $\pm$ STD (cycles)	RT-qLAMP Ct $\pm$ STD (min)
High	17–22.9	2×10^6 to 3×10^4	13	20.20 ± 1.82	10.08 ± 0.83
Upper-medium	23–28.9	3×10^4 to 5×10^2	36	26.51 ± 1.54	12.55 ± 1.41
Lower-medium	29–34.9	5×10^2 to 8	52	31.78 ± 1.69	16.47 ± 3.97
Low	>35	<8	26	36.51 ± 0.88	21.64 ± 4.17
NEG	NEG	NEG	56	NEG	NEG
TOTAL			183		

Table 7. Results obtained with RT-qPCR and RT-qLAMP. Values are classified based on RT-qPCR Ct values: high, upper-medium, lower-medium, low, and negative. RNA concentration (copies/ μ L of eluted sample volume) is based on RT-qPCR standard curve $y = -3.30x + 37.98$.

Sensitivity and specificity obtained were respectively 91% and 100% for RT-qLAMP. A cycle threshold value (Ct-value) < 37 was defined as a positive test result, while a Ct-value equal to or higher than 40 was defined as a negative test result. These diagnostic criteria were based on the recommendations of the National Institute for Viral Disease Control and Prevention (China). From a total of 183 clinical samples, both techniques, RT-qPCR and RT-qLAMP, detected 54 negative samples and 115 positive samples. A total of 12 samples were detected as positive only by RT-qPCR and negative by RT-qLAMP, while two samples were detected as positive only by RT-qLAMP and negative by RT-qPCR. A total of 14 misclassified samples clustered in the “low” concentration group, which could justify the discrepancy between the two assays.

Assay	RT-qPCR positive (n)	RT-qPCR negative (n)	Sensitivity (%)	Specificity (%)
RT-qLAMP positive (n)	115	0	90.55	100
RT-qLAMP negative (n)	12	54	90.55	100

Table 8. Sensitivity and specificity of RT-qLAMP compared to RT-qPCR to detect SARS-CoV-2.

The sensitivity and specificity shown in Table 8 were calculated as follows:

$$SEN (\%) = \frac{TP}{TP+FN} * 100, SPE (\%) = \frac{TN}{TN+FP} * 100$$

where the sensitivity % is equal to the number of true positives (TP) divided by the sum of true positives and false negatives (FN) multiplied by 100. The specificity % is equal to the number of true negatives (TN) divided by the sum of TN and false positives (FP) multiplied by 100.

The two samples (specifically samples 39 and 97) detected only by RT-qLAMP were confirmed as true positives by a melting curve analysis, as shown in Figure 42.

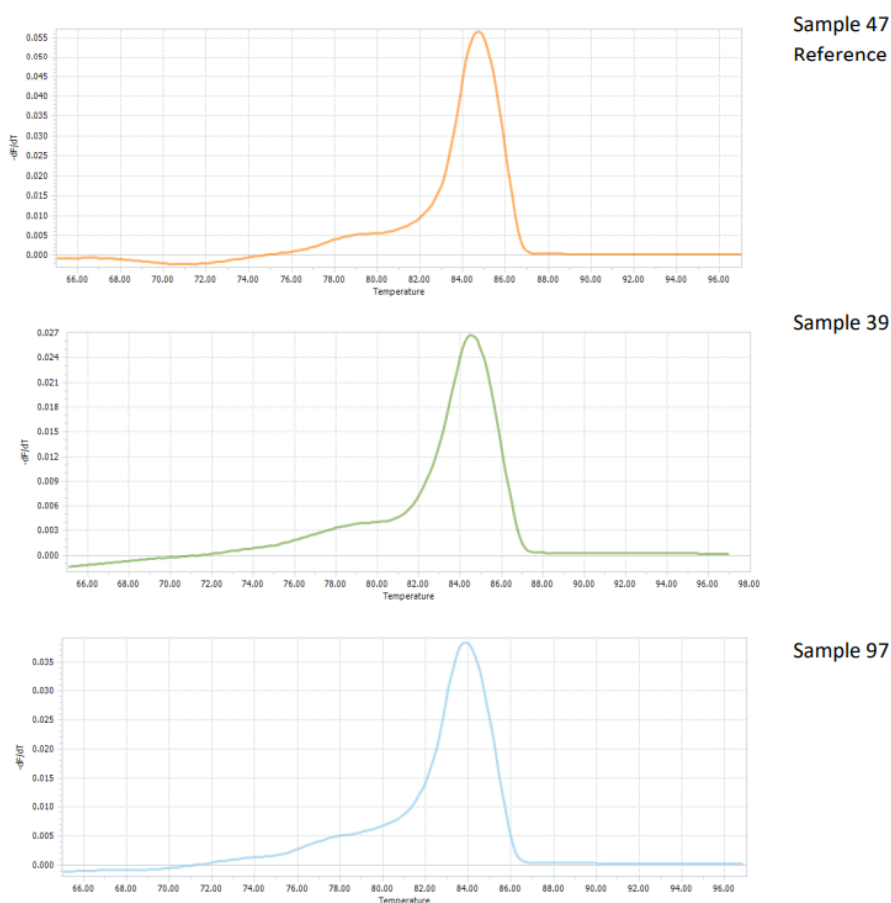


Figure 42. Melting curve analysis for the two misclassified samples.

The analysis confirmed the specificity of the product for two samples detected only by RT-qLAMP, omitted in the Table for sensitivity and specificity analysis. Sample 47 is included as a reference (detected by both RT-qPCR and RT-qLAMP) showing a melting peak at 84°C. Melting peaks of the samples (39 and 97) uniquely detected by RT-qLAMP but not by RT-qPCR show the same melting peak (84° C), confirming the specificity of the assay.

Correlation analysis between RT-qPCR and RT-qLAMP

To evaluate a direct comparison between the proposed RT-qLAMP assay and the gold standard RT-qPCR, a correlation study was performed by applying a linear regression method. A total of 115 samples were detected by both methods and were included in the analysis. Viral load was calculated based on standards built using RNA extracted from SARS-CoV-2 viral particles as control. Figure 43a summarises and plots the viral load calculated using the two methods. The x-axis corresponds to the viral load calculated using RT-qPCR and the y-axis corresponds to the viral load calculated using RT-qLAMP. The correlation showed using 115 clinical samples in both methods is 0.8, with an R^2 of 0.72.

The detection of the human gene RNase P was used as an internal control to evaluate the presence of human genes in the sample and to therefore provide information on the quality of the extracted RNA. RT-qPCR was used to target a positive control (Hs_RPP30) provided by IDT to build standards to evaluate the concentration of RNase P. RNase P concentration was homogeneously distributed across all patients positive by RT-qPCR ($n = 127$ samples), indicating similar extraction quality (Figure 43b).

A different distribution was observed when looking at RNase P gene ($p\text{-value}=8.09\times10^{-6}$) between RT-qPCR positive ($n = 127$) and negative ($n = 56$) samples for SARS-CoV-2 (Figure 43c). The lower concentration of human gene present in negative patients might cause an increased number of false negatives. The boxplot in Figure 43d is explained in the following section.

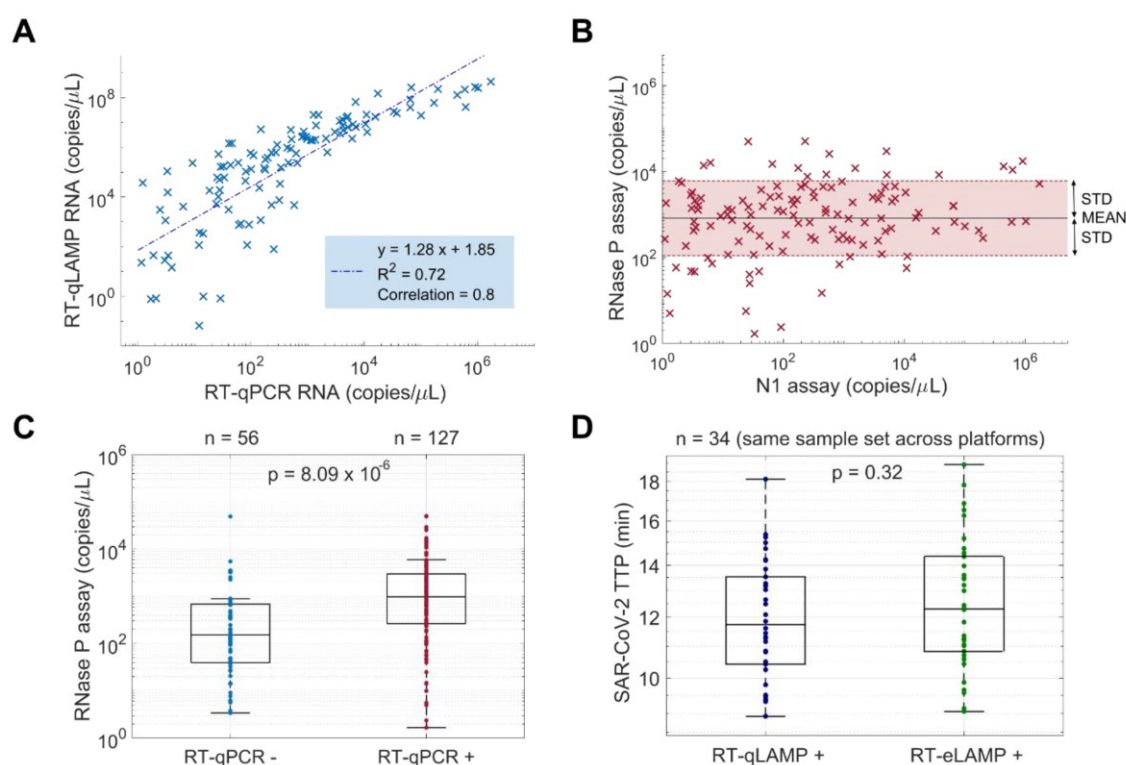


Figure 43. Correlation between RT-qPCR and RT-qLAMP. Rodriguez-Manzano et al. [79]

In (A), the correlation between qPCR and qLAMP is based on the 115 calculated sample concentration (copies/ μ L of eluted sample volume). (B) shows the correlation between RNase P (human gene) and N1 (viral RNA concentration) of the 127 clinical samples (copies/ μ L of eluted sample volume) to indicate the quality of extraction across the cohort. (C) shows the boxplot distribution of RNase P concentration (copies/ μ L of eluted sample volume) across negative (n = 56) and positive (n = 127) clinical samples by RT-qPCR. The calculated p-value between both groups was below 0.05 (p-value = 8.09×10^{-6}). (D) shows the boxplot distribution of TTP for the RT-qLAMP and RT-eLAMP, showing the performance of the subset cohort on the LoC platform. The calculated p-value between both groups was higher than 0.05 (p-value = 0.32).

Lab-on-a-chip platform: a new two-well manifold

A new two-well microfluidic manifold was designed by M. Cavuto to run two reactions in parallel. The RT-qLAMP assay was run using the ISFET array containing 4,368 sensors, and DNA amplification was monitored through the release of protons in the reaction.

During this experiment, the cartridges had a customised 3D printed microfluidic manifold bolted on. The manifold was tested using different resins for biocompatibility with LAMP reactions, and the final version was made using the Standalone 3D Printer from 3D Systems, in their transparent biocompatible (ISO 10993-5 and -10) MED-AMB-10 resin (Figure 44).

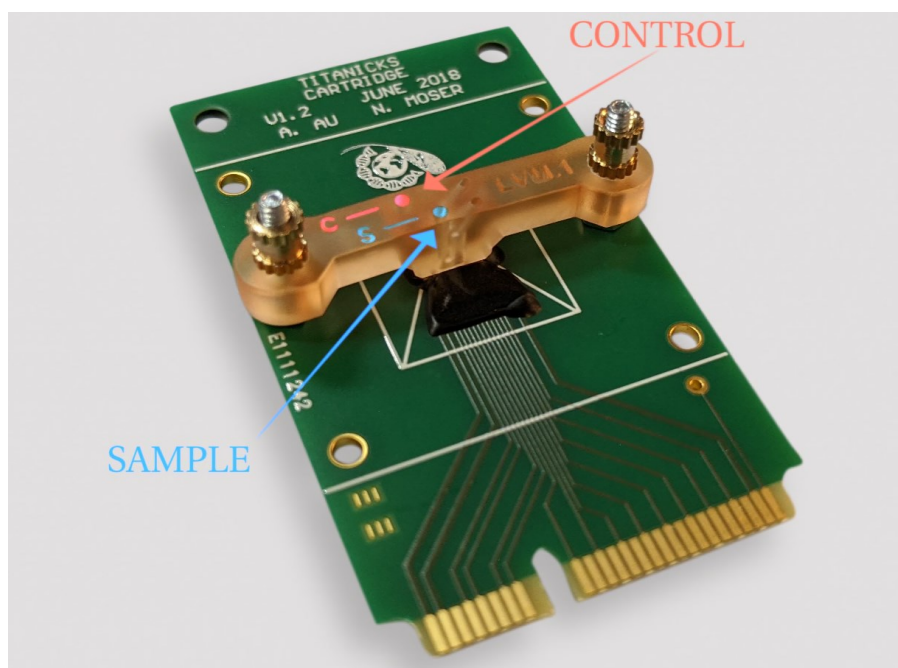


Figure 44. A new two-well manifold.

A new two-well manifold with inlets and outlet channels to allow two LAMP reactions to run in parallel on the same chip.

The manifold contained two internal microfluidic channels as shown in Figure 44 (sample well and control well), containing 5 μL of sample and control reaction mix, onto two separate designated sensing areas of the chip's surface. The sample and control mixes were loaded into each of the two wells. The sample reaction mix contained the reaction mix described for RT-eLAMP, including extracted RNA. The control reaction mix included nuclease-free water instead.

To seal between the manifold and chip surface, laser-cut double-stick Tessa® adhesive gaskets were used. An Ag/AgCl reference electrode (chlorinated 0.03mm Ag wire) across each reaction well between the chip surface and tape gasket was also used. The adhesive gasket and printed resin materials were chosen after being tested for their low reaction inhibition.

RT-eLAMP was performed on the battery-powered Lacewing platform, with a Peltier module in contact with the cartridge to ensure temperature regulation of the solution.

RT-eLAMP results using the lab-on-a-chip device to detect SARS-Cov-2

A subset of 40 clinical samples (34 positive and six negative) were tested on the LoC platform using RT-eLAMP to characterise the detection performance of the LoC technology compared to the benchtop instrument. The correlation between RT-qLAMP and RT-eLAMP techniques using the same sample set across platforms is represented in Figure 43d. The boxplots show equivalent performance in terms of average TTP values, 12.10 ± 2.20 min in RT-qLAMP and 12.68 ± 2.56 minutes in RT-eLAMP. A p-value above 0.05 indicates there is no statistical difference in performance for each of the platforms (p-value=0.32).

Interpreted together, these results showed a higher sensitivity of the RT-qPCR assay compared to the RT-qLAMP assay, suggesting more optimisation needs to be performed to enhance the LAMP assay performance.

Furthermore, the RT-qPCR technique required an additional 40 minutes when compared to the RT-qLAMP's execution time to perform the screening of the samples, therefore a total of 60 minutes was required to complete the minimum recommended 45 cycles, doubling the time required when performing RT-qLAMP instead.

Extracting TTP from the LoC platform

The ISFET array employed in this study, containing 78x56 electrochemical sensors, presented a new microfluidic module to accommodate the two wells illustrated in Figure 45, to run the no template control and sample reactions on the same sensing surface. An initial calibration was required to remove sensors that were not in contact with the solution, or sensors that were not in range (inactive sensors shown in Figure 45 in dark blue).

The sensors corresponding to the green area were in contact with the clinical sample, and the sensors corresponding to the red area were in contact with the reaction control. The algorithm calculated the averaged output of the sensors within each well and performed a drift compensation (dotted lines shown in the raw data). To reliably identify the voltage change (mV) in response to the pH variation, the time of maximum derivative was identified and compensation of sensor drift (based on exponential interpolation) was performed to extract information on NA amplification.

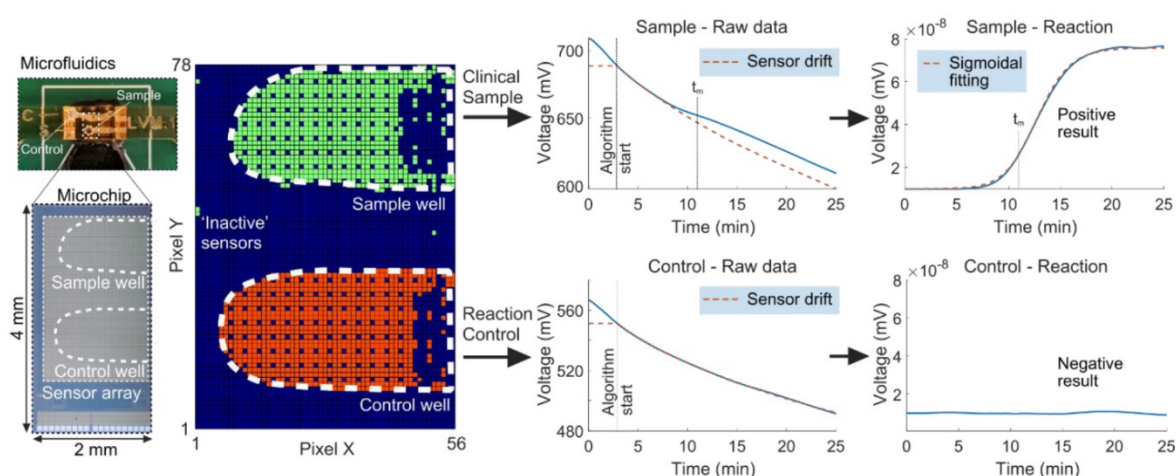


Figure 45. Overview of data processing for one clinical sample published in Rodriguez-Manzano et al.[79]

From left to right: Microfluidic manifold placed on the top of the sensor arrays containing sample inlet and control inlet (containing each 5 μL of reaction volume). The spatial image illustrates the ISFET array output containing 4,368 sensors on a 2 x 4 mm surface. The blue area contains inactive sensors, the green area contains the active sensors in contact with the sample reaction, and the red area contains active sensors in contact with the reaction control. Results extracted from the ISFET arrays during RT-eLAMP show, respectively, amplification with a TTP of 10.63 min (sample) and no amplification (control).

To reflect the dynamics of a typical NA amplification, a sigmoidal fitting was performed on the compensated sensor signal to detect any amplification reaction. Negative amplification reactions showed a flat curve (Figure 45). For each of the clinical samples tested, no amplification reaction occurred in the control well within 20 minutes, confirming no presence of contamination. The presence of SARS-CoV-2 in the clinical sample well was quantified by extracting the TTP based on 0.2 thresholding, measuring the ng/ μ L and the pH of the solution before and after the amplification reaction.

So far, the ISFET-based platform and algorithms employed have successfully detected and quantified the presence of SARS-CoV-2 viral RNA in the extracted clinical samples, showing similar performance between the benchtop RT-qLAMP and the RT-eLAMP-based platform. The proposed LoC platform, including both the microfluidic cartridge with control and sample inlets designed by M. Cavuto and the smartphone application designed by N. Moser to acquire and process the sensor data during the reaction to provide real-time monitoring of the amplification, has added additional benefits as compared to the LoC described in Chapter 3, including improved speed of detection, improved temperature and reaction control stability, portability, and real-time monitoring of two reactions.

Conclusion

The proposed study was developed during the first wave of COVID-19 by the CBIT team to detect the presence of viral RNA from SARS-CoV-2 using the handheld LoC technology. Its performance, screened on a total of 183 clinical samples, was compared to the gold standard RT-PCR technique using the recommended kit from the CDC. Comparing RT-qLAMP to RT-qPCR, the RT-qLAMP assay revealed a 91% sensitivity, suggesting that LAMP primers need to be further optimised. Furthermore, the significant difference between positive and negative samples ($p\text{-value} < 0.05$) when targeting RNase P provided awareness that the integrity of the samples was not optimal and could have led to an increase in the number of false negatives.

Overall, RT-qLAMP showed a faster turnaround time, with an average TTP of 15.45 ± 4.43 minutes. This provided confidence that the proposed assay represents a rapid alternative to RT-qPCR for SARS-CoV-2 POC diagnostic applications. The performance of the LAMP assay was also successfully tested on the LoC platform, testing a subset of clinical samples ($n=40$). RT-eLAMP showed a good correlation compared to the results on a commercial PCR instrument ($p\text{-value} > 0.05$), confirming the good performance of the LAMP assay alongside the robustness of the LoC platform.

Even if the RT-qPCR is considered the gold standard method for the detection of SARS-CoV-2, it cannot be considered a POC diagnostic system because of the need for a laboratory-based setting and equipment, expensive instruments, and dedicated personnel. This system would delay public health institutions, which have relied on rapid lateral flow tests to increase the speed of diagnosis, leading to a high rate of false negatives. This poses a big risk for the uncontrolled spreading of the virus and slows the healthcare system, which then needs to confirm the result with molecular methods.

I believe the potential of the LoC technology might have a positive impact on the diagnosis of infectious diseases. The technology has the potential to be implemented to detect more pathogens because of the versatility to adapt specific LAMP assays targeting either pathogens or biomarkers. When assessed together, all these characteristics offer a good opportunity for the platform to improve its scalability, offering a wide range of applications that can be easily scaled for the multiplex detection of pathogens. The main limitation of this study is represented by the important missing sample preparation step. How this limitation has been addressed will be explained in Chapter 5.

Chapter 5

A new sample preparation method to rapidly extract viral RNA from respiratory swabs

Chapter overview

The previous chapter showed the successful detection of SARS-CoV-2 using the LoC technology. However, the study was conducted from extracted RNA, representing a limitation in the development of a whole sample-to-result process. This chapter presents the work I have conducted with M. Cavuto to develop a new method to rapidly extract NAs from oral, nasal and nasopharyngeal specimens to integrate a sample preparation method into the existing diagnostic system. A brief overview of the existing extraction methodologies is presented to provide enough background to discuss the choices that motivated the development of the actual sample preparation method. I conceptualised the work, performed the laboratory work, and conducted the statistical analysis together with M Cavuto. The clinical validation was performed with the assistance of the entire team.

The concepts in this chapter resulted in the following journal articles and patents:

- Matthew L. Cavuto, **Ivana Pennisi**, Jesus Rodriguez-Manzano, Pantelis Georgiou, Lid assembly for a sample tube, method of using the same to collect magnetic beads, and sample processing kit, GB2102573.9 · Filed Feb 23, 2021.
- **I Pennisi***, M Cavuto*, L Miglietta, R Reid, O Stringer, KM Cardenans, N Moser, P Georgiou and J Rodriguez-Manzano. A simplified, power-free sample preparation method to allow direct detection of SARS-CoV-2 RNA from nasopharyngeal samples. In preparation.

Introduction

The need for sample preparation

The ongoing COVID-19 pandemic has led to an unprecedented need for diagnostic screening, causing a global shortage of otherwise commonplace laboratory consumables such as RNA extraction kits and has necessitated a proportionate response by the scientific community to find different solutions to allow sample extraction and detection methods [159]. Even before the pandemic, access to both manual and automated sample preparation methods in LMIC was severely limited [160]. With the intent to address this need, I co-invented and clinically validated a frugal, power-free, rapid, and simplistic method to extract NAs from nasopharyngeal swabs with the immediate purpose of detecting SARS-CoV2 RNA, named the SmartLid (SL) kit.

NAs extraction (NAE) represents an essential process in molecular biology as the primary step to allow successful downstream applications. The success of target detection depends on the purity of the analyte extracted [96]. However, obtaining pure NAs from crude, unprocessed samples is challenging. The first function of NA extractions is to eliminate inhibitors because if inhibitors are carried into the elution, they can delay the reaction, inactivate key enzymes or even lead to false-negative results, causing the failure of downstream molecular applications [141]. To remove these inhibitors, solid-phase extractions (SPE) are an effective choice because they have been found to yield higher purity compared to liquid-liquid extractions [161] [162]. The two most common solid-phase extraction methods use either spin columns or magnetic beads [163]. These involve chemical and mechanical processes, depending on the application and the specimen of interest. They usually rely on lab-based environment and skilled personnel although, with the advent of POC diagnostics, sophisticated analytical machinery is being replaced by microanalytical systems to be used outside lab environments [164]. To date, a simple and effective extraction method that can be universally applied at the POC seems difficult to achieve because of the complexity of the steps involved from the sample manipulation to the result (Figure 46).

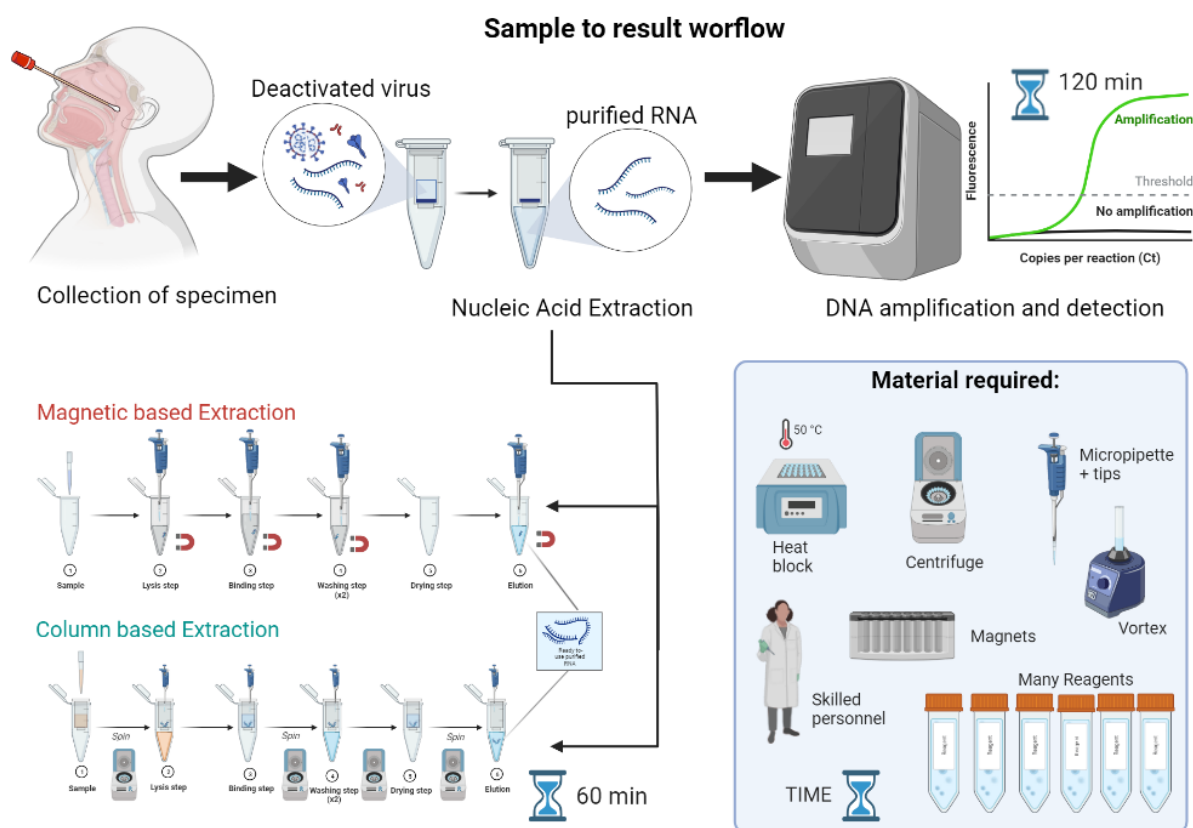


Figure 46. The typical workflow of an amplification-based SARS-CoV-2 diagnostic test. Samples are collected, and the virus is inactivated. NA extraction is performed using SPE, either magnetic or column based. NA extracted are detected using an RT-qPCR instrument and diagnostic results are analysed. The overall workflow is time-consuming (>180 minutes), and the material required for the extraction step relies on a laboratory environment, expensive equipment and reagents, and a highly skilled operator. Illustration created with Biorender.com.

Typically, extraction kits such as column-based extraction (QIAmp Viral RNA kit - Qiagen) or the magnetic bead-based method (MagNA Pure kit by ROCHE) are considered the gold standard for RNA purification [165]. These kits are expensive and unsuitable for POC applications because they rely on several complex steps, as illustrated in Figure 46. Both magnetic and column-based extraction take ~60 minutes to extract the NA of interest and the material required to perform the extraction is not easy to access in limited settings [166]. The global medical supply shortages caused by the pandemic have only exaggerated the need for laboratory supply, although similar challenges have been faced as well in more developed medical environments such as the UK, the EU and the USA [159], [167]. Therefore, sample preparation workflows that are less reliant on the above traditional supply chains and requirements are critical to improving healthcare around the world [168]. To overcome these limitations, several groups have developed approaches that include a direct addition of swab samples to RT-qPCRs [169]. This is a chemical or physical procedure that allows the lysis of cells, and the cell-free extract is used without any purification step. Single-tube indirect extraction of NA is achieved by the selective precipitation of protein and other contaminants or by chelation of inorganic substances. The advantages of these methods are that they are simple and quick and can be more easily scaled up and automated for high throughput. While this represents a significant reduction in terms of time and

costs, the absence of the purification step leads to less concentrated RNA, limiting the sensitivity of detection [170]. Moreover, sample pre-treatment involving preheating, alternating heating-cooling or various additives are still required and are time-consuming and cumbersome [141] [171]. Besides its suitability for high-throughput processing, the use of a one-step purification and amplification cannot be applied to all downstream molecular applications [172].

NA sample preparation process

The main purpose of the extraction process when targeting NAs is to disrupt the cell membrane, and nuclear membrane to release intact NAs in solution. The extraction process can be summarised in four main steps [173]:

- i. Cell disruption through lysis
- ii. NA binding to a functionalised surface (either a membrane or a magnetic bead)
- iii. Removal of cellular components (membrane lipids, proteins, cellular debris)
- iv. NA elution

The disruption of the cell wall or cellular membranes can be achieved by either chemical methods (chaotropic agents, detergents, lytic enzymes), mechanical methods (bead beating, shearing, grinding, sonication), heating methods or a combination of these and depends on the properties of the sample [174],[175],[176]. Integrating NAE methods into POC diagnostic tests represents a non-trivial challenge due to the limitations to adapt many laborious steps into a small, portable, and cost-effective platform.

Silica-based DNA/RNA extraction

Silica-based coating is a key technology for the purification of NA [177]. Double- and single-stranded DNA and RNA are all strongly negatively charged, with inter-phosphate charge separations. This high charge density causes the free energy of NA interactions to be strongly influenced by electrostatics. The unique properties of functionalised silica surfaces are used to selectively bind NAs because of the high affinity of the negatively charged DNA or RNA molecules for the positively charged silica particles [178].

Under high salt conditions and low pH, NAs are tightly bound to the silica surface and all contaminants such as binding salts, cell components, proteins, cell debris, lipids and cell walls are washed away during the washing step. Hydrophilic NA can leave the silica surface under low ionic strength and return to the solution (hypoosmotic solution, water or alkaline Tris-EDTA buffers). [111]

For RNA extraction specifically, chaotropic agents (i.e., phenol, ethanol, isopropanol, guanidine hydrochloride) play a key role when disrupting the macromolecules' structures, denaturing

RNases, and protecting RNA when binding to the silica-based surface. Guanidinium thiocyanate (GTC) or guanidinium isothiocyanate (GITC) is generally used as a protein denaturant, contributing to viruses' inactivation so that the sample can be safely handled [179]. In addition to their lysing action, being chaotropic agents, they prevent the activity of RNase enzymes and DNase enzymes by denaturing them and acting as NA protectors. When combined with IPA, the percentage of RNA molecules that can be bound to the silica surface increases by easing the water coat surrounding NAs.

Overall, silica-based extraction has revolutionised purification procedures for research and commercial use because of its adaptability in covering different materials' surfaces and its efficiency in selectively binding NAs, providing robust and high-quality purification. [180].

Magnetic beads-based and column-based NA extraction

Silica-based extraction methods commonly used in research and commercially available are based on magnetic nanoparticles or columns (Figure 47). Magnetic-based extraction is a practical emerging solution used to extract antibodies, antigens, oligonucleotides, aptamers and other molecules. Magnetic beads typically consist of two components: a magnetic material (often iron, nickel or cobalt) and a chemical component that functionalises the surface of the magnetic bead, thereby enabling the beads to attach to biomolecules or other chemical or biological species [181]. The technique involves the magnetic separation (through a magnet) of molecules of interest from complex mixtures using complementary hybridisation between the target and the functionalised surface of the magnetic bead [182],[183]. The extraction process consists of the attraction of the magnetic beads to the side of the tube by using a magnet while the solution present is replaced by the next solution involved in the extraction process. This method does not require any centrifuging step; therefore, longer fragments of genomic DNA are preserved and remain intact. The positive aspect of avoiding centrifuging allows us to use this technique as an automated procedure for large numbers of samples [184].

The other silica-based extraction technique involves the use of spin-columns, where a centrifuging step is essential to bring the solution through a functionalised membrane that allows the molecule of interest to be bound to it and consequently discard the flow-through [177]. The binding element is represented by a charged membrane, where NA can be bound in presence of specific buffer solutions at specific pH and salt conditions, while the rest of the contaminants are washed away before the membrane is washed with solutions where high-quality NA is recovered from the membrane and eluted[185]. So far, column-based NA extraction gives the highest purity rate of extracted NA due to the presence of the membrane that allows less carryover of contaminants into the elution step. Column-based methods are not easy to integrate into a POC test because of the presence of many steps, buffer solutions and the need for centrifuging steps to force the liquid through the membrane.

On the other hand, magnetic beads-based techniques require labour-intensive steps, the use of expensive micropipettes and trained operators [186].

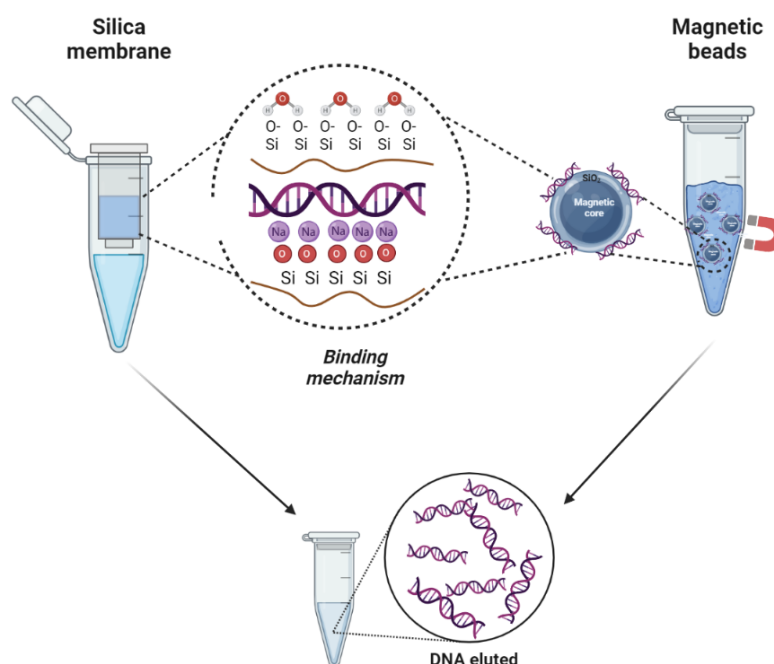


Figure 47. Silica-based membrane and magnetic beads with DNA sample in chaotropic buffer. Chaotropic salt disruption of the water structure around negatively charged silica allows a cation bridge to form between it and the negatively charged phosphate backbone of DNA; this reaction is reversible in low salt concentrations. Illustration created with Biorender.com

The SmartLid concept

A novel sample preparation method to extract the viral RNA for SARS-CoV-2 based on commercially available magnetic beads is presented in this chapter. Silica-coated magnetic beads are more affordable, faster and easier to manipulate compared to filter columns that require centrifuging steps to allow the fluid to go through the filter. To allow NA extraction avoiding the use of any electrical or expensive micropipette, a magnetic removable lid that enables the binding of magnetic particles and moving them from tube to tube was designed to eliminate the need for expensive micropipettes to move solutions in and out. A customised 3D printed lid with a removable magnet, named SmartLid, was developed together with an optimised extraction protocol consisting of three pre-filled tubes to perform three main extractions steps (lysis/binding, wash, elution). The SL kit was clinically validated during a clinical trial to evaluate its performance against the gold standard column-based QIAmp Viral RNA Mini Kit.

Materials and Methods

Clinical samples

A total of 410 archived clinical samples obtained from nasal, nasopharyngeal, or buccal swabs were collected from the Northwest London Pathology Laboratory (United Kingdom) and stored in 1mL of VTM at -80°C until further analysis. All samples were collected between October 2020 and January 2021 for testing from staff and patients undergoing routine SARS-CoV-2 diagnostic testing, including both symptomatic and asymptomatic patients. Patient-level data were not shared to protect patient privacy. All samples were inactivated by adding eNAT®Medium (#6U072S; 42.5-45% Guanidine Thiocyanate) at a 1:2 v/v ratio in BSL-3 facilities using standard BSL-3 work practices before transportation and were provided in an anonymised form with no associated data.

SARS-CoV-2 genomic RNA and viral particles

Synthetic SARS-CoV-2 Genomic RNA was purchased from Twist Biosciences. Genomic RNA vials were stored at -80°C, and stock volumes were diluted to concentrations ranging from 1x10⁵ to 50 copies/μl in TE buffer. To perform the initial validation, UV-inactivated viral particles were provided from the BLS-3 laboratory in the Virology Section. Inactivated viral particles were stored in aliquots at -80°C for further use.

RT-qPCR based on CDC assay

For RT-qPCR experiments, the molecular assay developed by the CDC and defined in “CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel” was used. The primers and probes for detecting SARS-CoV-2 were identified from genetic regions belonging to the nucleocapsid (N) gene, encompassing the usage of two primer/probe sets (Table 9). An additional primer/probe set can then be used for amplifying the human RNase P gene (RP) to confirm successful extraction. The CDC assays for SARS-CoV-2 detection were purchased from Integrated DNA Technologies (Catalogue #10006770). Ct values were used to calculate the amount of target NA in the sample. Lower Ct values (typically below 29 cycles) indicate high amounts of the target sequence. Higher Ct values (above 38 cycles) mean lower amounts of the target NA.

Name	Description	Oligonucleotide sequence (5'→3')	Label	Final conc.
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	GAC CCC AAA ATC AGC GAA AT	None	500nM
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	TCT GGT TAC TGC CAG TTG AAT CTG	None	500nM

2019-nCoV_N1-P	2019-nCoV_N1 Probe	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	FAM, BHQ-1	125nM
2019-nCoV_N2-F	2019-nCoV_N2 Forward Primer	TTA CAA ACA TTG GCC GCA AA	None	500nM
2019-nCoV_N2-R	2019-nCoV_N2 Reverse Primer	GCG CGA CAT TCC GAA GAA	None	500nM
2019-nCoV_N2-P	2019-nCoV_N2 Probe	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	FAM, BHQ-1	125nM
RP-F	RNAse P Forward Primer	AGA TTT GGA CCT GCG AGC G	None	500nM
RP-R	RNAse P Reverse Primer	GAG CGG CTG TCT CCA CAA GT	None	500nM
RP-P	RNAse P probe	FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1	FAM, BHQ-1	125nM

Table 9. CDC RT-qPCR panel: primers and probes [33].

SmartLid concept design

For the present study, each two-part SmartLid was produced through 3D printing (3D Systems MJP2500Plus) with a UPS Class VI biocompatible-capable material (VisiJet M2S-HT90). 5mm by 5mm cylindrical neodymium N42 grade magnets were then glued into the removable tab part with cyanoacrylate. To reduce waste, magnet tabs were reused from batch to batch, while each lid part was disposed of after each sample.

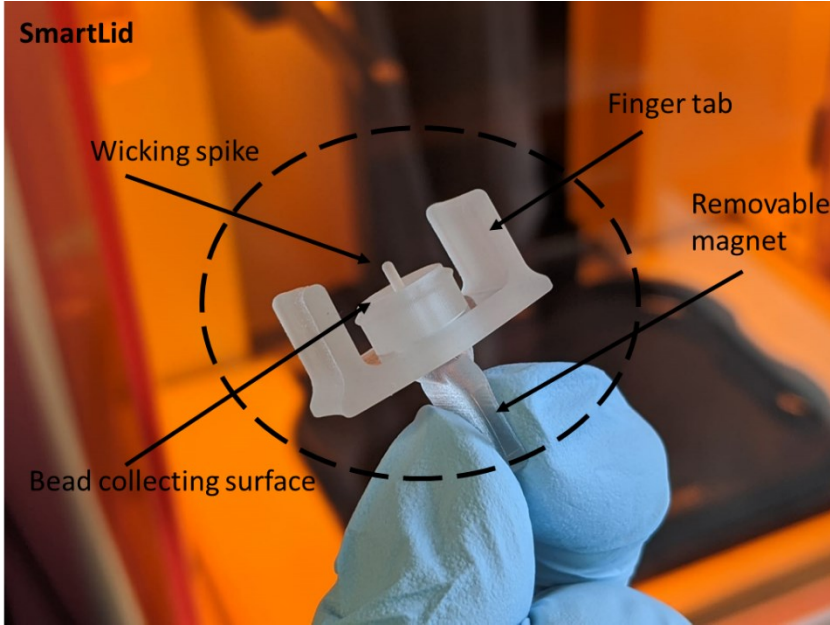


Figure 48. A perspective view of SmartLid developed by M. Cavuto and me. A removable magnet is engaged with the lid. Finger tabs are designed for the user to have a grippable part to which the magnet is attached and also to allow the SmartLid to rest on a surface without contact between the surface and the inner part of the lid. A bead-collecting surface allows the beads to be collected within the area and a wicking spike is designed to encourage liquid to come out from the lid.

SmartLid sample preparation protocol

The sample preparation kit included the contents listed in Table 10.

Sample preparation Kit (detailed content)	
Tube 1 Lysis	Contains Lysis/binding buffer ($\leq 61\%$ isopropanol and $\leq 25\%$ guanidinium thiocyanate) and magnetic beads
Tube 2 Wash	Contains Wash buffer (80% ethanol)
Tube 3 Elution	Contains Elution solution
SmartLid	Includes magnetic key and transparent base

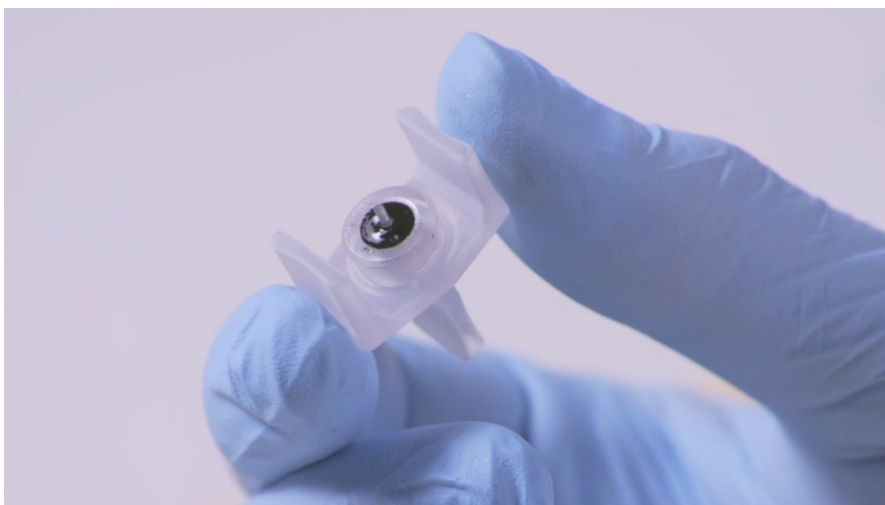
Table 10. Sample preparation kit contents.

To allow the full extraction of NA, optimised reagents were pre-dispensed into three tubes. Tube 1 was preloaded with lysis/binding buffer liquid (based on guanidinium thiocyanate, and isopropanol 99.97%) and magnetic beads (TurboBeads LLC). Tube 2 contained washing solution (80% ethanol). Tube 3 contained elution solution. The SmartLid allowed the common micropipette to be replaced by transferring NA from tube to tube.

Clinical isolates were extracted following the protocol developed:

1. A total of 400 μL of VTM/eNAT (COPAN) lysis buffer containing the clinical specimen was transferred into Tube 1 and gently shaken for 60 seconds. Tube 1, with the SmartLid attached, was inverted four to six times to collect the magnetic beads on the bead-collecting surface via magnetic attraction (Figure 49).
2. The magnetic beads having a silica coating that binds NAs from the viral particles were transferred to Tube 2, and the magnet was removed from the lid. The magnetic beads were resuspended in the washing liquid by gently inverting or shaking Tube 2 for 60 seconds, thereby washing the beads and removing contaminants and chemicals from previous steps and unwanted biological molecules. The magnet was reinserted into the lid and the tube was inverted to collect the magnetic beads. The magnetic beads on the detached lid were exposed to the air for 30 seconds to allow the washing liquid to evaporate.
3. The lid containing the magnet was moved to Tube 3. The magnet was disengaged from the lid so that the magnetic beads were no longer magnetically held against the bead-collecting surface and were resuspended in the elution liquid by gently shaking Tube 3 for 60 seconds. The final step was to recollect the magnetic beads through the insertion of the magnet tab and gentle inversion. The SmartLid kit was then disposed of, and the magnet tab was either reused or disposed of along with the SmartLid kit.

The overall extraction process took 20 minutes per batch of 18 samples. The purified NAs were stored at -80°C until further analysis.



*Figure 49. SmartLid with magnetic beads.
Close-up of magnetic beads collected on the bead-collecting surface when a magnet is inserted on the lid.*

Comparator QIAamp Viral RNA Mini Kit protocol.

A total of 140 μL of COPAN eNAT lysis buffer containing VTM was extracted in parallel to the SmartLid kit according to the manufacturer's instructions. Sample manipulations (mixing with viral RNA extraction lysis buffer) were done in a Class II Biosafety Cabinet using BSL-2 precautions. For the viral RNA extraction kit, the nasopharyngeal swab diluent was added directly to the lysis buffer, vortexed, and incubated for ten minutes to allow for complete viral inactivation. After a two-step washing process, the RNA was eluted in a total volume of 80 μL . The overall extraction process took one hour per batch of 18 samples. The purified NAs were stored at -80°C until further analysis [187].

RT-qPCR reaction conditions

Experiments were carried out in duplicate with a final volume of 20 μL per reaction. The above primers were utilised with the GoTaq Probe 1-Step RT-qPCR (Promega), recommended by the CDC. Each well contained the following: 10 μL of 2 $\times$ GoTaq Probe qPCR master mix, 0.4 μL of 50 $\times$ GoScript RT mix for 1-Step RT-qPCR, 1.5 μL of N1/N2/RNase P assay primer/probe mix, 5 μL of extracted RNA, and enough nuclease-free water to bring the volume to 20 μL . Reactions were performed following the recommendations of the manufacturer: one cycle of 15 minutes at 45°C , one cycle of two minutes at 95°C , and 45 cycles at 95°C for 30 seconds and 55°C for 30 seconds. Reactions were plated in 96-well plates and loaded into a LightCycler 96 Real-Time PCR System (LC96) (Roche Diagnostics). Amplification signals were absent in all negative controls.

Data analysis

Specificity, sensitivity and accuracy of the 410 samples used were calculated based on the hypothetical test statistic published by Baratloo et al. (2015) and $P < 0.05$ was considered significant (24). Moreover, the linear regression method to assess the performance of the SmartLid kit extraction compared to the Qiagen kit (Ct values correlation) was performed in Python (version 3.8) using the Statsmodels package (version 0.11.0), and the correlation coefficient was calculated using the statistic functions of SciPy package (version 1.7.3).

Results and Discussion

Development of SmartLid

SmartLid was designed to work with most flip-top 1.5-2mL tubes currently on the market. The removable magnet was designed to rotate 90° clockwise once inserted on the lid to lock it into place and prevent it from falling out during the shaking and inverting steps. As Figure 50 shows, a 5mm x 5mm cylindrical N42 grade neodymium magnet (in red), press-fitted into the lid, was selected to achieve the strongest possible magnetic field while still ensuring sufficient structural integrity of the lid side walls.

The bowl wall of only 0.5 mm was designed to allow the magnet to sit near the buffer, to ensure a strong magnetic attraction of the suspended beads. When inserted, magnetic particles are rapidly collected onto the underside of the lid through gentle inversion of the tube (Figure 49). Collected beads settle in the shallow bowl feature (Figure 49), which concentrates the beads into the centre, preventing accidental contact with the tube rim during the transfer process.

In the centre of the lidded bowl, a thin spike allows each buffer to be efficiently released from the beads and collecting surface, minimising the carryover of the buffer from tube to tube (Figure 48). SmartLid has two finger tabs for gripping and handling the lid, protecting the bead-collecting surface from accidental contact. Furthermore, the finger tabs act as a stand for the lid, allowing it to be placed on the surface and preventing contamination issues. Figure 50 illustrates the magnetic beads transfer when using SmartLid on a common 1.5 mL Eppendorf tube.

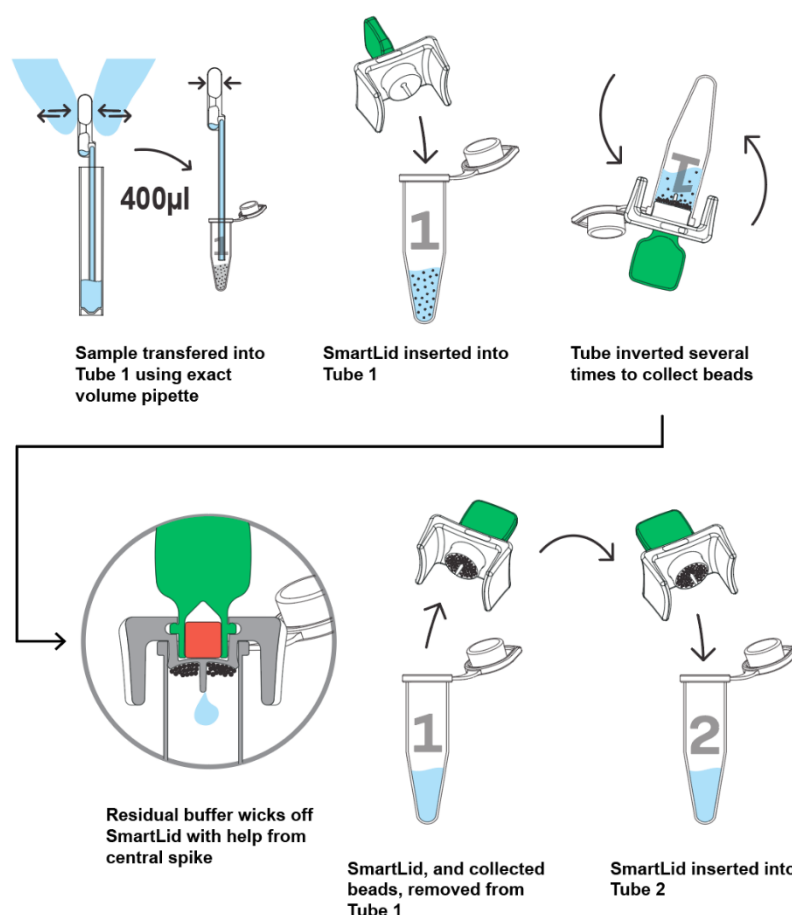


Figure 50. Magnetic bead transfer process.

The illustrated process shows the initial transfer of clinical sample from the eNAT collection tube into the lysis tube allowed by a disposable 400 µL exact volume pipette. Then comes a series of steps where magnetic beads are collected onto the lid and transferred to the second tube. Illustration kindly provided by M. Cavuto.

Development of the sample preparation

Five different extraction kits (column-based and magnetic-based) were evaluated and tested to understand which step was more essential to prioritise and which one could be avoided while obtaining the same performance. The RNAmix kit from Qiagen was used as a comparator kit during the clinical validation as the gold standard column-based extraction method, while the magnetic-based MM Viral RNA Kit was studied as a kit of reference (approved by the Food and Drug Administration of Canada for diagnostic testing) to optimise a new extraction protocol compatible with SmartLid.

The MM kit was chosen as it can efficiently isolate NA (> 75% RNA recovery) from clinical specimens. The protocol followed to extract NA is reported in Table 11. A summary of the main results obtained during the optimisation of the SmartLid protocol appears below. All experiments were performed using RT-qLAMP or RT-qPCR assay. A pool of eNAT containing human background and spiked RNA or SARS-CoV-2 viral particles were used in the different experiments. All experimental

conditions were carried out in triplicate across multiple days and targeted either the human gene, the COVID gene or both.

1. The input sample was increased from 200 μ L (as suggested in point 1 of Table 11, following the MM kit) to 400 μ L to obtain a higher recovery of the extracted NA, as shown in Figure 51a. By doubling the input sample (400 μ L) of an eNAT pool spiked with 3X LoD of viral particles, approximately tenfold more concentration of product was recovered.
2. The MM reagents preparation protocol consisting of 12 steps was replaced by prefilling the tubes with three optimised reagents. The overall performance of the two kits is shown in Figure 51e.
3. The MM extraction protocol was optimised and the steps were reduced from the 24 suggested to four (lysis/binding, wash, dry, elution). The overall performance between the two methods is shown in Figure 51e. Standard curves were generated to compare the two methods with a control (elution directly spiked with a known amount of RNA as reference). RT-qPCR (CDC) was used as a detection method when analysing the results from the two extraction strategies. N1/N2 genes were targeted, showing a high correlation among the two methods with R^2 0.87 and 0.89 for MM extraction and SmartLid extraction, respectively, compared to an R^2 0.92 for the spiked elution control, showing comparable RNA recovery and performance when using the SmartLid kit compared to the commercial extraction kit MM.
4. Vertexing steps in MM protocol (> 12 minutes total) were replaced with a total of three minutes of shaking by hand (one minute per step) as the performance observed was equivalent; the same TTPs were obtained (14.92 vs 15.26 minutes), as shown in Figure 51b. Various degrees of tube mixing and shaking were explored, and it was determined that manual shaking for 30 to 60 seconds was sufficient for lysis/binding and wash steps, while slightly more vigorous shaking (still for 30 to 60 seconds) was required for the elution step (due to the higher surface tension of water).
5. Thermofisher Magnetic beads were replaced with paramagnetic (TurboBeads LLC) because of the speed performance observed when collected using a common magnet (three minutes versus 20 seconds). Beads from Thermo Fisher, Beckman and TurboBeads were compared using SmartLid extraction. Serial dilutions of synthetic RNA ranging from 1×10^5 to 5×10^1 were spiked into Tube 1 and results showed no variation among the different conditions, with a linear correlation (R^2 of 0.98) when using three different magnetic beads. TurboBeads were chosen for their purely metallic core, and thin functionalised carbon outer layer, which allowed them to provide the fastest magnetic separation (< 10 seconds) compared to the other magnetic beads (five to ten minutes) (Figure 51c).
6. A total of four washing steps (point 3 in Table 11), two washing steps using wash buffer 1 and two washing steps using wash buffer 2, were replaced with one single washing step using 80%

EtOH after testing different washing solution concentrations in RT-qPCR (Figure 51d). 70% EtOH did not perform well, causing a four-cycle delay (Cq 29) compared to the MM wash (Cq 25.1), consisting of more than a tenfold difference. 60% EtOH was not suitable as no amplification happened. 80% EtOH and a mix of EtOH and DMSO provided similar Ct (26.4 and 25.9 respectively) as the MM kit. 80% ethanol and EtOH/DMSO were chosen based on the lower Ct value. DMSO was only evaluated as a secondary wash step to avoid the drying step when using alcohol-based washes; therefore it was excluded to avoid adding an extra step to the sample preparation kit. The 60/40 EtOH/DMSO wash was considered as an alternative to avoid the 80% ethanol in the kit, which is not allowed by regulation during aircraft transportation.

7. The drying step of five minutes was replaced by 30 seconds because of the SmartLid design, which exposed the beads' surface directly to the air, achieving rapid ethanol evaporation within seconds. This process takes far less time than other conventional kits (~15 minutes).
8. The elution buffer was replaced by molecular-grade water. A change in elution volume was a limitation of the SmartLid kit because compared to the 80 μ L used in the MM kit, the minimum volume required for the SmartLid to get the beads equally resuspended in the Eppendorf tube was 150 μ L, resulting in a more than ten-times-diluted RNA.

Overall, these optimisations allowed us to propose a simple extraction protocol, minimising the number of steps, the reagents, the costs, and the extraction time and keeping the same performance as a commercial kit. An overview of the extraction kits tested alongside SmartLid and their characteristics compared to the SmartLid kit is presented in Table 12.

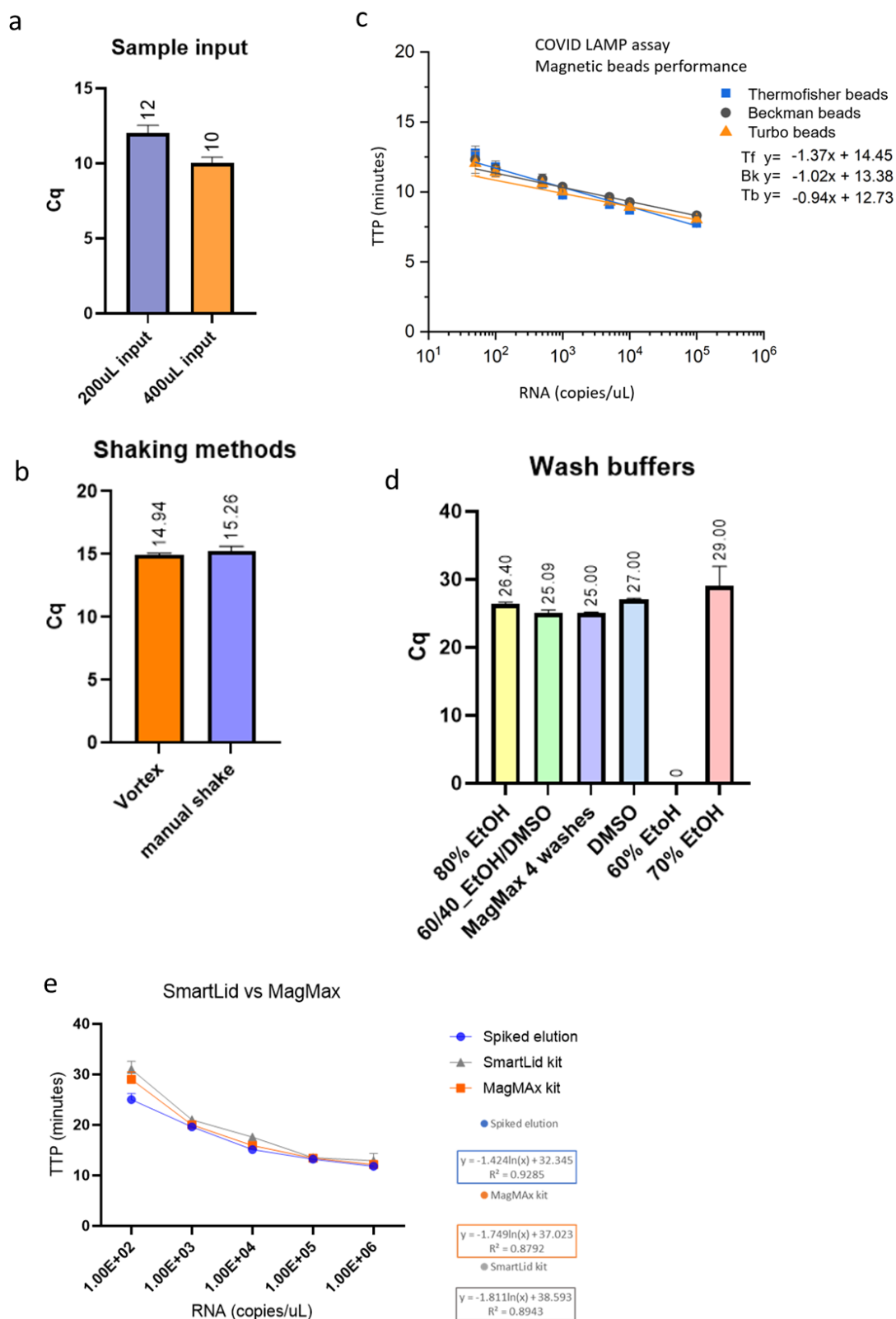


Figure 51. Optimisation of the SmartLid protocol.

Samples containing cultured SARS-CoV-2 using different concentrations were analysed by both methods and extracted RNA was quantified by RT-qPCR targeting the N1/N2 gene and by RT-qLAMP targeting the N12 gene. Recovery results showed during the optimisation process demonstrated equivalent performance with comparable RNA copies for MM and the SmartLid methods. These promising results indicate that the frugal extraction method can compete with lab-based extraction protocols that require expensive equipment and delays over 30 minutes.

Original protocol MM kit for Viral RNA (45 minutes per extraction)	Optimised SmartLid protocol (5 minutes per extraction)
1. Reagents preparation (10 min) a. Mix LB and carrier RNA then add 100 uL IPA and votex. b. Vortex the RNA Binding Beads at moderate speed to form a uniform suspension before pipetting. c. Prepare Bead Mix by combining the volumes of RNA Binding Beads and Lysis/Binding Enhancer Mix thoroughly by gently vortexing. d. Place the prepared Bead Mix on ice until it is needed.	1. Reagents preparation (2 mins) a. In one processing tube add 20uL of magnetic beads, 300 uL of LB buffer and 400uL of IPA. b. In one processing tube add 300uL of 80% EtOH c. In one processing tube add 150uL of nuclease-free water
2. Sample and Lyse (10 min) a. Pipet 802 µL prepared Lysis/Binding Solution (Carrier RNA and isopropanol added) into each Processing Tube. b. Add 400 µL sample to 802 µL Lysis/Binding Solution c. Mix by gently vortexing for 30 sec or by flicking the tube several times. Then centrifuge briefly (~2 sec) to collect the contents at the bottom of the tube. d. Vortex the Bead Mix at moderate speed to create a uniform suspension before pipetting e. Add 20 µL Bead Mix to each sample. f. Gently shake the sample for 4 min on a vortex mixer to fully lyse viruses and bind RNA to the RNA Binding Beads. g. Centrifuge briefly (~2 sec) to collect tube contents. h. Capture the RNA Binding Beads and carefully discard the supernatant i. Leave the tube on the magnetic stand for at least 3 min. j. Carefully aspirate and discard the supernatant without disturbing the beads and remove the Processing Tube from the magnetic stand.	2. Sample and Lyse (1 min) a. Pipette 400uL of sample into tube a: shake by hands 30-60 seconds, capture beads with SL and transfer into tube b.

3. Washes (10 min) Wash twice with 300 µL Wash Solution 1 a. Add 300 µL Wash Solution 1) to each sample and vortex at moderate speed for 30 sec. b. Centrifuge briefly (~2 sec) to collect tube contents. c. Capture the RNA Binding Beads on a magnetic stand for 3–5 min. d. Carefully aspirate and discard the supernatant without disturbing the beads and remove the Processing Tube from the magnetic stand. It is critical to move the Processing Tube off the magnetic stand before the subsequent wash. e. Repeat above steps to wash a second time with 300 µL Wash Solution 1. Wash twice with 450 µL Wash Solution 2 a. Add 450 µL Wash Solution 2 to each sample and vortex at moderate speed for 30 sec. b. Centrifuge briefly (~2 sec) to collect tube contents. c. Capture the RNA Binding Beads as in the previous wash. d. Carefully aspirate and discard the supernatant without disturbing the beads and remove the Processing Tube from the magnetic stand. is critical to move the Processing Tube off the magnetic stand before the subsequent wash. e. Repeat above steps to wash with a second 450 µL of Wash Solution 2.	3. Washes (1 min) Shake 30-60 seconds and capture beads into SL.
5. Dry (10 min) a. Leave the tube open at room temp for 2-5 min	5. Dry (30 sec) Leave the SL at room temperature for 30 seconds then transfer into tube c.
6. Elution (5 min) a. Add 50 µL Elution Buffer (room temp or warmed to 37–65°C) to each sample, and shake or vortex vigorously for 4 min Capture the RNA Binding Beads as in the previous steps. The purified RNA will be in the supernatant. c. Transfer the supernatant, which contains the RNA, to a nuclease-free container appropriate for your application, and store the purified RNA at –20°C.	6. Elution (1 min) Shake 30-60 seconds tube c and capture beads into SL.

Table 11. MM protocol was followed as a reference to optimise the SmartLid protocol.

Extraction kit	Extraction type	Total time per extraction	Electricity	Laboratory environment	Cost
MM Viral RNA	Magnetic-based	45 minutes	Yes	Yes	263 (GBP) 50 preps
Bk viral RNA	Magnetic-based	30 minutes	Yes	Yes	2443 (GBP) 768 preps
RP Viral RNA	Column-based	40 minutes	Yes	Yes	unknown
Q viral RNA	Column-based	45 minutes	Yes	Yes	495 (GBP) 50 preps
TB viral RNA	Magnetic-based	30 minutes	Yes	Yes	206.53 (GBP) 100 preps
SmartLid	Magnetic-based	5 minutes	No	No	100 (GBP) 50 preps

Table 12. Extraction kits assessed compared to the SmartLid in terms of extraction time, use of electrical equipment, need for a laboratory environment and cost.

Note: The cost proposed for the SmartLid kit refers to the whole kit without the need to add anything else, while for the other kits the cost refers only to the reagents provided, excluding all the non-included material required.

Development of the SmartLid kit

The SmartLid kit has been designed to be used with any commercially available magnetic bead-based sample extraction kit by simply prefilling 1.5-2 mL Eppendorf or cryovials tubes with prescribed buffers and using the SmartLid to transfer the magnetic beads between tubes and steps. However, to take full advantage of the method, an optimised single-use POC kit (Figure 52) has been developed containing everything required to extract a single sample in under five minutes with no need for electrical equipment.

Each kit contains pre-filled tubes with all the necessary reagents, simplifying the process and mitigating user error when preparing sample extraction. The three Eppendorf tubes, Tubes 1, 2, and 3, contain respectively magnetic beads with lysis and binding buffer, wash buffer, and elution buffer. The kit includes a disposable exact volume pipette, used for transferring the precise amount of inactivated patient sample into the first tube. As shown in Figure 52a, all required kit components, including the SmartLid kit itself, come packaged securely in a disposable plastic tray, which doubles as a preparation stand for the process, allowing

each reagent tube to stand upright and providing a safe place for the SmartLid between steps. This is in contrast to commercial kits where the sample collection tubes and media are intended to be sourced and/or packaged separately.

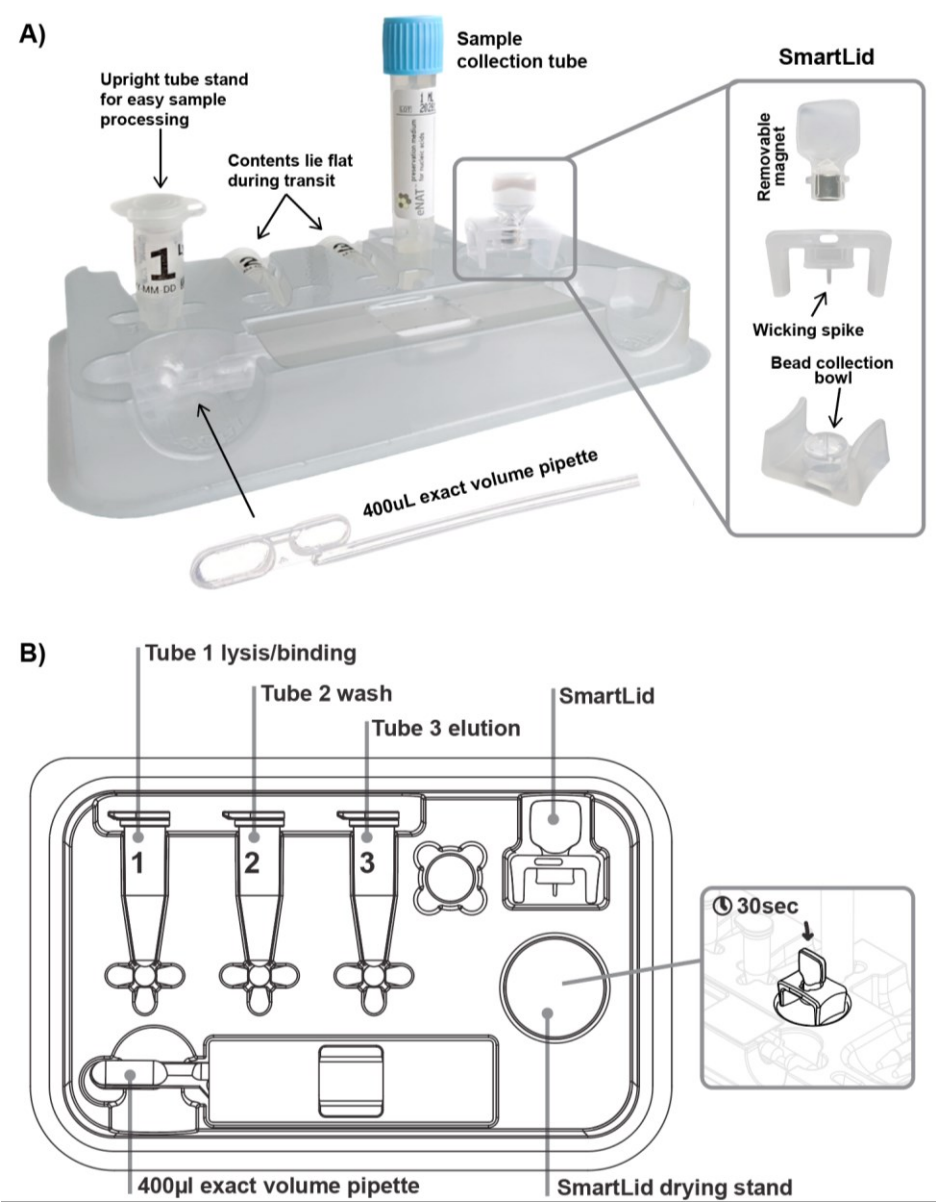


Figure 52. SmartLid kit sample preparation. (A) The illustrated extraction kit contains a lid assembly as described above, three sample tubes and a fixed volume pipette. (B) The first tube contains a lysis/binding buffer liquid, the second tube contains a washing solution, and the third tube contains an elution solution. The kit includes an inexpensive and useful packaging tray, made of thermoformed plastic, which also functions as a stand for the sample tubes and lid assembly. Illustration kindly provided by M. Cavuto.

Figure 52b shows a schematic representation of the kit. In detail, Tube 1 contains a lysis and binding buffer (guanidine thiocyanate) together with magnetic beads (TurboBeads LLC) to allow cell lysis and binding of NAs to the beads. Furthermore, Tube 1 can include isopropanol, which aids in sample breakdown, and NA binding. Tube 2 contains a washing solution (80% EtOH) to remove contaminants, chemicals, and unwanted biomolecules. Tube 3 contains an elution solution (molecular grade nuclease-free water), which releases captured NAs from the beads. As described in the previous section, the SmartLid is used to collect, transfer,

and resuspend the magnetic beads when moving them from tube to tube, with the magnet being inserted or removed. Upon collecting the washed beads, one 30-second drying step is required to let the ethanol evaporate. Therefore, a specified location in the tray was designed to allow complete evaporation.

After the final elution step, the magnetic beads can be collected and disposed of along with the SmartLid kit. The eluted viral RNA and human genomic DNA may then be used for downstream molecular applications such as RT-qPCR and RT-qLAMP, according to the user’s requirements.

Characterisation of clinical samples

To characterise the 410 clinical samples, extraction was performed using the Qiagen QIAmp Viral RNA Kit, and viral load was determined following the CDC protocol for RT-qPCR assay. A specimen was classified as positive when both the N1 and N2 assays were positive within 40 cycles, which is the lower LoD for the CDC RT-qPCR assay. The human RNase P assay was used as a control to reduce false negative rates that can occur due to insufficient numbers of cells collected and/or poor sample quality.

Of the total 410 clinical isolates, 163 were determined as SARS-CoV-2 positive and 247 as negative. The RT-qPCR Ct values for N1, N2 and RNase P assays ranged between 14.0 and 37.6, 14.3 and 40.0, and 19.6 and 40.0 Ct, respectively. All samples tested positive by the RNase P assay, confirming the presence of genomic material. The Ct was calculated by the cycle-threshold method using 0.2 as the fluorescence cut-off value. RT-qPCR results obtained with the N1 assay were used to classify the samples into five categories (high, upper-medium, lower-medium, low, and negative). Estimated RNA concentration ranged from 10⁹ to 10¹ copies per 500µL of lysis buffer, covering all clinically relevant ranges of viral loads. Ct results are summarised in Table 13 and the sample concentration distributions are visualised in a histogram plot (Figure 53). Concentrations (copies per reaction) were estimated based on RT-qPCR standard curve $y = -3.30x + 37.98$.

Categories ^a	RT-qPCR Ct range N1 assay (cycles)	RNA concentration (copies/reaction)	RNA concentration (copies/500µL of Lysis Buffer)	Samples per category (n)
High	14 – 20.9	1 x 10 ⁷ – 1 x 10 ⁵	1 x 10 ⁹ – 8 x 10 ⁶	41
Upper-medium	21 – 24.9	1 x 10 ⁵ – 8 x 10 ³	8 x 10 ⁶ – 5 x 10 ⁵	47
Lower-medium	25 – 30.9	8 x 10 ³ – 1 x 10 ²	5 x 10 ⁵ – 8 x 10 ³	39
Low	31 – 37.6	1 x 10 ² – 1 x 10 ⁰	8 x 10 ³ – 7 x 10 ¹	36
Negative	Not detected	–	–	247

Table 13. Sample characterisation using Qiagen extraction kit with CDC RT-qPCR. All samples tested positive by the RNase P assay. Estimated concentration (copies per reaction) based on RT-qPCR standard curve $y = -3.30x + 37.98$. Estimated concentration (copies/500µL of Lysis Buffer) considering 140µL of sample input and 80µL of eluted volume.

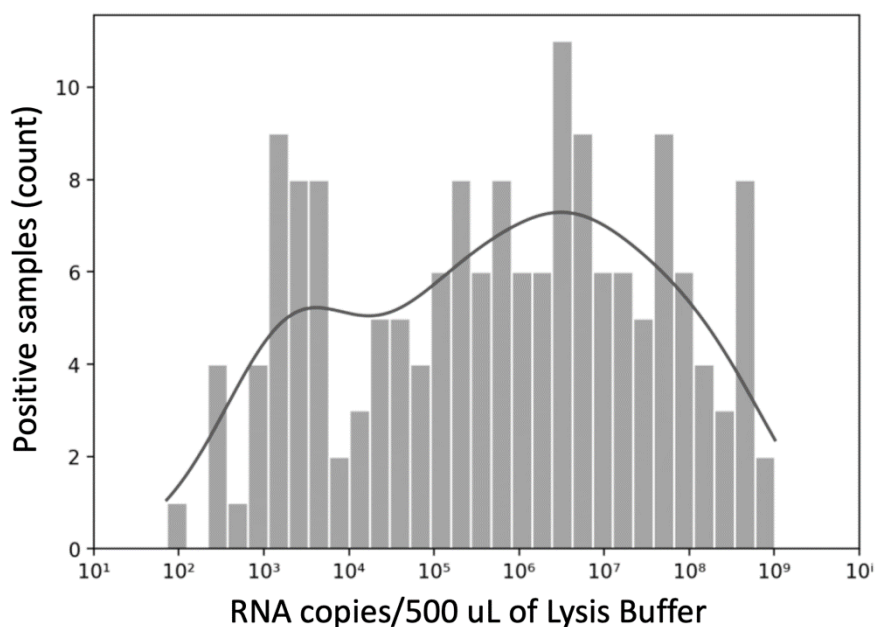


Figure 53. Estimated RNA concentration across positive samples.

RNA recovery performance

The recovery of viral RNA was assessed in the SmartLid and Qiagen methods by RT-qPCR following the current CDC 2019-nCoV guidelines [168]. The RT-qPCR protocol targeted the N1 and N2 genes for specific detection of the viral nucleocapsid genes. To validate the RNA recovery of the SmartLid kit extraction method compared to the column-based Qiagen method, a commercially available SARS-CoV-2 synthetic RNA (Twist Biosciences) was spiked at different concentrations (ranging from 10^5 to 50 copies/ μ L) in eNAT inactivation buffer containing human sample as a background. RNA extraction was performed in parallel using the QIAamp Viral RNA Mini Kit and SmartLid. As shown in Figure 54, the RT-qPCR evaluated for each assay resulted in efficiencies of 3.65 and 3.54 for the N1 assay and 3.91 and 3.78 for the N2 assay, respectively, in the Qiagen and SmartLid kit showing linear correlation among the two different techniques when assessing for RNA recovery.

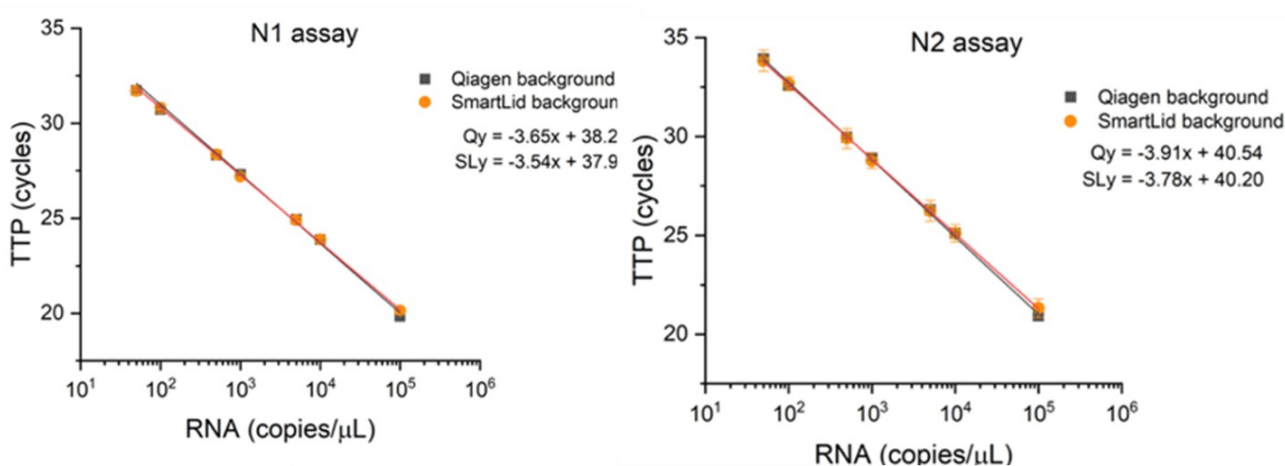


Figure 54. Standards using the CDC RT-qPCR assays with healthy human sample background. Standards using the CDC RT-qPCR N1 and N2 assays with Qiagen and SmartLid kit extractions.

Clinical performance of the SmartLid kit compared to the Qiagen extraction kit

A total of 410 clinical isolates were extracted by the SmartLid kit method and tested for the presence of SARS-CoV2 using the CDC RT-qPCR assay. The RNase P assay was used to check the sample integrity. The samples were aliquoted and frozen at -80°C previous being tested in RT-qPCR. Using a disposable micropipette, 400 µL of the inactivated clinical sample was added to the lysis buffer, then washed and finally eluted in 150 µL using the above-mentioned SmartLid kit extraction workflow. Following the CDC protocol, RNA extracted was tested and the resulting Ct values from LC96 were compared to those obtained using the gold standard Qiagen extraction. As shown in Figure 55, the SmartLid kit method identified a total of 155 true positives (TP), 246 true negatives (TN), eight false negatives (FN) and one false positive (FP) when compared to the Qiagen method, which translates to an overall 95.1% sensitivity (SEN), 99.6% specificity (SPE) and 97.8% accuracy (ACC).

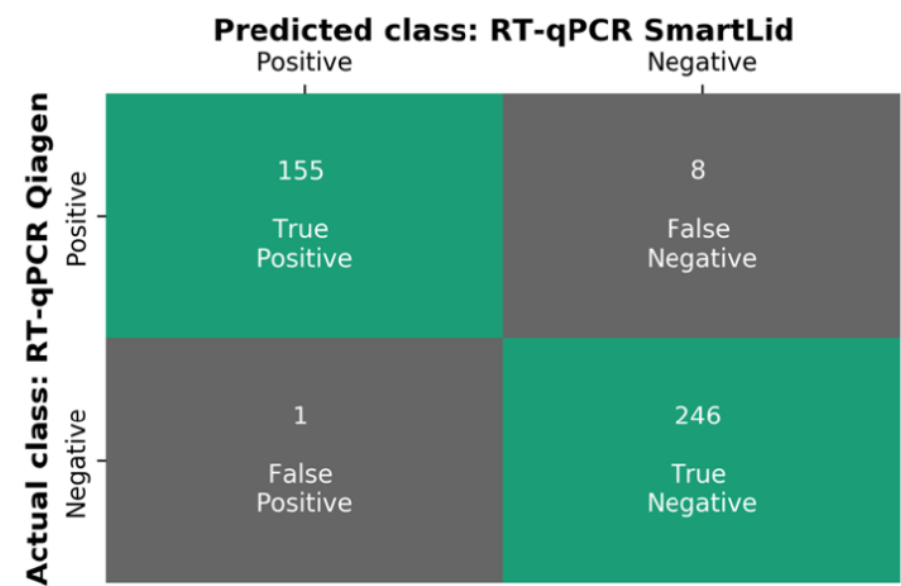


Figure 55. Confusion matrix for Qiagen and SmartLid methods. Sensitivity (%) was calculated as true positives/(true positives+ false negatives) *100. Specificity (%) was calculated as true negatives/(true negatives + false positives) * 100. Accuracy (%) was calculated as (true positives + true negatives)/total.

As reported in Table 14, the SmartLid method showed a 100% SEN, 99.6% SPE and 99.6% ACC for samples with high, upper-medium and lower-medium concentrations ($1 \times 10^9 - 8 \times 10^3$ RNA copies/500µL of lysis buffer); and 81.8% SEN, 99.6% SPE and 96.8 ACC for samples with low concentration ($8 \times 10^3 - 7 \times 10^1$ RNA copies/500µL of lysis buffer).

Categories	RT-qPCR Ct range N1 assay (cycles)	Sample (n)	TP	TN	FP	FN	SEN (%)	SPE (%)	ACC (%)
High	14 – 20.9	41	41	-	-	0	100	-	99.6
Upper-medium	21 – 24.9	47	47	-	-	0	100	-	99.6
Lower-medium	25 – 30.9	39	39	-	-	0	100	-	99.6
Low	31 – 37.6	36	28	-	-	8	81.8	-	96.8
Negative	Not detected	247	-	246	1	-	-	99.6	-
Total		410	155	246	1	8	95.1	99.6	97.8

Table 14. SmartLid kit method performance based on sample category. Categories are defined based on the Qiagen method and CDC RT-qPCR N1 assay values.

To further evaluate the performance of the SmartLid kit method compared to the Qiagen method when using the CDC RT-qPCR assay, the correlation between Ct values obtained by both methods was calculated by applying linear regression. Figures 56a and 56b show a total of 155 positive samples detected by the N1 and N2 with $R^2 > 0.99$. Figure 56c illustrates the correlation between the Ct values of RNase P assay, comparing all 410 clinical samples. The SmartLid and Qiagen methods showed an excellent correlation for the RT-qPCR Ct values, with an $R^2 > 0.99$ across the three assays.

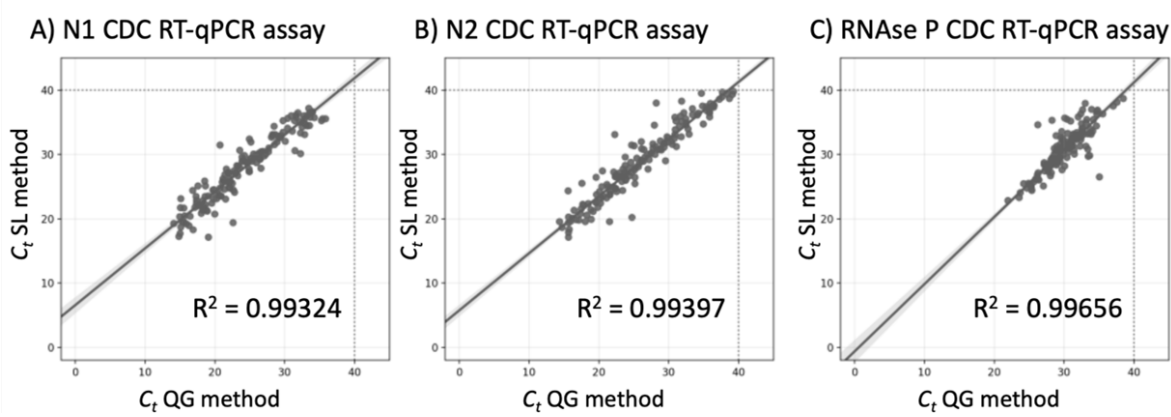


Figure 56. Correlation between SmartLid kit and Qiagen methods based on CDC RT-qPCR Ct values. A) Correlation between the two methods based on N1 assay. B) Correlation between the two methods based on N2 assay. C) Correlation between the two methods based on RNase P assay.

Designing an affordable POC kit

NA extraction of 100 samples using the SmartLid in-house protocol had an overall cost of £300 (£200 reagents and £100 SmartLid) with a hands-on time of <5 minutes (a shaker was designed and 3D printed by M. Cavuto to shake nine samples by hand simultaneously, bringing to a total of 18 samples extracted in < 20 minutes). The Q kit cost was >£900 (reagents and spin columns, excluding extra solutions and labware) with a

hands-on time of 45 minutes for 18 samples. To make an extraction kit that was for routine testing, the price of every constituent part of this sample preparation kit had to be factored in and minimised where possible. To achieve the lowest cost possible, multiple companies were approached and engaged in conversations regarding long-term partnerships to negotiate reduced costs at scale, so that the sample preparation kit would be accessible to all. The final cost of £2.20 at a large scale is the first step towards making an affordable technology that could provide a true POC extraction method for use in LMIC.

Conclusion

This chapter presented a novel sample preparation method that has been developed to simplify the extraction process of NA, for use in conjunction with molecular-based detection methods such as PCR and LAMP. While the presented kit and protocol have been validated for manual and rapid isolation of RNA and DNA from buccal, nasal, and nasopharyngeal swabs, isolation of NA or other biomolecules such as proteins from other sample types (blood, stool, tissues, saliva, urine) will also be explored and validated.

During this clinical validation, SmartLid demonstrated fast (<5 minutes) and frugal (<£3 per sample at scale) RNA extraction from clinical specimens without necessitating trained personnel or expensive laboratory equipment such as micropipettes, centrifuges, heaters, or vortex mixers. To my knowledge, no other commercially available extraction kit requires less than 45 minutes for performance without relying on electrical equipment in a controlled laboratory environment. The SmartLid method obtained comparable performance when compared to the QIAamp Viral RNA Mini Kit, with an overall 95.1% sensitivity, 99.6% specificity and 97.8% accuracy when using RT-qPCR. The sensitivity of the SmartLid method increases to 100% with samples with medium/high viral load ($Ct < 31$). While achieving comparable performance to a column-based kit, a few positive samples were not detected after preparation with the SmartLid kit. This could be due to several factors, such as opting for shorter periods of manual shaking instead of longer powered vortex mixing (perhaps decreasing the degree of cell lysis, NA binding, washing, and eluting), or eluting in a volume three to five times larger than standard. Any one of or a combination of the above factors could have resulted in these observed samples with extremely low viral loads ($Ct > 31$) not being detected by RT-qPCR when extracted by the SmartLid kit. However, published works have shown that samples with a Ct above 30 are unlikely to be culturable and patients are not likely to be infectious[188]. Thus, the benefits in cost, convenience, simplicity, time, and mobility outweigh the drop in extraction efficiency.

In comparison to the high standard Qiagen kit, the SmartLid method has multiple advantages, especially in LMIC and POC settings: The QIAamp Viral RNA mini-Kit kit requires an expensive and bulky benchtop centrifuge, multiple micropipettes, and detailed laboratory training, in addition to a multi-page protocol lasting over 45 minutes for a single sample. While this extraction time per sample can be decreased through batch processing, the average extraction time per sample at best only decreases to around five minutes with a "24 place centrifuge" (assuming a skilled and trained user).

The MM magnetic-based kit scale is far less efficient with quantity due to the time-intensive (24 steps protocol) and technique-sensitive process of collecting beads against the tube walls using a magnetic stand and removing each buffer completely without disturbing the beads. When prioritising high throughput, it was not possible to batch process samples because they had to undergo the vortexing process manually one by one.

SmartLid allowed batch process samples using a 3D-printed handheld multi-tube shaking tool, allowing a user to shake or invert up to 13 tubes per hand. During the study, a minimum per-sample extraction time of just under two minutes was managed using this tool, without any drop in performance or convenience. With the proposed single-use kits (in which the packaging/tray serves as a sample processing station) there is no need for any other tube rack or stand and no need for the user to place anything on an external surface, thus reducing the risk of contamination of the test apparatus and final elution. Furthermore, all buffers and components of the kit were chosen for their stability at room temperature, with no cold chain requirement for storage or transport.

For future iterations, a few alterations to the described kit are being explored. First, as the kit is intended to be used in a POC setting, everything is disposable. Given the magnitude of the pandemic, this creates a potential for a large amount of plastic waste. To offset this, new iterations of the packaging/preparation stand made from 100% recycled cardboard have been employed, leaving only the SmartLid kit, buffer tubes, and disposable exact volume pipette as plastic waste. Furthermore, the magnet tab can easily be reused from sample to sample, as there is minimal risk of exposure to patient samples during processing. Other improvements are being considered concerning long-term storage and shipping. It has been observed that the ethanol in Tube 2 can experience significant evaporation (20-30% of the volume lost after three to four months) when stored at room temperature. Moreover, ethanol (in concentrations above 10%) is considered a flammability risk according to most international shipping guidelines. To mitigate both concerns, the use of different types of tubes has been investigated (CRYOVIAL® from Simport Scientific), some of which have shown little to no evaporation loss and would decrease the chance of leakage during shipping.

In the next chapter, I will show the results that have been achieved by combining the SmartLid kit with a colourimetric RT-LAMP detection developed by Rodriguez Manzano.

I hope this will rapidly extend beyond the current pandemic to future infectious disease outbreaks (e.g., monkeypox), enabling prompt, optimised care, minimising the use of unnecessary treatments such as antibiotics, and instead guiding rapid decisions on the use of appropriate treatments. Ideally suited to applications in both low- and high-resource settings, the SmartLid kit might represent a step-change in POC and molecular testing anywhere in the world.

Part II

Chapter 6

From the lab to the clinic:

Design and validation of a true POC test to detect respiratory viruses in under 30 minutes

Chapter 4 showed the results from the first clinical validation conducted with the CBIT team to detect the presence of SARS-CoV-2 using the electrochemical-based platform. It was intended to conduct a second clinical validation of the SmartLid sample preparation method in conjunction with the on-chip detection in order to advance towards a true POC solution, however, the COVID-19 pandemic created unprecedented challenges in the process. Semiconductor shortages, in addition to longstanding issues within worldwide industries producing essential electronic components, caused an inevitable pause in the on-chip experiments, preventing validation of the sample to result on the Lacewing platform. Therefore, to validate the extraction method at the POC, an alternative LAMP technique based on colourimetric detection described in *Rodriguez-Manzano et al. [124]* was utilised.

Using the work detailed in the previous chapter, in agreeance with work being developed by the CBIT team on lyophilised reaction mixtures, a novel POC diagnostic test is described. During the pandemic, the research group became part of a start-up called ProtonDX, hence obtaining the necessary funding to conduct a clinical validation. I contributed to conceptualise the work, perform the laboratory work, and partially conduct the statistical analysis. The clinical validation was performed with the assistance of the entire team.

My thesis concludes with this chapter, which has been a significant milestone in my personal development, since it enabled me to acquire the knowledge to contribute to the development of diagnostic solutions closer to the patient.

Introduction

In vitro diagnostics (IVDs)

Diagnosis is a driver of a patient, financial and health systems impact and a critical enabler of universal health coverage, but it is also the weakest link in the care chain [189]. This is especially true in primary healthcare settings in LMIC. These countries often lack an integrated network of laboratories used for such diagnoses; however, a broad set of IVDs is available for testing patients in the primary care setting where laboratories are not available. Access to good quality, affordable, and appropriate health products is indispensable to advance universal health coverage, address health emergencies, and promote healthier populations. Without access to IVDs, health providers cannot diagnose patients effectively and promptly or provide appropriate treatments.

The WHO Essential Diagnostic List provides evidence-based guidance and sets a reference for the development of essential IVD tests [190], [191] with the goal to contributing toward health systems strengthening and releasing universal health coverage, ensuring healthy lives and promoting well-being for all people of all ages [192].

In vitro simply means 'in glass', meaning these tests are typically conducted in test tubes and similar equipment, as opposed to in vivo tests, which are conducted in the body itself. In vitro tests can detect diseases, conditions, and infections and may be performed in laboratories, health care facilities or even in the home. The tests themselves can be performed on a variety of instruments ranging from small, handheld tests to complex laboratory instruments. They can be used to monitor a person's overall health allowing doctors to diagnose patients effectively and provide appropriate treatments.

To demonstrate that the developed POC test meets the expected WHO requirements, [193] a clinical validation was conducted as part of the ProtonDx team.

Respiratory viruses

In the last two decades, respiratory viruses have become increasingly recognised as serious cause of morbidity [194]. The incidence of acute respiratory diseases in high-income countries accounts for 75% of all acute morbidities, with approximately 80% of these infections being viral [195]. According to the Centers for Disease Control and Prevention's national vital statistics report, infections of the upper respiratory tract, such as rhinitis, pharyngitis, and laryngitis, are among the most common infections in children. In infants and young children, this occurs three to eight times every year, leading to an acute asthma exacerbation, acute otitis media, and lower respiratory tract infections such as pneumonia, bronchitis, and bronchiolitis. Children and infants who suffer from acute viral respiratory tract infections are most likely to be hospitalised [194].

The presence of more than one respiratory pathogen in a mixed infection is not uncommon and is likely underreported due to the limited sensitivity of the current diagnostic methods. As a result of insufficient sensitive tests or the presence of as-yet-unknown pathogens, it is often difficult to identify a particular virus in clinical practice [196].

It is important to identify the pathogen responsible for the symptoms and provide timely results to guide clinician's decision, institute appropriate antiviral or antibiotic therapy as well as isolate infected patients to prevent health-care-associated infections which can have devastating effects on immunocompromised patients rendering them highly susceptible to infectious pathogens [197].

Viruses responsible for respiratory infections include influenza viruses A and B, respiratory syncytial virus (RSV), parainfluenza viruses 1,2 and 3, human metapneumovirus (hMPV) and picornaviruses (rhinoviruses and enteroviruses) [194].

The influenza virus is an enveloped single-stranded RNA virus with a negative sense. It belongs to the Orthomyxoviridae family and has a negative sense genome. There are three types of influenza viruses: influenza virus types A, B, and C. Of the three types, influenza virus types A and B are most commonly isolated, whereas influenza virus type C is less common and is not routinely tested for [198].

It is common for influenza viruses type A to cause seasonal epidemics in the northern hemisphere between December and May, characterized by an increase in the frequency of upper respiratory symptoms. Most influenza infections remain confined to the upper respiratory tract in most patients, however complications have been reported with lung transplants recipients being particularly at increased risk.

RSV is a single-stranded negative-sense, RNA virus belonging to the family Paramyxoviridae family. In the northern hemisphere, the incidence of RSV increases during the winter months in accordance with the seasonal patterns of influenza viruses [199]. There can be serious complications associated with RSV infection in transplant patients and in the young and elderly population with an increased risk for these patient groups to develop pneumonia. RSV it is indeed considered the most important etiological agent causing respiratory disease in infancy and major cause of pneumonia and bronchiolitis in infants under 2 years of age [200].

Rhinoviruses are positive-sense single-stranded RNA viruses that belong to the Picornaviridae family. Rhinoviruses were considered to be the most common cause of the "common cold" and therefore, neglected by the medical community until recent years, when their clinical spectrum of disease expanded. Infections caused by rhinoviruses can occur throughout the year, with a peak incidence in September [194]

When individuals are healthy, rhinovirus infections are usually self-limited, lasting between seven and fourteen days. According to several studies, rhinoviruses can cause more severe infections including otitis media, bronchiolitis, and exacerbation of asthma in children. However, severe complications, including pneumonia, can occur especially in immunocompromised individuals.

The POC test presented in this chapter is intended to enable rapid diagnosis of respiratory pathogens using a multipathogen approach. In addition to focusing on the presence of SARS-CoV-2 virus, the simultaneous extraction and detection of Influenza A/B, Rhinovirus, and RSV (subtypes A and B) viruses provide valuable information about the individual's pathological state.

State-of-the-art POC molecular technologies

The volume of NA testing for infectious diseases at the point of care has steadily increased since its widespread introduction [201]. POC tests entered clinical practice in developed and developing countries, especially for applications requiring fast turnaround times, and in settings where a centralised laboratory approach faces limitations.

Current systems for clinical diagnostic applications are mainly PCR-based, can only be used in hospitals, and are still relatively complex and expensive [71]. Integrating sample preparation with NA amplification and detection in a cost-effective, robust, and user-friendly format remains challenging. Examples of fully or partially integrated POC platforms authorised by the FDA are listed in Table 15. FilmArray Respiratory Panel 2.1 (RP2.1) allow the simultaneous qualitative detection and identification of multiple respiratory viral and bacterial NAs in nasopharyngeal swabs (NPS) obtained from individuals suspected of respiratory tract infections, including COVID-19 (<https://www.biofiredefense.com/covid-19test/>). Xpert® Xpress SARS-CoV-2 (Cepheid) is an automated molecular test for the qualitative detection of SARS-CoV-2, also offering a cartridge where multiple regions of the viral genome are targeted in approximately 45 minutes with less than a minute of hands-on time to prepare the sample (<https://www.cepheid.com/en/systems/GeneXpert-Family-of-Systems/GeneXpert-Omni>). Abbot ID NOW™ COVID-19 (Abbot) is instrument based, using an isothermal system for the qualitative detection of infectious diseases although the cost is still very high (\$8,500 cost of the instrument and \$70-85 cost per cartridge) and no multipathogen detection can be performed (<https://www.globalpointofcare.abbott/en/product-details/id-now.html>). These systems have an integrated sample preparation step and provide results within one hour, which is very convenient. However, they require a dedicated specialist to operate the instrument, and even if they represent a reliable tool to diagnose SARS-CoV-2 they do not meet all of the criteria to be considered a true POC test.

The reliance on the optical detection methodology limits the scalability and accessibility, and the resulting bulky and expensive instruments require complex internal mechanics that are prone to failure in the field if moved or not set on a stable surface, which represents a limiting factor when using these POC devices, preventing them from being used anywhere in the field. Furthermore, the cost-effectiveness of these devices is far from affordable in LMIC, with costs ranging from \$17,000 to \$85,000 (considering only the instrument). Improvements over the years have been made in terms of total time from sample to result from a minimum of 20 to a maximum of 120 minutes.

Manufacturer & product name	Multi-pathogen	Sample prep methodology	Detection process	Cost	Total time
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Abbott ID Now	No	Heat Chemical	NEAR (isothermal) amplification real time fluorescence	Cartridge: US\$70–85 Instrument: US\$8,500	23 min
bioMérieux FilmArray	Yes - up to 43 targets on one test, with space for more	Chemical Bead beating Magnetic extraction	Nested multiplex RT- PCR	Instrument: \$49,000 Cartridge: \$129	62 min
Cepheid GeneXpert Xpress	Yes - up to 4x on current Flu/RSV/COVID array	Chemical ultrasonic horn Magnetic extraction	RT- PCR amplification Fluorescence	Omni: <US\$3,000 GeneXpert IV: US\$17,000 Cartridge: US\$40-60	26-37 min
CerTest SARS- CoV-2 Test	Yes - up to ~20 single tests in stool panel	Chemical Heat Filter/columns	External - RT- PCR fluorescent dyes	unknown	120 min
DNA Nudge CovidNudge	Yes - a single cartridge detects up to 8 genes	Chemical Silica filter based	RT- PCR amplification real- time fluorescence	Cartridge: US\$130 Instrument: US\$40,000	90 min
Roche Diagnostics - cobas liat (lab in a tube)	Yes - up to 6 fluorescent wavelengths	Chemical Magnetic extraction	RT- PCR amplification real time fluorescence	Cartridge: €55 Instrument: €25,000	20 min
Visby Medical	No	unknown	RT - PCR	\$250/test (@200qty)	30 min

Table 15. POC platforms that are commercially available.

<https://www.globalpointofcare.abbott/en/product-details/id-now.html>. <https://www.biofiredefense.com/covid-19test/>.
<https://www.cepheid.com/en/systems/GeneXpert-Family-of-Systems/GeneXpert-Omni>. <https://www.covidnudge.co.uk/en/COVID-Bubble-test>. <https://www.certest.es/products/sars-cov-2-n1-n2/>. <https://diagnostics.roche.com/us/en/products/instruments/cobas-liat-ins-6050.html>. <https://www.fda.gov/media/142228/download>

The DF concept

An innovative and rapid POC test is described in this chapter and called DF. This test enables the rapid preparation of samples and detection of NAs using RT-LAMP within 30 minutes. DF has been developed to be used in a POC setting or in a decentralised setting or a limited resource area. The intention to create such a test was motivated by the need to rapidly detect the presence of individuals affected by SARS-CoV-2 efficiently and rapidly. The qualitative detection of SARS-CoV-2 RNA as a single target is an option that can be also combined with the extraction and detection of other respiratory pathogens such as Influenza A, Influenza B, RSV and human rhinovirus from nasopharyngeal, nasal and buccal specimens, regardless of gender or age, and with or without signs and symptoms suggesting infection. Each test comprises of two main components, a disposable NA extraction kit and an eight-tube strip of dried primers and LAMP reagents to allow multi-pathogen detection.

Respiratory viruses are responsible for a variety of clinical syndromes, ranging from mild upper respiratory tract infection to severe complicated lower respiratory tract infection, particularly in patients with chronic lung disease or immunosuppression [202]. There is a considerable challenge in diagnosing suspected

lower respiratory tract infection in immunocompromised individuals due to the wide spectrum of infectious and non-infectious etiologies that may present in a similar manner [203].

Infections caused by respiratory viruses can occur through direct contact with an infected individual. When infected hosts breathe, splash, cough, and sneeze, they release viruses from the environment, causing particle-sized particles that contain viruses to form sprays, which then cause infection [204].

Due to the similar symptoms and signs associated with respiratory viral infections, it may be difficult to identify the causative virus from the clinical features alone, and because the contagious power is very high, it may cause a pandemic in a short period of time. Therefore, respiratory viruses pose a significant threat to infection spread both inside and outside of hospitals. By detecting infectious causes rapidly, it is possible to obtain diagnostic clarity, receive targeted and timely treatment, and implement infection control measures in order to limit transmission of infections [205].

It is therefore imperative that infection control guidelines take into account respiratory infections strictly in order to protect the health and safety of the population especially given the lack of effective antiviral therapy [206]. Recent developments in molecular diagnostic platforms have enabled detection of a wide range of pathogens at the POC.

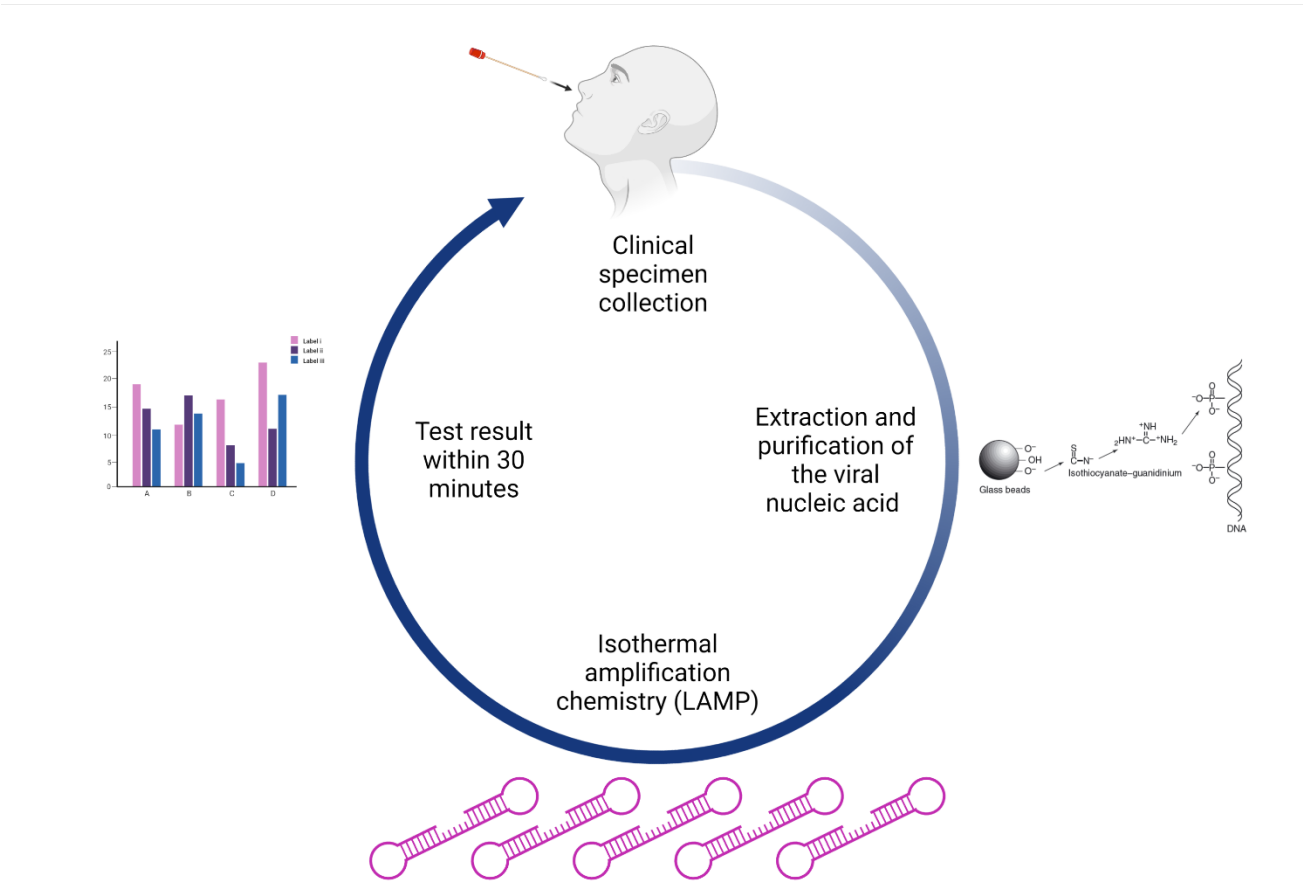


Figure 57. The DF concept. Created with BioRender.com

The early diagnosis of respiratory virus infections is essential in order to prevent unnecessary antibiotic use as well as to take appropriate precautions against infection. In this chapter, a miniaturised new test equipment that has the advantages of existing molecular diagnostic tests has been developed to simultaneously detect multiple respiratory viruses. This may assist in reducing antibiotic prescriptions for outpatients suffering from respiratory tract infections and anti-influenza prescriptions for patients suffering from undetected pathogens. Extraction of viral NAs from respiratory swabs is performed as explained in the previous chapter, through magnetic separation and manual manipulation of three buffer tubes (lysis-binding, wash, and elution). The elution solution containing purified NA is transferred into the test panel, which comprises a strip of tubes, each of which contains lyophilised reagents designed to detect the different pathogens and controls. The reaction is heated for 25 to 30 minutes at 63.5°C, and if amplification occurs, the pH of the solution changes, causing a colour change from pink to yellow and offering visual detection of the results within thirty minutes (Figure 57).

Colourimetric LAMP as a naked-eye detection method

LAMP stands out as a simpler and more powerful alternative to other amplification methodologies for its exceptional speed and capability of being performed at one single temperature without the requirement of optical detection, eliminating the need for thermal cycling. As an alternative to optical detection, researchers have been developing assays based on naked-eye observation with endpoint colourimetric LAMP recently becoming more popular [207].

To obtain a colourimetric reaction, a simple and cheap heater is the only requirement to heat the solution to 60-65°C and visual inspection of colour change is possible by looking at small reaction tubes after the amplification terminates. During DNA synthesis as a response to the DNA polymerase adding bases to the growing DNA products, a direct visual colour change from pink to yellow takes place. Figure 58 illustrates the colour change mechanism [208].

Similarly to on-chip amplification, the DNA polymerase adds dNTP to the 3' end of the DNA strand and consequently, a pyrophosphate ion and a proton H⁺ are released. The exponential production of amplicons during LAMP causes a proportional release of protons that consequently allows the pH to drop by two pH units. The incorporation in the reaction mix of a metal indicator or pH indicator allows the formation of a reversible complex with a metal ion, enabling colour change from pink to yellow. Phenol red is a pH-sensitive dye responding to the production of protons by DNA polymerase under non-buffered conditions, therefore used as a good candidate for colour discrimination between negative and positive results. Based on an exponential amplification, the higher the amount of target NA in the sample, the faster a change in colour due to the amplicons produced during the reaction will be observed.

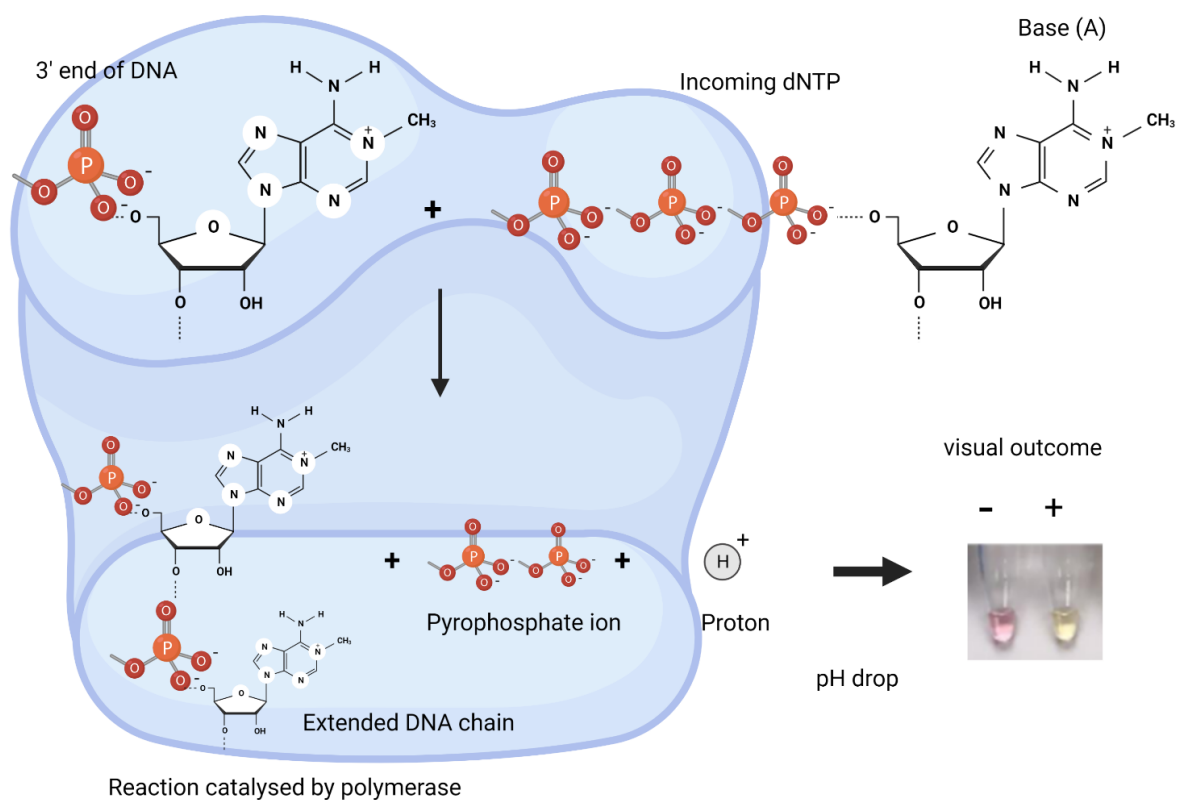


Figure 58. Schematic illustrating the properties of pH-sensitive (phenol red) colourimetric readout for LAMP. Phenol red interacts with protons (H^+) generated during DNA amplification, which causes a colour change from pink/red to yellow. Illustration made with Biorender.com

Materials and Methods

Clinical samples

To conduct the clinical validation, 435 archived clinical samples were acquired by PDx from Imperial College Innovations Limited, acting on the behalf of Imperial College London, under a Material Transfer Agreement (Internal Reference 9012929) to complete this CE Marking Validation Study only. All original samples were obtained from nasal and throat swabs during routine asymptomatic testing across Imperial College London locations and collected and stored in Sigma Virocult® media between July 2021 and May 2022. Locations included clinics in South Kensington, Hammersmith, Charing Cross, White City, Saint Mary's and Silwood Park. Patient-level data were not made available for this study to protect patient privacy.

Residual samples were inactivated by adding eNAT®Medium (#6U072S; 42.5 –45% Guanidine Thiocyanate) at a 1:2 v/v ratio in BSL-3 facilities using standard BSL-3 work practices before transportation and were provided in an anonymised form with no associated data.

A total of 925 sub-lineages of SARS-CoV-2 lineages were present in the database, accounting for 2,632,504 genomes with >99% coverage. Lineages of interest such as Omicron, Omicron BA.2, Omicron BA.4, Omicron BA.5, Alpha, Beta, Gamma, Delta and Lambda are present. Moreover, between 1 July 2021 and 30 April 2022 (when the samples used in this study were collected), a total of 71,416 cases were reported in the UK. Among these cases, SARS-CoV-2 variants with a prevalence above 1% were AY.4, AY.4.2, BA.1.1, BA.1.15, BA.1.15.1, BA.1.16, BA.1.17.2, BA.1.1.15, BA.1.20, BA.2, BA.2.20, BA.2.12, BA.2.16, BA.2.3, BA.2.5 and BA.2.9. Prevalence above 1% is considered based on the total number of clinical samples tested positive and the associated possibility of our cohort containing the specific variants previously described.

The total 435 clinical samples were characterised as 162 positive and 265 negatives using the (CE-IVD alphaPrep™) Viral DNA/RNA Extraction Kit and RT-qPCR assay (CE-IVD PROmate COVID-19) in a UKAS accredited independent laboratory. Clinical samples were tested to validate the performance of the SmartLid kit NA extraction method in conjunction with the DF Respiratory Panel using colourimetric LAMP. The results were captured using the software provided with the companion app, and data were stored in the database.

Viral particles and synthetic RNA

Quantified reference viral particles purchased from Vircell and AB Scientific were used to evaluate the analytical performance for SARS-CoV-2 (Vircell, #MC127), Flu A (AB Scientific, #NATFLUAH1(2009)-S), Flu B (Vircell, #MC052) and RSV (AB Scientific, #NATRSVA-STQ and #NATRSVBSTQ, mixed at ratio 1:1). Vircell stocks were provided in a lyophilised format and the viral concentration was estimated in copies/mL after adding molecular biology grade water. AB Scientific stocks were provided in a wet format and viral concentration was

also provided in copies/mL. Different viral stock concentrations were obtained by spiking negative eNAT® Medium. Quantified synthetic RNA was used to perform experiments for HRV.

The NCBI BLAST version 2.12.0 (ncbi-blast-2.12.0+) was used for all the in-silico analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastDocs&DOC_TYPE=Download). Blast hits were reported for each of the five targets (SARS-CoV-2, IAV, IBV, HRV and RSV) to provide coverage results. Statistical analysis was conducted in Biopython v1.79.

Comparator RT-qPCR method

For the comparator RT-qPCR test, a nine-point dilution standard was generated to correlate the Ct value with the estimated viral load of each tested sample (in copies/mL). A total of ten negative clinical samples were pooled together and mixed with eNAT (COPAN) buffer in a 1:2 ratio. 1mL of this mixture was then dispensed into nine separate tubes, each simulating an inactivated negative clinical sample. Each tube was then spiked with different concentrations of inactivated viral particles [AmpliRun Total SARS-CoV-2 Control (SWAB) from Vircell]. Each dilution point was extracted and tested in triplicate and then averaged to obtain the standard. Figure 63 shows the dilution range extracting the concentration standard with relative Ct values.

RNA from all specimens (n= 435) was extracted using the NC15, CE IVD marked NA extraction method combined with the CE IVD alphaPrep™ extraction kit. Post-extraction samples were analysed to identify the presence or absence of SARS-CoV-2 using the PROmate™ RT-PCR Kit, in combination with the Genesig® q32Real-time PCR instrument. The test targeted the ORF1ab genomic region and could detect all variants of concern of SARS-CoV-2 to date.

The experimental workflow

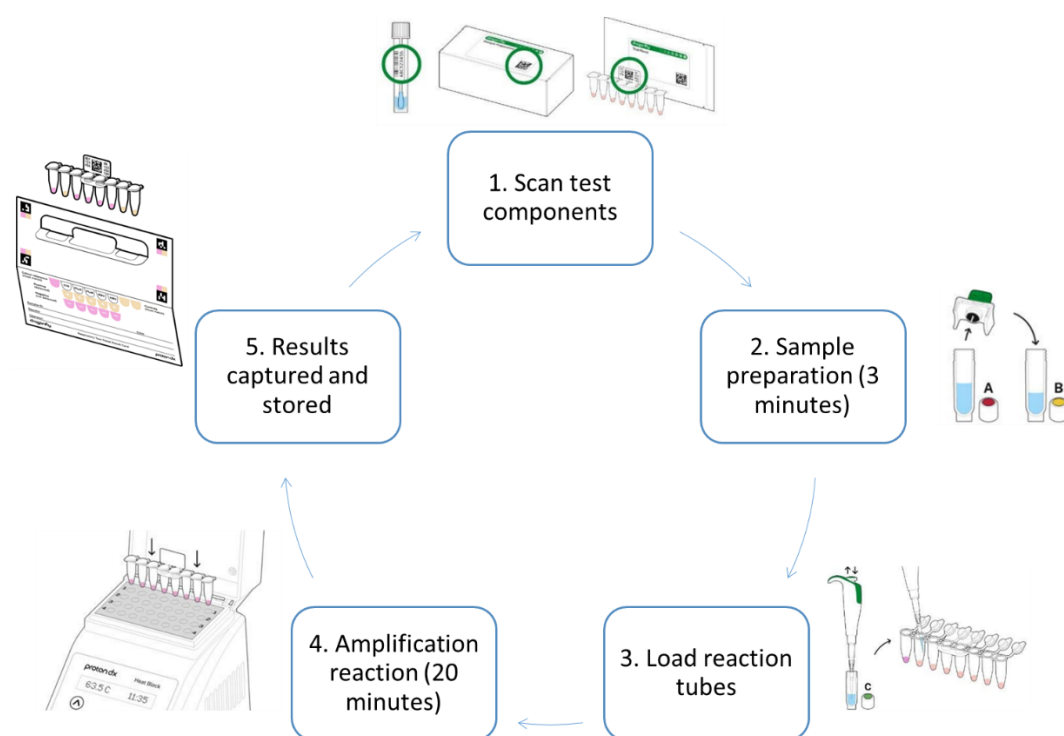


Figure 59. DF experimental workflow.

The DF experimental workflow illustrated in Figure 59 consisted of a preliminary recording of patients sample and kits (1), an extraction step (2) where the NA is extracted from the patient sample and an incubation step where the purified sample is dispensed into the test panel (3) containing lyophilised LAMP reagents and incubated for 20 minutes (4). The heat block was designed to host a maximum of four strips of eight tubes, enabling testing of four patient samples simultaneously while providing a constant temperature of 63.5°C to perform the LAMP reaction. The DF Software was designed to guide the user in the workflow of the DF Kit and store results data on a cloud server.

During the clinical validation, DF testing was performed with the 'Advanced Users' setting to enable faster throughput of assays.

Materials required for DF testing were:

- 63.5°C dry block heater
- Tablet with DF app set to "Advanced Users"
- Single-volume reusable micropipette and pipette tips
- DF diagnostic kit (Sample Preparation Kit and DF Respiratory Test Panel)

LAMP primers

GAPDH LAMP primers were designed to target the presence of human genomic DNA in the sample as a reference to the quality of the collected sample. The non-amplification of the human control determined invalid the result. Other primers were designed by members of the team: two sets of different LAMP primers

(tandem) targeting each specific pathogen were resuspended at a final concentration of 400 μ M stocks using nuclease-free water and included in the lyophilisation process. A list of the primers is illustrated in Table S6 in the Appendix. One LAMP assay is used for the specific detection of SARS-CoV-2, targeting the open reading frame that encodes polyproteins PP1ab (ORF1ab gene).

Lyophilised reaction conditions

A panel consisting of LAMP assays developed by members of the team for the detection of five respiratory pathogens is presented. The targets were selected based on the impact of these pathogens to cause similar flu-like symptoms.

The LAMP reaction mix contained all reagents prepared and lyophilised by Biopharma. A total of eight lyophilised reagent cakes were prepared according to the master mix below, dispensed and freeze-dried into a strip of eight microcentrifuge tubes 0.2mL containing from left to right the following targets: (1) colour control, (2) SARS-Cov-2, (3) Influenza A, (4) Influenza B, (5) RSV, (6) rhinovirus, (7) positive control, and (8) internal control. No enzymes were included in the first reaction tube, known as the colour control, ensuring it never changed colour (pink) during incubation. All other reaction tubes contained a DNA polymerase. All the tubes targeting viral RNA also contained reverse transcriptase in the lyophilised reaction. Lastly, the target-specific LAMP primers were included in each lyophilised reaction; six to eight primers were used per target gene, allowing target-specific amplification to occur. Human control and internal control should always be positive to determine a valid test. The human control targets a human gene and is used to validate the successful extraction of the sample and its quality (not degraded). The internal control consists of a non-human assay and includes its respective non-human template; therefore, this is used to validate that the reagents are working as expected and the reagents are stable under the stored conditions.

The LAMP reaction mix contained the following stock reagents in a total volume of 25 μ L: custom isothermal buffer 10X containing the cofactors KCl, $(\text{NH}_4)_2\text{SO}_4$, Polysorbate 20 required for optimal activity of the reaction enzymes, trehalose 50% enhancing the lyophilisation process and maintaining the long-term stabilisation of the reagents, dextran 20% enhancing the lyophilisation process and maintaining the long-term stabilisation of the reagents, MgSO_4 100mM, NaOH 500mM useful for the lyophilisation process and to maintain the reaction pH stable, dNTP mix 25mM, BSA glycerol-free 40mg/mL for LAMP enzyme stability, Phenol-red 2500uM as pH indicator dye (red at pH 8.0 and yellow at pH 7.6), DNA polymerase 120U/ μ L and Retro transcriptase 25U/ μ L.

The lyophilisation process and freeze-drying microscopy (FDM) were performed by Biopharma in the UK. The FDM analysis provided the critical temperature to design the freeze-drying protocol. LAMP formulations were dispensed to form lyophilised cakes of 25 μ L volume. After the lyophilisation process, cakes were obtained as shown in Figure 60.

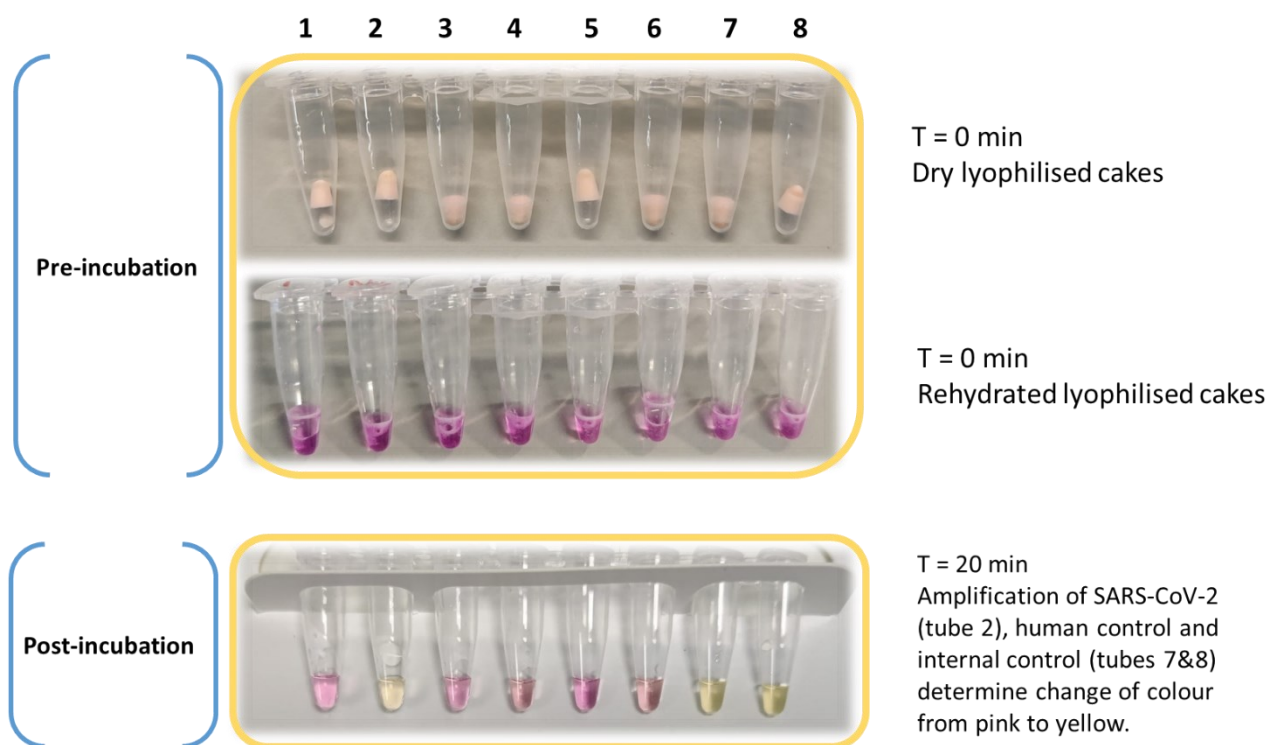


Figure 60. Lyophilised cakes pre/post-resuspension and pre/post-incubation.

The figure shows the respiratory panel and its physical appearance when cakes are in a dry format and rehydrated with an eluted sample. The post-incubation panel shows a valid result for the presence of SARS-CoV-2 after 20 minutes of incubation in a heat block at 63°C.

Sample preparation

The 435 clinical samples provided were extracted using the Sample Preparation Kit following instructions visualised in the companion app. The first step consisted of the registration of the patient ID and test panel. Specimen ID on the patient sample was tracked together with the test panel ID and reported on the result card to ensure tracking of each test (Figure 61).

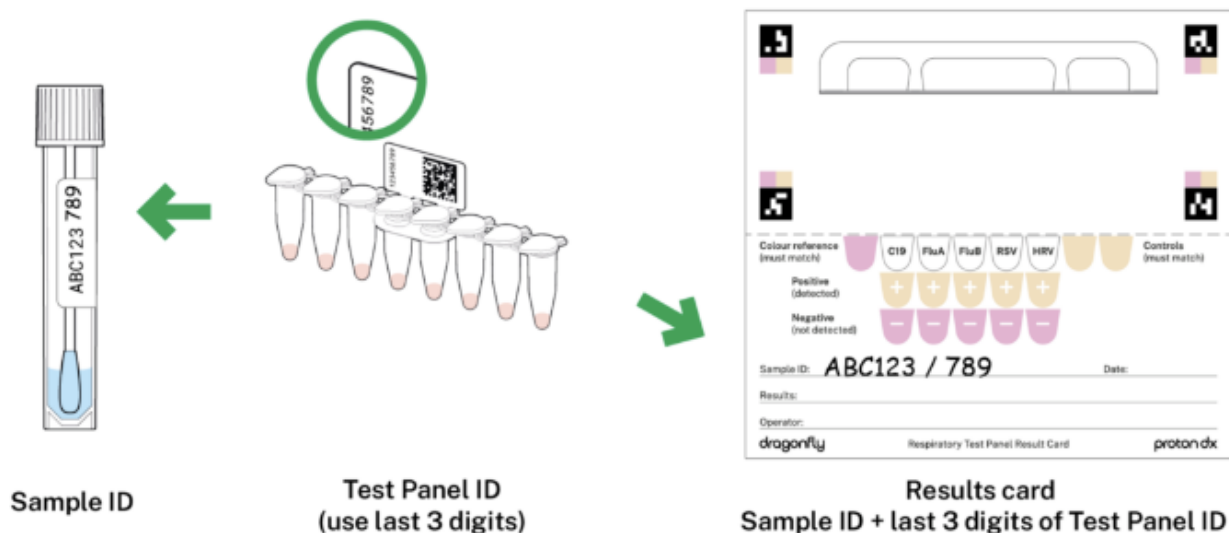


Figure 61. eNAT tube containing patient sample and test panel tracking system.
Illustration kindly provided by D. Gratiot.

The sample preparation step was performed following three main steps:

i. Lysis step

- Remove the SmartLid magnet (green) by turning clockwise at 45° and place it in the tray holder.
- Shake Tube A for 30 seconds to lyse the cells and bind NAs (DNA and RNA) on the magnetic beads. The liquid should be dark grey/black.
- Re-insert the SmartLid magnet into the SmartLid and turn 45° clockwise to lock.
- Invert until the liquid is clear to collect the magnetic beads onto the SmartLid.
- Remove SmartLid from Tube A and insert it into Tube B. Close and dispose of Tube A.

ii. Wash step

- Remove the SmartLid magnet to release the magnetic beads. Place the magnet in the tray holder.
- Shake Tube B for 30 seconds to wash contaminants from the NAs. The liquid should be dark grey/black.
- Re-insert the SmartLid magnet into the SmartLid and lock. Invert until the liquid is clear to collect the magnetic beads onto the SmartLid.
- Remove SmartLid from Tube B and place it on the drying stand for 30 seconds to dry the beads.

iii. Elution step

- Remove the SmartLid from Tube B and insert it into Tube C.
- Remove the SmartLid magnet to release the magnetic beads. Place the magnet in the tray holder.
- Shake Tube C for 30 seconds to release the NAs from the magnetic beads. The liquid should be dark grey/black.
- Re-insert the SmartLid magnet into the SmartLid and lock. Invert until the liquid is clear to collect the magnetic beads onto the SmartLid.
- Remove the SmartLid with the magnet and magnetic beads and dispose of it appropriately. Close Tube C with the cap. The sample is now purified and ready for analysis.

Additionally, NEC (negative extraction controls) were run at the beginning, middle, and end of each testing block, to ensure the absence of environmental contamination and ensure the validity of the results. This was done by processing the test following the SOP from start to finish but extracting from negative (un-swabbed) eNAT (COPAN) sample collection tubes. A result where amplification was observed only in the final tube (8), the internal assay control, was considered a valid NEC result and indicated a lack of environmental

contamination. This tube acts as the positive control reaction and ensures that the reagents performed as expected.

The test panels were loaded with 20 µL of a purified patient sample using the fixed volume pipette and incubated into the heat block for a maximum of 35 minutes. Results read beyond 35 minutes were considered invalid.

Results capture and interpretation

After 20 to 35 minutes, test panels were unloaded from the heat block and tubes were placed on the result card. Incubated test panel tube colours were compared to the corresponding printed reference colours in the result card. Tubes 1, 7 and 8 were compared with the colour key to guarantee the validity of the results. Tubes 2, 3, 4, 5 and 6 were then compared against their colour key to confirm if the colour indicated a positive (yellow) or negative (pink) result. Any change from pink to yellow was considered positive since any amplification is evidence of the presence of the target.

By holding the folded result interpretation card with the test panel in front of the camera, the software confirms the correct test panel, and an image is captured automatically and shown on the result interpretation screen. An illustration of the capture and interpretation process is shown in Figure 62.

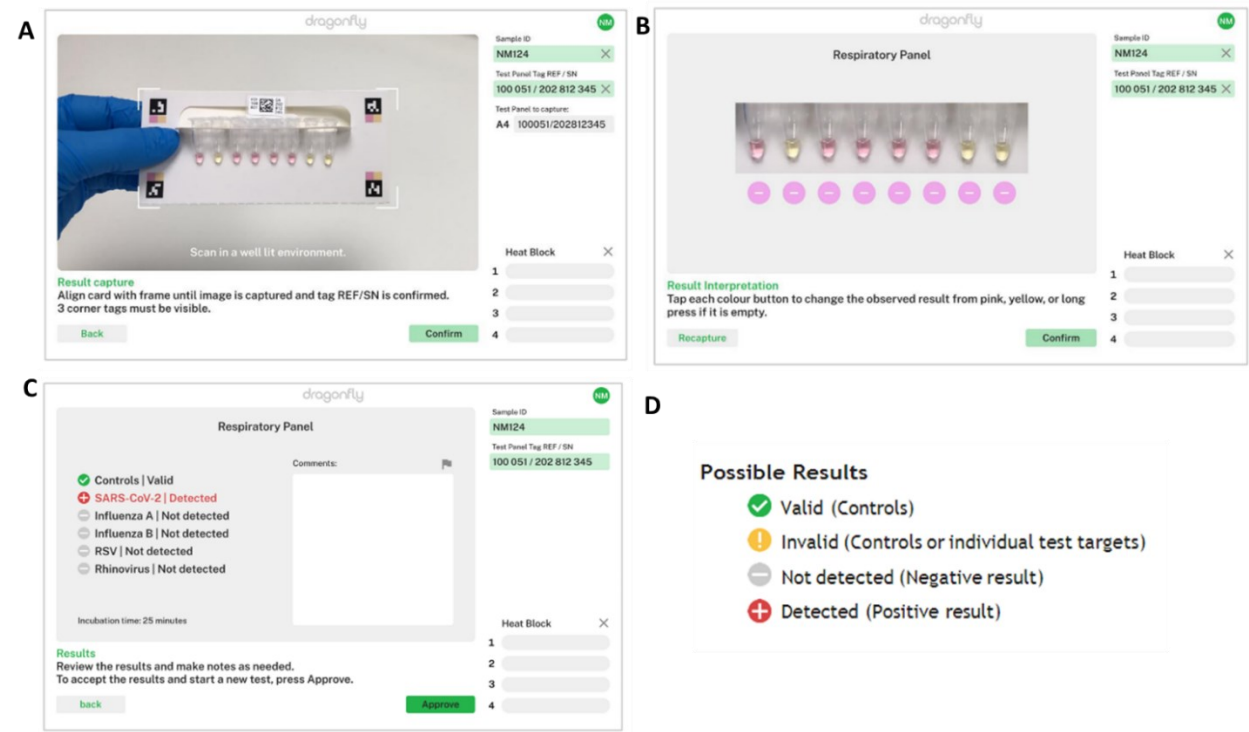


Figure 62. Results interpretation. By holding the test panel on the card result in front of the camera an image is captured (A) and automatically the software advance to the result interpretation screen (B), where the user can assess the colour in each tube and press the corresponding button to save the results. The result screen (C) confirms the sample and panel tested with results. Results include verification that the controls are valid. If any of the control is invalid the entire test is invalid, and no result will be shown. If the controls are valid, the results for each target will be shown as negative, positive or invalid.

User interface

The companion Application developed offers two modes of use: guided and advanced. Each mode provides integrated sample and consumable assay, tracking timers for sample preparation steps and incubation and digital capture of the results. The guided mode is meant for new users, providing illustrated step-by-step instructions from start to results. The advanced mode provides a more flexible streamlined interface for experienced users. Both modes use the sample protocol and therefore deliver the same results. Multiple samples can be run simultaneously at the same time, tracking the incubation time. Real-time advice is provided as support for the user to minimise errors as they occur.

Results and Discussion

Viral load distribution in clinical samples

To validate the performance of the DF Diagnostic Kit a UKAS accredited independent laboratory performed the clinical validation comparing the DF kit to an authorised extraction (CE-IVD alphaPrep™Viral DNA/RNA Extraction Kit) and RT-qPCR assay (CE-IVD PROmate COVID-19 RT-qPCR assay). A total of 435 archived clinical nose and throat swabs, stored in VTM, were obtained and subsequently characterised as 163 positive and 265 negatives by the comparator RT-qPCR.

Based on the nine-point dilution standard generated using inactivated SARS-CoV-2 viral particles (Vircell, #MC127) shown in Figure 63, the following final concentrations were selected in order to obtain a wide range of Ct values: 1x10⁵copies/mL, 1x10⁴copies/mL, 5000 copies/mL, 2250 copies/mL, 1500 copies/mL, 1000 copies/mL, 750 copies/mL, 500 copies/mL, 250 copies/mL.

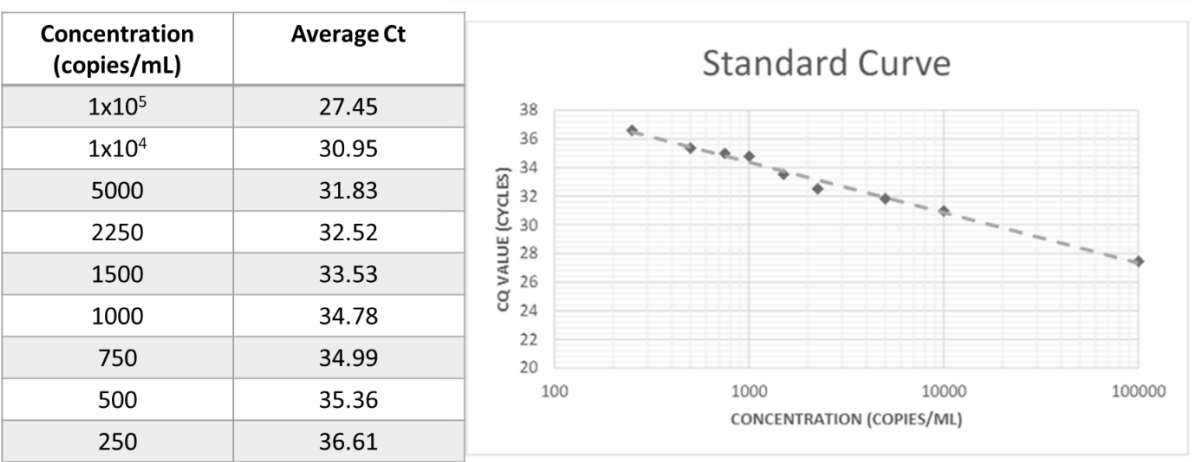


Figure 63.Viral load distribution in clinical samples using inactivated viral particles. Standard curve and concentration (copies/mL) based on a nine-point dilution generated using inactivated SARS-CoV-2 viral particles (Vircell, #MC127). The standard curve equation was $y = -1.53\ln(x) + 44.94$ with $R^2 = 0.989$.

Clinical samples were categorised into four classes based on their viral load (high, upper-medium, lower-medium, and low). Out of a total of 163 positive samples, five were categorised as having high viral load (Cq range 14-20.99 and 6.1x10⁸–6.2x10⁶ copies/mL). 46 samples were categorised in the upper-medium range (Cq range 21-24.99 and 6.2x10⁶–4.6x10⁵ copies/mL). 70 samples were categorised into the lower-medium range (Cq range 25-30.99 and 4.6x10⁵–9.1x10³ copies/mL). 42 samples showed low viral load with a Ct range of 31-37.66 and 9.1x10³–1.2x10² copies/mL (Table 16).

Of the total analysed samples, 163 were identified as positive with RT-qPCR cycle threshold (Ct) values for the PROmate®COVID-19 CE-IVD RT-PCR ranging between 19.55—36.26, with a median Ct of 27.45 (Figure

64). Estimated RNA concentration ranged from >10⁷ to 291copies/mL of eNAT® Medium, covering a clinically relevant range of viral loads.

Category (viral load)	RT-qPCR Ct Range	Viral concentration (copies/mL)	Samples per category (n)
High	14 – 20.99	6.1 E+08 – 6.2E+06	5
Upper-Medium	21 – 24.99	6.2E+06 – 4.6E+05	46
Lower-Medium	25 – 30.99	4.6E+05 – 9.1E+03	70
Low	31 – 37.66	9.1E+03 – 1.2E+02	42
Total			163

Table 16. Viral load distribution in clinical samples..
SARS-CoV-2 clinical samples Ct range and viral load (copies/mL) distribution.

Based on the standard curve generated, the positive clinical samples analysed were shown to have more low viral loads corresponding to high Ct value, than high viral loads (low Ct). This skewed the distribution towards lower-medium viral loads as there were not enough high viral loads available to normalise the distribution. However, this was found to be acceptable due to the high vaccination rate in London, providing an accurate representation of the current prevalence of viral loads in the population. Lower viral loads are also closer to the LoD of the DF assay, presenting more of a disadvantage than an advantage in this study. Another limitation factor in this study is that clinical samples were stored in VTM media rather than the one designed for this kit (eNAT). Samples where user error was reported were retested, with the confirmed result reported (n = 16, 3.84%).

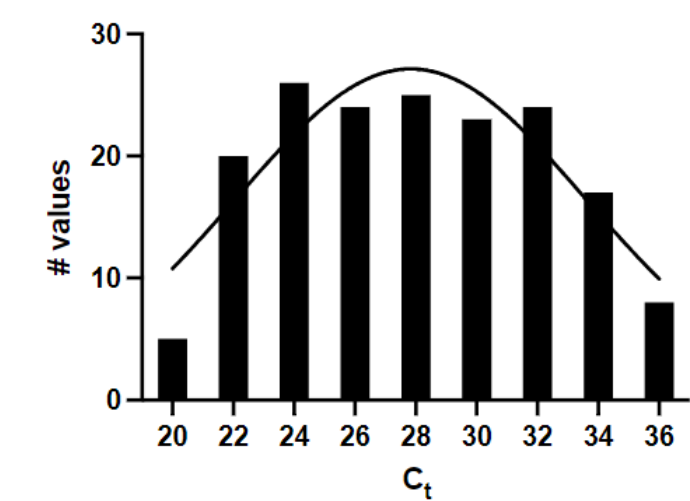


Figure 64. Ct distribution in positive samples.

Clinical performance of the DF assay compared to the PROMate RT-qPCR

A total of 162 valid positive samples were detected by RT-qPCR, while 158 were detected by DF, highlighting a discrepancy of four samples.

Ct < 34.71		CE-IVD RT-qPCR Comparator	
		Positive	Negative
DF test	Positive	154	0
	Negative	1	265
	Total	155	265
Sensitivity (PPA)		99.35% (95% CI: 96.46% - 99.98%)	
Specificity (NPA)		100.00% (95% CI: 98.62% - 100.00%)	

Table 17. Clinical performance characteristics in DF when Ct is <34.71.
Clinical performance characteristics of SARS CoV-2 positive samples in the DF SARS-CoV-2 Panel when compared to the comparator RT-qPCR for COVID-19. Clinical samples with Ct < 34.71 (N=420) show sensitivity (positive percentage agreement) of 99.35% and a specificity (negative percentage agreement) of 100%.

Seven clinical samples had a Ct >34.71 and were deemed inconclusive, as recommended by the independent UKAS accredited lab. Excluding these inconclusive samples (Ct > 34.71, n=7), DF resulted in a clinical sensitivity of 99.35% (95% CI: 96.46% - 99.98%) and a clinical specificity of 100% (95% CI: 98.62% - 100.00%), as shown in Table 17. However, if the samples determined to be inconclusive are considered SARS-CoV-2 positive, the clinical sensitivity across all samples tested is adjusted to 97.35% (Table 18).
Sensitivity was calculated as:

Sensitivity (%) = True Positives / (True Positives+ False Negatives) *100

Ct < 37.66		CE-IVD RT-qPCR Comparator	
		Positive	Negative
DF test	Positive	158	0
	Negative	4	265
	Total	162	265
Sensitivity (PPA)		97.35% (95% CI: 93.80% - 99.32%)	
Specificity (NPA)		100.00% (95% CI: 98.62% - 100.00%)	

Table 18. Clinical performance characteristics in DF when Ct is <37.66.

Clinical performance characteristics of SARS CoV-2 positive samples in the DF SARS-CoV-2 panel when compared to the comparator RT-qPCR for COVID-19. Clinical samples with Ct < 37.66 (N= 427) show sensitivity (positive percentage agreement) of 97.35% (95% CI: 93.80% - 99.32%) and a specificity (negative percentage agreement) of 100% (95% CI: 98.62% - 100.00%).

Of the 435 clinical samples tested, 265 valid negative results were detected by both DF and PROMate RT-qPCR, and no samples amplified past the 37.66 Ct limit of blank cut-off.

A total of eight (1.84%) out of 435 results were identified on DF as having an invalid human genomic DNA control due to a lack of amplification in Tube 8. These eight samples were excluded from the final sample population as the comparator assay (PROMate) cannot confirm the presence or lack of human genomic DNA. A third comparator RT-qPCR assay (2019-nCoV RUO Kit 500 rxn, #10006713, IDT) was used to confirm the absence of human genomic DNA, showing that three out of the eight invalids were due to unreported user error (0.69%) whereas the other five were confirmed to not contain human DNA or SARS-CoV-2 RNA. Specificity was calculated as follows: specificity (%) = True Negatives / (True Negatives + False Positives) * 100. Therefore, the calculated specificity for DF was 100.00% (95% CI: 98.62% - 100.00%).

Disagreement between DF test and RT-qPCR

Discrepancies between the DF test panel and the RT-qPCR results obtained from the PROMate® COVID-19 CE-IVD RT-PCR kit could present in multiple ways, such as an invalid test result in DF due to the human extraction control reaction failure or SARS-CoV-2 being detected in RT-qPCR but not in DF or vice versa. Regardless of the cause or type of discrepancy, all were verified with a third RT-qPCR reaction method using the recommended CDC assays targeting the SARS-CoV-2 N1, N2 genes and the human RNase P gene (Primer Design, Novacyt, #D00070). During the clinical validation, four DF tests showed disagreement with the qPCR PROMate RT-PCR kit, all of which were positive in RT-PCR although negative in DF. Eight samples failed to amplify the human extraction control in DF, therefore producing an invalid test result. Of these eight, seven

were negative for SARS-CoV-2 and one was positive according to DF. All discrepancies, whether valid or invalid, were retested by the third RT-qPCR comparator (2019-nCoV RUO Kit 500 rxn, #10006713, IDT). While all invalid discrepancies were excluded from the sensitivity and specificity calculations, all valid discrepancies were included if the result agreed with the PROMate® COVID-19 CE-IVD RT-PCR result.

Analytical performance

The analytical performance of the DF kit was studied to evaluate the capability of the diagnostic device to correctly identify or measure a specific analyte. The analytical performance was performed on the following pathogens stored in COPAN tube: SARS-CoV-2, Flu A, Flu B, RSV and HRV across multiple days and using different DF kits batches.

LoD analysis was studied to determine the lowest detectable concentration of the pathogens of interest at which at least 95% of all replicates test positive. The analysis was run in triplicate using six dilution points for each pathogen.

The SARS-CoV-2 analytical sensitivity was assessed further, using ten dilution points and a total of 24 replicates at the lowest LoD. Dilutions were prepared in eNAT buffer along with a negative COPAN swab and extracted using the Sample Preparation Kit. The extracted DNA was detected using the Respiratory Panel. The analytical sensitivity was calculated independently for each DF viral target with the lowest viral input successfully detected in all three replicates, as a measure of viral copies/mL of sample. The limit of quantification for SARS CoV-2 has been calculated from the 24 using the calculation: SARS-CoV-2 LoD (%) = (positive SARS CoV-2 / 24) * 100.

After running triplicates at each concentration level, the LoD was determined to be 500 copies/mL. After this, DF was run a further 21 times to assess the LoD for SARS-CoV-2, resulting in 23 out of the 24 replicates displaying positive results (95.8% accuracy at 500 copies/mL) (Table 19).

	LOD of SARS-CoV-2 assessed with viral particles									
SARS-CoV-2 (copies/mL)	3.00E+05	1.50E+05	3.00E+04	7.50E+03	4.0E+03	2.50E+03	2.0E+03	1.0e+03	5.0E+02	2.5e+02
Positives/ Total	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	23/24 (95.8)	1/3 (33%)

Table 19. Analytical LoD of SARS-CoV-2 in the DF Respiratory Panel.

The LoD for the rest of the respiratory pathogens was characterised by dilution studies using quantified inactivated viral particles (Flu A, B, RSV) or synthetic RNA (RHV) and is summarised in Table 20.

Varying viral concentrations were used to determine the LoD for Flu A. Samples were spiked with viral particles: NATtrol Influenza AH1N1pdm Stock (AB Scientific, #NATFLUAH1(2009)-S). After running three DF tests at each concentration level, the LoD for Flu A was determined to be 25,000 copies/mL. Similarly for Flu B, samples were spiked with IBV viral particles: INFLUENZA B VIRUS 100K TOTAL CONTROL (Vircell, #MC052). After running three DF tests at each concentration level, the LoD for Flu B was determined to be 25,000 copies/mL. For RSV, the following samples were spiked with RSVA/B viral particles: NATtrol Respiratory Syncytial Virus Type A Stock (AB Scientific, #NATRSVA-STQ) and NATtrol Respiratory Syncytial Virus Type B Stock (AB Scientific, #NATRSVB-STQ). After running three DF tests at each concentration level, the LoD for RSV was determined to be 25,000 copies/mL. For HRV, samples were spiked with in-vitro transcribed and quantified RNA by digital PCR. After running three DF tests at each concentration level, the LoD for HRV was determined to be 822 copies/mL.

	LoD of respiratory pathogens assessed with viral particles/RNA					
Flu A concentration (copies/mL)	3.00E+05	1.50E+05	3.00E+04	2.50E+04	1.50E+04	7.50E+03
Positives/Total	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	2/3 (67%)	2/3 (67%)
Flu B concentration (copies/mL)	3.00E+05	1.50E+05	3.00E+04	2.50E+04	1.50E+04	7.50E+03
Positives/Total	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	2/3 (67%)	2/3 (67%)
RSV concentration (copies/mL)	3.00E+05	1.50E+05	3.00E+04	2.50E+04	1.50E+04	7.50E+03
Positives/Total	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	1/3 (33%)	1/3 (33%)
HRV concentration (copies/mL)	9.86E+03	4.93E+03	9.86E+02	8.22E+02	4.93E+02	2.46E+02
Positives/Total	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	1/3 (33%)	1/3 (33%)

Table 20. Flu A, Flu B, RSV and HRV LoD.

Cross-reactivity and cross-contamination test

A total of 49 DF tests were performed, including cross-reactivity and cross-contamination studies. For cross-reactivity, a quantified panel of synthetic DNA/RNA (TwistBioscience, #103730) was used, containing 13

viral respiratory pathogens, including Influenza A H3N2, Influenza A H1N1 (2009), Influenza B, human bocavirus 1, human enterovirus 68, human rhinovirus 89, mumps virus, human parainfluenza, human coronavirus 229E and human coronavirus NL63. Cross-reactivity testing was performed by the addition of synthetic viral RNA into a negative COPAN swab in an eNAT® medium for a final concentration of 10^7 copies/mL. The samples were subsequently extracted through the Sample Preparation Kit. None of the synthetic DNA/RNA resulted in unexpected positive results in the Respiratory Test Panel. In all positive results (Influenza B, Influenza A H3N2 and Influenza A H1N1 (2009)), amplification was due to the presence of pathogens that are targeted by the DF test. It should be noted that for these experiments, the application resulted in an 'invalid' test result, as no human genomic DNA was spiked into these samples, and no RNA target cross-reacted with the human extraction control primers. Furthermore, it is important to note that the three repeats for human rhinovirus 89 (NC_001617.1; HRV-A; as specified in Twist Bioscience Respiratory Virus Control, #103730) were not detected by the HRV assay as it is specific to HRV B.

Cross-contamination experiments were performed on a total of ten consecutive tests alternating expected positive results using a high concentration of the reference pathogen (SARS-CoV-2) and negative samples constituted by eNAT medium.

Cross Contamination of SARS-CoV-2 at 10,000 copies/mL		
Test	Target	Result
1	Sars-CoV-2	POSITIVE
2	Negative	NEGATIVE
3	Sars-CoV-2	POSITIVE
4	Negative	NEGATIVE
5	Sars-CoV-2	POSITIVE
6	Negative	NEGATIVE
7	Sars-CoV-2	POSITIVE
8	Negative	NEGATIVE
9	Sars-CoV-2	POSITIVE
10	Negative	NEGATIVE

Table 21. Cross-reactivity results alternating SARS-CoV-2 spiked samples and negative samples.

Results are shown in Table 21 and demonstrate the ability to work with high concentrated positive samples and negative samples without interfering with each other and showing no sign of cross-contamination.

Reproducibility and whole system failure

The whole system failure rate was examined over five days at 3X LoD for SARS-CoV-2 (1,500 copies/mL), displaying DF’s ability to perform consistently over time, as shown in Table 22. Viral particles SARS-CoV-2 1M TOTAL CONTROL (Vircell, #MC127) were diluted to a maximum of 3X the SARS-CoV-2 LoD (not more than 1500 copies/mL) in COPAN eNAT, made fresh for each time point. The complete end-to-end DF system successfully detected SARS-CoV-2 in 100/100 of the tests performed over five days by independent users, with an accuracy of 100%.

	Whole System Failure				
Replicates of detected SARS-CoV-2	Day 1	Day 2	Day 3	Day 4	Day 5
	20/20 (100%)	20/20 (100%)	20/20 (100%)	20/20 (100%)	20/20 (100%)

Table 22. Whole system failure at 3X LoD of SARS-CoV-2.

Quantified reference viral particles were used to estimate the repeatability of the test. A total of 30 experiments per target (N=150 DF diagnostic tests) at 3X the LoD concentration of each target were used to assess how reproducible the results were. All tests achieved an accuracy of 100% when detecting their respective target at 2X or 3X LoD.

Evaluation of stability study

To assess the robustness of the methodology, all different stability tests were conducted by analysing SARS-CoV-2 specimens (Vircell, #MC127) at 3X LoD (1,500 copies/mL). I will summarise the results obtained below.

Elution stability: To evaluate the stability of the NAs in the elution, experiments were run at 4°, 20° and 30°C at three different time points per temperature (three, ten and 30 minutes). The sample preparation kit elution was stable for at least half an hour, even at the highest temperature tested (30°C).

Stability of lyophilised reagents: To determine the maximum length of time after which the test panel would become unusable due to lyophilised reagent moisture absorption and degradation, lyophilised reagents were exposed to the atmosphere for 30 minutes and the DF test panel performed as expected. However, after 60 minutes of exposure, only two of the three test panels produced a positive SARS-CoV-2 result (67%).

Stability of reagents after rehydration: Reactions were rehydrated and subsequently left at 20°C for two, five and 15 minutes. Results show that is safe to wait up to five minutes after rehydration before loading a panel in the heat block 3/3 (100%). However, accuracy cannot be guaranteed if users wait longer, given the drop to 67% accuracy after 15 minutes of exposure. Furthermore, carrying out the DF test at 4°, 20° and 30°C does not affect the performance of the assay 3/3 (100%).

Interfering substances study

A wide range of substances known to be inhibitory for molecular diagnostics was tested to demonstrate the resilience of the product in detecting SARS-CoV-2 at a concentration of 3x LoD (1,500 copies/mL). The following substances resulted in 3/3 (100%) replicates being detected: ethanol, coffee, coke, throat spray (Benzocaine), nasal spray (Oxymetazoline), and saline nasal spray (sodium chloride). Only mouthwash containing antiseptic substances resulted in one replicate failing to produce a positive SARS COV-2 result (2/3, 66.67%). Table 23 shows the results of the interference substances study.

Substance	Description	Concentration stock	Concentration tested	Volume (µL)	Volume final (µL)	Spiked eNAT volume (µL)	SARS-CoV-2 replicates (3xLOD) detected
Ethanol	N/A	100%	7%v/v	140	2000	1860	3/3 (100%)
Coffee	N/A	N/A	7%v/v	140	2000	1860	3/3 (100%)
Coke	N/A	N/A	7%v/v	140	2000	1860	3/3 (100%)
Throat spray	Benzocaine	0.71%w/v (1mg per spray)	1.7 mg/mL	359.15	2000	1640.85	3/3 (100%)
Nasal spray	Oxymetazoline	0.15mg/mL	15%v/v	300	2000	1700	3/3 (100%)
Saline nasal spray	Sodium chloride	100% natural micro-filtered sea water	15%v/v	300	2000	1700	3/3 (100%)
Mouthwash		N/A	7%v/v	140	2000	1860	2/3 (66.67%)

Table 23. SARS-CoV-2 detection in the presence of inhibitory substances.

Conclusion

This chapter presents the successful integration of the developed sample extraction method into a LAMP-based colourimetric detection approach to reliably detect the presence of a panel of respiratory pathogens at the POC.

As SARS-CoV-2 continues to spread globally at a high rate, the prompt and accurate laboratory detection of infections is essential to guide the control strategies. Therefore, confirmation methods have become a public health policy in all affected countries. The confirmation of cases can be determined using both direct (viral RNA or antigens) and indirect (specific antibodies) methods. SARS-CoV-2 infection can be detected through different methods in clinical settings but the detection of the viral RNA using reverse transcriptase PCR (RT-PCR) remains the gold standard laboratory test for the health authorities. However, the limitations of RT-PCR have been well identified along the thesis, for example the need for skilled personnel and expensive equipment, the long wait time (more than three hours) for results, and limited access to remote areas.

On the other hand, scientific literature and current health guidelines (CDC, WHO) recommend the NA amplification technologies (NAAT) as the main approach for screening and diagnosing infectious respiratory diseases due to their accuracy and quick turnaround result time.

The choice of RT-LAMP as an amplification technique represents an alternative RNA detection method that can overcome the limitations of RT-PCR.

Particularly, the use of the colourimetric LAMP has great potential for POC applications; as demonstrated, this technique only needs lyophilised reaction mixtures containing all reagents necessary to allow a colourimetric amplification to happen. The stability of the reagents at different temperatures combined with the long shelf life of the tests enables its use where needed. The only source of power needed is for the heat block, which will be substituted with rechargeable solutions. An electronic device such as a mobile phone can be employed to follow the instruction steps and send the recorded data directly to a cloud if desired. The power-free sample preparation offering high yield NA extraction contributed to the high sensitivity and specificity when successfully combined with multipathogen LAMP detection of SARS-CoV-2, Influenza A and B, RSV and human rhinovirus RNAs.

In conclusion, the results obtained showed comparable performance of the DF diagnostic platform when compared to PROMate® COVID-19 CE-IVD RT-PCR kit, with a 99.35% (95% CI: 96.46% -99.98%) sensitivity for Ct values < 34.71 (< 800 copies/mL) and 97.35% (95% CI: 93.80% -99.32%) sensitivity including Ct values <37.66 (>120 copies/mL). Of the 435 clinical samples tested, 265 valid negative results were returned by both DF and PROMate RT-qPCR, and no samples were amplified past the 37.66 Ct limit of blank cut-off. Therefore, the calculated specificity for DF was 100.00% (95% CI: 98.62% -100.00%). Precision of measurement experiments show a LoD of 500 copies/mL for SARS-CoV-2, 25,000 copies/mL for Flu A/B and RSV, and 822 copies/mL for HRV.

The clinical validation conducted provided evidence that the DF Respiratory Kit can be used in the diagnostic field, offering precise information about the clinical conditions related to respiratory infections by causative agents such as SARS-CoV-2, Influenza A and B, RSV and human rhinovirus.

Chapter 7

Conclusion and Future Perspective

In Chapter 7, I present the achievements and conclusions of my thesis by bringing together the results and discussions from the previous chapters. I discuss future and ongoing research routes along with limitations and lessons learnt in this PhD thesis. Potential implications in the field and a personal perspective are also discussed.

What this thesis has achieved

Part I

In Chapter 1 I provided an overview of the diagnostic landscape, underlining the existing gaps and limitations associated with current diagnostic tools. The urgent need to develop new methods to provide better diagnostic guidance when it comes to distinguishing between bacterial and viral infections is still there. To date, there is no fast, cheap, robust, molecular method that can simply discriminate between different diseases. A lack of timely diagnosis risks patients receiving inappropriate treatments or wrong diagnosis. Rapid diagnostics can facilitate the process of providing the right treatment to the right patient at the right time.

Among the main classes of diagnostic tools used for the detection of infectious diseases, only NAAT provides a quantitative and accurate output, which is the reason for focusing my PhD on developing diagnostic solutions by employing these technologies. Unfortunately, the available diagnostic methods are not easy to implement as they rely on expensive and complex instruments, all based on proper laboratory environments and limited in being miniaturised into portable devices. RNA-seq, gene expression microarrays, and qPCR are all gold-standard instruments with powerful analysis capabilities, but they can only be used by trained operators and the data extracted is becoming more complex and advanced, presenting further challenges. CMOS ISFET-based technology is showing great potential for employment in the diagnostic field due to its capability to be miniaturised, providing a low-cost, and efficient sensing system to allow molecular reaction detection.

In Part I, I have moved the promising transcript signatures closer to a diagnostic application by studying the translation process of the two-transcript using CMOS ISFET-based platform suitable for implementation as POC technology. The advantage of adapting isothermal chemistries with a label-free detection method based on scalable and inexpensive microchip semiconductor technology is the strategy employed to meet the suggested ASSURED criteria.

Chapter 2 proposes the experimental design of the translation process. This included the experimental conditions to adapt the isothermal chemistry to be compatible with the electrochemical

detection. The optimisation of the LAMP assays was conducted to achieve high sensitivity and specificity of the assay to increase the discrimination power of the classification of the two infectious groups on-chip. A LoD of ten RNA copies was achieved for the two assays within 23 minutes, much faster than using microarrays.

Chapter 3 explored the potential and bottlenecks of translating host-gene expression signature into a POC test using ISFETS for quantitative analysis, and I offered a successful solution to translate the two-gene signature to RT-eLAMP, demonstrating sensitivity and specificity of 100% (95% CI, 96.0%-100%) and 100% (95% CI, 96.0%-100%), respectively, with AUC of 100% (95% CI, 92.2%-100%) discriminating bacterial and viral infections on-chip.

This study also explored new methods to extract TTP values from ISFET arrays to determine a diagnostic outcome, allowing me to learn about some aspects that should be taken into consideration when future studies and improvements are performed using this approach.

Limitations

- Some of the limitations faced are due to the complexity of the operations; therefore, performing complex data analysis from ISFET might not be trivial due to the heavy computational and memory operations, especially when larger arrays are employed. That said, having each sensor analysed independently indeed allows them to be all treated in parallel, yielding the same complexity as the conventional method. However, with advanced computational power these operations may not represent a significant limitation.
- This study included a relatively small sample size, although confidence intervals were used to estimate the upper and lower bounds of the metrics of classification accuracy. A larger number of samples would have improved confidence in confirming these important findings. So, the next step would be to scale up and repeat the study on much larger numbers of children with fever and suspected infection. This would underline eventual challenges when other contexts are considered such as the robustness of the signature when applied to a wider population with geographical heterogeneity or the presence in the samples of endemic underlying co-infections as well as phenotype differences. The use of transcriptomics is expanding across a wide variety of fields and as a researcher considering integrating this technology into my diagnostic research program, I consider of critical importance knowing about the limitations and biases, in addition to the substantial benefits that transcriptomics offers. It is essential to determining what types of experimental questions transcriptomics can be applied to, enhancing experimental designs, and wisely employing significant sample size as well as accurately interpreting transcriptomic data.
- The focus of infections is based on definite bacterial and viral patients, which helped to minimise the effects of data discrepancies and better understand the characteristics of the model to train the algorithm. In the next step, a larger sample size for a randomised patient study would be required to ensure there is no unintended sampling bias. As a matter of fact, it is well known that in clinical practice, extremes of definite bacterial/viral infection are uncommon. A major challenge in the

diagnostic process is the inherent uncertainty associated with diagnosis. In light of this, a diagnosis does not have to be absolutely certain in order for treatment to be initiated. The focus would be on reducing the level of diagnostic uncertainty to make the most effective therapeutic decisions possible. An option that can be explored as a means of addressing clinical uncertainty is by optimising molecular assays to enhance the sensitivity to detect bacterial infections, while ensuring not to miss patients with life-threatening bacterial infection and therefore providing the antibiotic therapy necessary. On a related note, when developing a diagnostic test in absence of perfect reference standards, it would be interesting to see how patients classify when training the algorithm based on a pure statistical model. For instance, latent class models (LCM) may constitute a promising alternative by statistically defining the true disease status. However, the limitation of these statistical model is represented by the complexity of the methodology relying on parametric assumptions, as well as the limitations due to the existing software.

- The clinical samples provided in this study were extracted using a commercial extraction kit; therefore, this study does not include a sample preparation module integrated into the diagnostic platform to extract NAs from whole blood.
- RT-eLAMP experiments presented have been performed in single-well microfluidic manifold while the microarray technique can simultaneously detect or analyse many targets. The work focused on the proof of concept that host-response signatures can be effectively detected using RT-eLAMP, rather than competing with high-throughput platforms. However, I believe it is necessary to increase the number of genes and samples per reaction to detect multiple signatures and allow higher throughput analysis. As a team, we are currently working on the implementation of a larger ISFET array (12-8 mm²) with 59,160 sensors coupled to a 12-well microfluidic manifold.

In conclusion, in part I of my thesis I have demonstrated for the first time a method to discriminate bacterial and viral infections using host-RNA signatures integrated into a LoC technology. I have patented a new method to determine a diagnostic outcome from NA amplification on ISFET, and I have successfully obtained a high correlation between conventional lab-based RT-qLAMP and the ISFET-based LoC platform, yielding 100% accuracy in discriminating viral from bacterial isolates targeting the two-gene signature. Additionally, the entire workflow, from expensive microarray analysis to affordable LoC devices, allowed me to study the translation process to better guide its future implementation in a sample-to-result system.

Part II

With 550,218,992 confirmed cases, including 6,343,783 deaths, reported to the WHO through July 2022, COVID-19 represents one of the most serious challenges we have faced in recent history. Many strategies have been employed across various countries to mitigate the spreading of SARS-CoV-2; strict social restrictions

such as lockdowns and mandatory use of face masks were employed to prevent devastating peaks of hospitalisation from the virus. Some individuals have first been infected with the original strain of the virus, others with the Alpha, Beta, Gamma, Delta or Omicron variant. Others may have never been infected but have developed protection from one, or more doses of different vaccines. Those experiences will likely have an impact on our immune response to future variants of COVID, but it will take time to fully understand what the implications of those combinations are in terms of protection.

Meanwhile, the active testing of symptomatic and asymptomatic people is one of the tools we have available to control the spread of COVID-19, which is continuing to be characterised by this uncontrolled rate of person-to-person transmission, bringing an ongoing increase in cases [209]

Laboratories had to compete with priorities of increased testing volume while managing costs and doing so all in the middle of a workforce shortage of laboratory reagents and professionals. During the whole pandemic, POC diagnostics have been more critical than ever in help identifying and tracking the spread of the disease.

In this pandemic context, my PhD was influenced by the choice to become an active member of the COVID-19 response and **Part II** of the thesis expanded from gene-based detection to direct-pathogen detection using developed methodologies.

Chapter 4 of the thesis presented a clinical validation study performed with the team as part of the COVID-19 response. The detection of SARS-CoV-2 from extracted RNA samples on RT-qLAMP and semiconductor technology was achieved in under 20 minutes. The on-chip detection of SARS-CoV-2 was validated against 183 clinical samples including 127 positive samples (screened by the CDC RT-qPCR assay).

Results showed 90.55% sensitivity and 100% specificity when compared to RT-qPCR and average positive detection times of 15.45 ± 4.43 min. For validating the translation RT-LAMP assay into the POC platform (RT-eLAMP), a subset of samples was tested ($n=40$), showing average detection times of 12.89 ± 2.59 minutes for positive samples ($n=34$), demonstrating a comparable performance to a benchtop commercial instrument. Paired with a smartphone for results visualisation and geo-localisation, the on-chip platform enables real-time case identification and epidemiological surveillance.

Limitations

- The clinical samples provided in this study were already extracted using a commercial extraction kit, and the performance of the analysis when using freeze-thawed RNA decreased compared to a freshly extracted sample. Therefore, it is unknown if the sensitivity percentage could have been higher.
- Similar to the previous study, integrated sample preparation is missing on the on-chip platform; therefore, it can only be considered a detection platform.
- The new manifold containing the two wells reaction represents a step forward compared to the previous one-well manifold. Therefore, a control can be run in parallel to assess the lack of contamination in the reagents. However, both internal control and human control should be included to provide a further confirmation of the validity of the results.

- Although the SEN, SPE and ACC achieved using clinical samples is above 90%, the lower sensitivity of the assay compared to the PCR assay may be tackled from a molecular perspective by optimising the assay design and targeting multiple genes to increase the detection accuracy without compromising the performance of the reaction (e.g., speed, sensitivity, specificity).

To address one of the above-mentioned limitations, **Chapter 5** presents the development of a novel sample preparation methodology to rapidly and cost-effectively extract viral RNA from clinical specimens.

During the ongoing COVID-19 pandemic, the subsequent global shortage of otherwise commonplace laboratory supplies, such as DNA and RNA extraction kits, has necessitated a rapid response by the scientific community, bringing academia, healthcare, and industry together to address the public health emergency. This need was especially felt in LMIC, where even before the current pandemic, access to both manual and automated extraction equipment alike was severely limited.

With all commercially available sample extraction methods requiring electricity and/or expensive benchtop lab equipment, COVID-19 diagnostics in LMIC are rarely achievable at a POC level, and instead relegated to remote centralised laboratories.

To overcome this need, I have co-invented and patented a new extraction method, to bring the sample preparation concept to a new level where no electricity, expensive instruments and reagents, or trained personnel are required to extract NAs from clinical specimens.

The extraction method named SmartLid sample preparation kit utilises a custom 3D-printed magnetic lid, to transfer magnetic nanoparticles and attached RNA through three sample preparation steps.

This contrasts with all other manual extraction methods, which require expensive micropipettes, lab training and electrical power. The whole extraction process is performed within five minutes, providing pure RNA adequate for downstream applications. A total of 410 NPS from positive and negative patients were tested (including 150 COVID-positive subjects). All clinical isolates were extracted by the SmartLid kit and the gold standard QIAmp Viral RNA methods and tested using the CDC RT-qPCR assays. The SL kit method achieved 93.9% sensitivity and 99.5% specificity compared to the column-based QIAmp Viral RNA demonstrating equivalent performance.

Limitations

- While achieving comparable performance to the gold standard, eight samples were not detected using the SmartLid kit. This could be due to the absence of a powered vortexing step replaced by manual shaking that represents a variable among different users.
- Another aspect is the limited choice to have a large elution volume (three to five times larger than standard) that from one side may benefit by decreasing the concentration of potential inhibitors, however causing the dilution of the end purified product, increasing the chance to miss positive results.

- The SmartLid kit proposed is intended to be used in a POC setting. Therefore, the plastic tray would contribute to the increase in plastic waste. In future iterations, it would be beneficial to only use packaging stands made of recycled cardboard.
- The wash buffer made of 80% ethanol loses 20-30% of its volume after three to four months, thus lowering the shelf life of the reagents. Furthermore, international shipping guidelines and aircraft consider it a flammable risk. Future iterations to mitigate these concerns will involve the use of cryovial to decrease the evaporation rate and different wash buffer formulations.

Taken together, these limitations were of valuable importance as they taught me how to optimise the SmartLid kit to make it a commercially available extraction method.

As a conclusion to Part II of the thesis, **Chapter 6** shows how the optimised SmartLid sample preparation kit was employed as the extraction method of a novel proposed POC test based on colourimetric isothermal detection of multiple respiratory pathogens.

The legal COVID restrictions have attempted to minimize the transmission and severity of COVID-19, preventing the spread of infection caused primarily by Delta and Omicron variants. As of July 2022, restrictions appear to have been lifted, however, daily infections have resumed in some countries [155]. COVID-19 is indeed still infecting tens of thousands of people a day and putting thousands in the hospital everywhere. This reinforces the importance of ongoing research into patient care for infectious diseases. Enrolling an active surveillance system based on accurate diagnostic tools is needed to control epidemiological developments.

To overcome this need, the DF POC test was validated with a batch of 435 clinical samples achieving appropriate clinical (diagnostic) sensitivity (or Positive Percent Agreement). DF performance was compared with CE-marked kits in a UKAS accredited laboratory. For Ct values under 34.71 (800 copies/mL), DF showed a sensitivity of 99.35% (95% CI: 96.46% -99.98%), specificity of 100% (95% CI: 98.62% -100%) and accuracy of 99.76% (95% CI: 98.68% -99.99%). For Ct values under 37.66 (120 copies/mL), the calculated sensitivity was 97.35% (95% CI: 93.80% -99.32%), specificity 100.00% (95% CI: 98.62% -100.00%) and accuracy 99.06% (95% CI: 97.62% - 99.74%). Therefore, DF was proven to extract and detect NAs sufficiently to mitigate the risk of false negatives, successfully satisfying the performance requirements to become a commercial product.

Limitations

- Similar to the previous study, SmartLid elution volume was increased to 250uL (>5 times larger than standard) to allow a 20uL elution volume to be dispensed into each of the eight strip tubes. Once again, this represents a limitation of the system that requires big elution volumes to allow resuspension of the lyophilised cakes, causing dilution of the end purified product. A future iteration will involve the decrease of the concentrations of the reagents to allow a smaller elution volume and hence more concentrated NA extracted.

- The optimised wash buffer and use of cryovials allowed to achieve a six-month shelf life guaranteeing safe handling of the ethanol-based solution. However, to obtain a longer shelf-life, ethanol might need to be substituted with solvent-free reagents to prevent the phenomenon of evaporation.
- The DF kit relies on an inexpensive pipette that in combination with tips releases an exact volume of 20uL elution into reagent strips. However, an alternative has not yet been found that will eliminate the need for a micropipette.
- Although the sample preparation process happens in minutes by employing three simple steps, hands-on time is critical when offering a POC solution to minimise the error rate. Therefore, a parallel extraction cartridge is being developed to remove all the manual steps and integrate them into a mechanical device that will miniaturise the sample prep into a microfluidic format and will allow microchip-based detection by bringing back the LoC platform described in Chapter 3 of the thesis.
- The DF test has been validated on nasal, oral and nasopharyngeal clinical samples. However, additional clinical specimens such as urine, blood, saliva, stool, tissues will need to be explored to allow this to become a universal extraction method.

In addition to providing a new option for high purity NA extraction, SmartLid coupled to the colourimetric detection will facilitate the sample to result diagnostic process anywhere with minimal requirements, thereby reducing the need for laboratories.

Establishing the utility of an affordable and accurate POC extraction kit will lead to a shift in handling sample preparation for the diagnosis of infections, inflammatory diseases, and other conditions. I hope this impact will extend beyond the current pandemic to future infectious disease outbreaks, enabling prompt, optimised care, minimising the use of unnecessary treatments such as antibiotics, and instead guiding rapid decisions on the use of antiviral or adjunctive treatments including anti-inflammatory drugs. There is certainly a clear interest in implementing new diagnostic tools this not only in a domestic clinical/laboratory setting (NHS), but also in a remote POC setting in a low- and middle-income country. Ideally suited to both applications in low- and high-resource settings, SmartLid as a standalone or combined with a detection test could represent a step-change in POC molecular testing, anywhere in the world.

Future Directions

The use of host gene expression signatures represents a great potential for the diagnosis of infectious disease, and I believe it might quickly move towards use in clinics. As part of the National Institute of Health NIH project: 5R01AI128765-02, (<https://grantome.com/grant/NIH/R01-AI128765-02>) with the Section of Paediatric Infectious Diseases at St. Mary's huge amount of work have been done on the discovery and validation of a variety of RNA transcripts for discriminating active TB from latent TB infection and other diseases in African settings. So far, microarrays and RNA-seq have successfully addressed the identification of a subset of six differentially expressed genes that discriminate TB versus non-TB patients in children, and I am in the process of develop LAMP assays to translate those transcripts into a new version of the Lacewing platform to be used at the POC.

The new Lacewing platform is a prototype that as a team we are currently working on to implement a larger ISFET array (12-8 mm²) with 59,160 sensors to overcome the limitations due to the use of a two-well manifold and a built-in sample preparation module. The larger array designed by Dr. Moser is coupled to a 12-well microfluidic manifold (designed by M. Cavuto) enabling multipathogen detection by targeting ten different pathogens and two controls. The new Lacewing concept miniaturising the detection platform and incorporating the manual SmartLid extraction into an integrated microfluidic system aims to solve the limitations due to manual extraction and loading steps by fully automating the process with less than one minute of hands-on time.

A novel NA extraction method adapted to extract from multiple specimens (whole blood, saliva, stool, tissue) coupled with optimised LAMP primers deposited directly on-chip will be combined into a test cartridge, designed to generate less waste, with a single test cartridge doing extraction and multiple detections simultaneously.

Although the new system might seem similar to what other POC diagnostic tests propose, it offers some technical advantages such as a smaller and lighter system (<2Kg allowing it to be easily handheld and stackable to save space opposite to the >5Kg offered by other systems), increased functionality with a multiplex/multipathogen detection, lower cost with a cartridge designed to be 50% cheaper than alternative tests and a reader with a predicted cost of <1K compared to the 15-46K presented from the competitor analysis in chapter 6.

As part of its mission to promote the quality of care for vulnerable individuals, the novel approach will be robust enough to provide adequate differential pathogen discrimination for different infectious diseases in order to make it an applicable diagnostic tool in low-resource settings.

Closing Remarks

Pathogen-based detection and host gene-based detection hold great potential when diagnosing infectious diseases in the field. They both have pros and cons but combined they might be an impactful resource in the clinical diagnostic field, especially in LMIC.

While new numbers of transcripts are explored and validated, direct pathogen detection can still prepare the ground for a rapid diagnostic solution that can be translated from the bench to the bedside. The approaches presented in this thesis can be applied to any other infection or condition that is seeking urgent diagnostic solutions. The major advantage of easily implementing the detection of any target from different specimens makes these methods a great potential for clinical management of infectious diseases in any setting.

A simple transcriptome or pathogen-based test that reliably excludes or detects the presence of pathogens such as TB, malaria, cholera or hepatitis from a single drop of a blood sample would add significant value to the diagnostic scenario, and with an acceptable safety and efficacy profile, the test would have an early detection advantage, deploying treatments sooner to help contain the spread of infectious diseases worldwide.

This PhD journey allowed the realisation of a possible diagnostic solution, making it appear a straightforward goal. However, I must acknowledge all the efforts that have been made in several years, especially when discovering and validating new biomarkers on multiple cohorts from various countries worldwide in order to obtain a small set of gene candidates that could remarkably represent a wide range of different conditions.

In addition, I must recognise all the efforts that have been made to enable unprecedented integration of amplification detection using microchip-based technology, in order to translate gene expression signatures into a cost-effective point-of-care diagnostic tools.

In light of this, I believe that the multidisciplinary approach used during my PhD has been an invaluable factor to achieve fruitful milestones by driving diverse perspectives and solutions to real-world problems.

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Appendix

Table S1. PAXgene extracted RNA and concentration in definite viral samples.

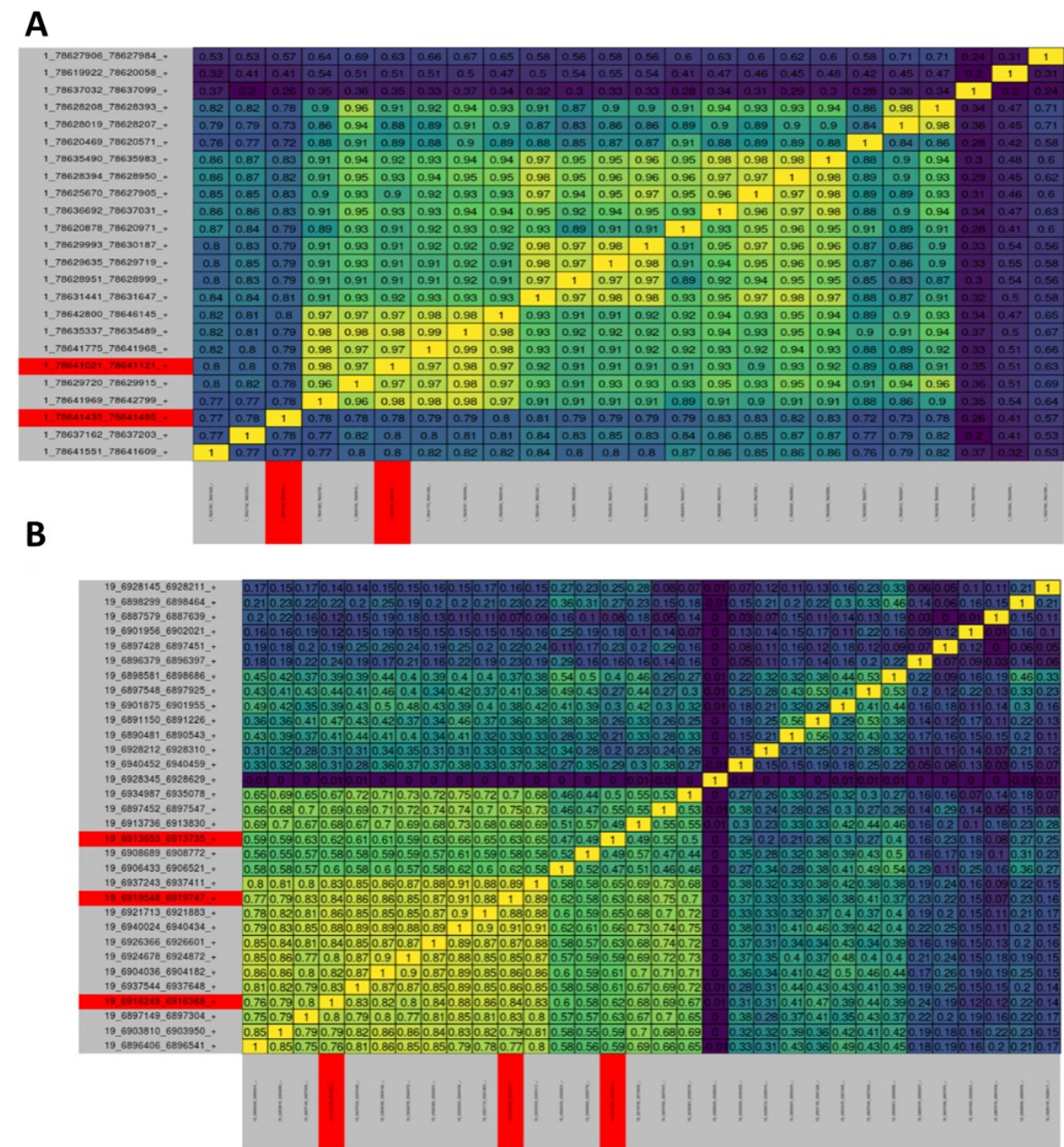
Site-number-episode	Age (months)	Sex	PAXgene extrac. kit type	IFI44-FAM89A DRS score	DRS call	RNA stock ng/ul	260/280	260/230
SMH-299-1	84	Male	mRNA	3.949022413	Bacterial	381.13	2.12	2.03
SMH-91-1	87	Female	mRNA	3.163780468	Bacterial	374.9	2.09	2.03
SMH-109-1	46	Female	mRNA	1.677361544	Bacterial	368.91	2.1	2.06
SMH-288-1	12	Female	mRNA	-2.432024213	Bacterial	338.46	2.14	2.06
SMH-111-1	58	Female	mRNA	2.260474805	Bacterial	304.08	2.1	2.05
SMH-176-1	10	Male	mRNA	1.477119479	Bacterial	256.1	2.11	2.07
SMH-188-1	22	Male	mRNA	-0.036868271	Bacterial	231.33	2.13	2.14
SMH-302-1	24	Female	mRNA	0.020543421	Bacterial	192.07	2.13	1.56
SMH-23-1	59	Female	mRNA	-0.268454514	Bacterial	183.3	2.11	1.95
SMH-122-1	22	Female	mRNA	2.616924067	Bacterial	169.11	2.12	2.04
SMH-11-1	10	Male	mRNA	1.221070599	Bacterial	136.59	2.08	1.41
SMH-290-1	22	Female	mRNA	-0.252993052	Bacterial	129.42	2.14	0.51
SMH-301-1	15	Female	mRNA	1.079695939	Bacterial	116.82	2.11	1.86
SMH-292-1	7	Male	mRNA	-1.122034297	Bacterial	115.91	2.15	1.97
SMH-61-1	25	Female	mRNA	-0.960019995	Bacterial	114.47	2.06	1.85
SMH-116-1	36	Female	mRNA	0.699885241	Bacterial	102.46	2.14	1.18

Table S2. PAXgene extracted RNA and concentration in definite bacterial samples.

Site-number-episode	Age (months)	Sex	PAXgene extrac. kit type	IFI44-FAM89A DRS score	DRS call	RNA stock ng/ul	260/280	260/230
SMH-113-1	63	Male	mRNA	- 5.16759	Viral	67.1	2.09	1.72
SMH-115-1	13	Female	mRNA	-2.9698	Viral	73.91	2.11	0.68
SMH-121-1	24	Male	mRNA	-4.9608	Viral	40.83	2.03	0.97
SMH-133-1	16	Male	mRNA	- 4.46682	Viral	184.7	2.13	1.5
SMH-143-1	10	Male	mRNA	- 5.09973	Viral	119.26	2.1	1.99
SMH-18-1	6	Female	mRNA	- 3.28713	Viral	23.16	1.95	1.2
SMH-32-1	102	Female	mRNA	- 4.18583	Viral	79.45	2.11	1.97
SMH-47-1	57	Male	mRNA	- 4.59235	Viral	49	2.05	1.91
SMH-50-1	172	Male	mRNA	- 5.49762	Viral	98.7	2.11	1.75
SMH-51-1	29	Female	mRNA	- 4.04263	Viral	103.82	2.11	1.47
SMH-52-1	33	Female	mRNA	- 5.41639	Viral	102.72	2.09	1.63
SMH-55-1	49	Male	mRNA	- 5.44295	Viral	47.81	2.03	1.79

SMH-68-1	162	Male	mRNA	-4.63768	Viral	80.6	2.08	1.95
SMH-73-1	6	Female	mRNA	-4.04693	Viral	96.99	2.11	1.92
SMH-80-1	48	Female	mRNA	-4.0122	Viral	108.11	2.06	1.68
SMH-95-1	7	Male	mRNA	-4.19738	Viral	50.85	2.1	0.46

Figure S1. Heatmap showing correlation of the exons counts for IFI44L (A) and EMR1 (B).



The matrix shows the correlation between the exons (underlined in red on the column) and the primers (underlined in red on the row). The values are indicative of the correlation between exon counts. The primer span and the exon-exon boundary are between the exons. Each splice junction perfectly correlates with the respective primer, showing an AUC of 1 indicated in yellow.

Table S3. Microarray and dPCR values for clinical samples when targeting *IFI44L*, *EMR1* and *PPIB*.

Gene	Clinical samples	Digital estimated count Fluidigm	Microarray expression level
<i>PPIB</i>	18	1874	9.59
	47	120	55.77
	51	2327	48.32

	95	222	43
	113	101	31
	32	136	29
	55	135	38
	52	271	30
	50	125	17
	115	305	37
	80	433	113
	68	258	57
	73	328	19
	121	221	28
	143	387	54
	133	608	27
	11	32	69.32
	23	1277	40.77
	111	1272	62.26
	122	867	35
	109	886	12
	301	359	29
	292	206	31
	288	1165	43
	188	1063	44
	302	682	67
	299	772	11
	61	724	47
	91	1958	187
	116	721	54
	176	1840	88
	290	411	39
	100	214	32
	172	327	36
	177	353	50
	184	495	11
	186	646	70
	210	453	43
	74	127	21.65
EMR	18	240	95.18
	47	92	252.31
	51	1933	118.18
	95	63	89
	113	182	317
	32	34	92
	55	60	127
	52	261	168
	50	63	27
	115	85	227
	80	85	108
	68	177	154
	73	294	162

	121	144	184
	143	107	72
	133	304	90
	11	182	404.13
	23	437	98.91
	111	183	1261.18
	122	435	356
	109	2053	543
	301	456	401
	292	409	468
	288	696	185
	188	1100	371
	302	691	412
	299	2145	582
	61	107	24
	91	2327	1268
	116	2011	703
	176	2327	1138
	290	1095	727
	100	190	143
	172	118	77
	177	101	59
	184	380	22
	186	146	62
	210	225	69
	74	361	52.61
IFI44L	18	619	2872.63
	47	397	6511.32
	51	1434	5073.45
	95	146	5731
	113	557	10436
	32	240	4940
	55	430	10741
	52	1156	10127
	50	714	3913
	115	103	4523
	80	321	7190
	68	379	5850
	73	808	6447
	121	340	10958
	143	1100	7106
	133	1256	2675
	11	1226	78.77
	23	816	320.31
	111	639	34.86
	122	13	125
	109	628	1216
	301	67	575
	292	206	2345

	288	1337	2441
	188	555	939
	302	334	2132
	299	7	9
	61	70	472
	91	47	78
	116	12	77
	176	114	196
	290	445	3656
	100	21	263
	172	77	645
	177	22	237
	184	52	124
	186	20	179
	210	24	273
	74	433	292.93

Table S4. LAMP primer sets developed and tested in this thesis.

PPIB LAMP Primer mixes								
ID:1		dimer(minimum)dG=-2.18						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	200	218	19	59.29	-4.85	-6.69	0.58	GGACTTCATGATCCAGGGC
B3	389	408	20	60.89	-5.05	-4.67	0.55	CCACATGCTTGCCATCTAGC
FIP			40					GAAGTTCTCATCGGGGAAGCGC- GGAGACTTCACCAGGGGA
BIP			40					CTGGGTGAGCATGGCCAACG- GCTGTCTTGACTGTCGTGAT
F2	219	236	18	59.4	-5.2	-6.04	0.61	GGAGACTTCACCAGGGGA
F1c	269	290	22	65.33	-4.01	-6.92	0.59	GAAGTTCTCATCGGGGAAGCGC
B2	366	385	20	59.42	-5.75	-5.23	0.5	GCTGTCTTGACTGTCGTGAT
B1c	314	333	20	65.69	-5.84	-5.94	0.65	CTGGGTGAGCATGGCCAACG
LF	237	257	21	62.7	-5.09	-5.75	0.57	GCTCTTTCCTCCTGTGCCATC
LB	334	352	19	60.82	-6.25	-5.94	0.58	CAGGCAAAGACACCAACGG
ID:4		dimer(minimum)dG=-1.18						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	210	227	18	60.17	-4.74	-4.36	0.61	ATCCAGGGCGGAGACTTC
B3	389	408	20	60.89	-5.05	-4.67	0.55	CCACATGCTTGCCATCTAGC
FIP			40					GGCCCGTAGTGCTTCAGTTTGA- GGAGATGGCACAGGAGGA
BIP			40					CTGGGTGAGCATGGCCAACG- GCTGTCTTGACTGTCGTGAT
F2	234	251	18	59.84	-4.59	-5.55	0.61	GGAGATGGCACAGGAGGA
F1c	289	310	22	64.79	-7.97	-4.18	0.55	GGCCCGTAGTGCTTCAGTTTGA
B2	366	385	20	59.42	-5.75	-5.23	0.5	GCTGTCTTGACTGTCGTGAT
B1c	314	333	20	65.69	-5.84	-5.94	0.65	CTGGGTGAGCATGGCCAACG
LF	252	271	20	61.42	-6.43	-5.09	0.55	CGCTACCGTAGATGCTCTT
LB	334	352	19	60.82	-6.25	-5.94	0.58	CAGGCAAAGACACCAACGG

ID:7		dimer(minimum)dG=-2.00						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	246	265	20	59.05	-5.55	-5.35	0.55	GGAGGAAAGAGCATCTACGG
B3	445	462	18	60.38	-6.41	-5.3	0.61	TGGTGCTCTCCACCTTCC
FIP			38					GTGTCTTTGCCTGCGTTGGC-CGCTTCCCCGATGAGAAC
BIP			40					TCACGACAGTCAAGACAGCCTG-CCACCTCCATGCCCTCTA
F2	270	287	18	59.3	-6.03	-4.36	0.61	CGCTTCCCCGATGAGAAC
F1c	327	346	20	64.44	-4.9	-6.01	0.6	GTGTCTTTGCCTGCGTTGGC
B2	424	441	18	59.57	-5.84	-4.27	0.61	CCACCTCCATGCCCTCTA
B1c	367	388	22	64.11	-5.6	-6.08	0.55	TCACGACAGTCAAGACAGCCTG
LB	389	408	20	60.89	-4.67	-5.05	0.55	GCTAGATGGCAAGCATGTGG

<i>EMR1 LAMP Primer mixes</i>								
ID:1		dimer(minimum)dG=-2.16						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	37	56	20	60.21	-5.5	-4.59	0.55	CCACAGTCTTCTGGAGAGT
B3	243	262	20	59.25	-5.5	-4.36	0.55	CCAGTGGTCTCTGTGGATTC
FIP			41					CGTCCGAACAGCCGGAGTGATA-TGGAAAGCATGACACTGGC
BIP			42					TGTGACGTTGGACTTGGTAGCC-CCTCAATTGTGGAACACCCG
F2	58	76	19	60.01	-4.53	-6.24	0.53	TGGAAAGCATGACACTGGC
F1c	101	122	22	65.93	-6.96	-3.64	0.59	CGTCCGAACAGCCGGAGTGATA
B2	218	237	20	60.69	-4.86	-6.78	0.55	CCTCAATTGTGGAACACCCG
B1c	176	197	22	64.46	-5.07	-5.42	0.55	TGTGACGTTGGACTTGGTAGCC
LF	77	98	22	60.33	-5.66	-3.4	0.41	TGCTGAGGGTTTCAAAAAGAT
ID:4		dimer(minimum)dG=-1.74						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	194	213	20	59.92	-5.75	-4.15	0.5	AGCCAAGGGGGATAAGATGA
B3	394	413	20	59.5	-7.03	-5.19	0.55	GCCGTCTTTCTTCTCTCCAG
FIP			42					AGGAGACAAAAGCCACACCACTG-CGGGTGTTCCACAATTGAGG
BIP			40					CTTCAAAGACCACAGGCTCCC-TATGCCCCAACGACTCG
F2	218	237	20	60.69	-6.78	-4.86	0.55	CGGGTGTTCCACAATTGAGG
F1c	258	279	22	64.07	-4.94	-5.39	0.5	AGGAGACAAAAGCCACACCACTG
B2	372	389	18	60.75	-4.98	-5.53	0.61	TATGCCCCAACGACTCG
B1c	314	335	22	64.94	-4.02	-6.54	0.59	CTTCAAAGACCACAGGCTCCC
LB	336	357	22	61.8	-5.02	-5.26	0.5	TTGACCACCTCTGAGATCAAGC

<i>GAPDH LAMP Primer mixes</i>								
1	ID:1		dimer(minimum)dG=-1.67					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	386	405	20	59.52	-5.18	-4.41	0.5	AGAACGGGAAGCTTGTCATC
B3	575	592	18	59.74	-5.35	-4.4	0.61	CGAACATGGGGGCATCAG

FIP			40					GACGTA	CTCAGCGCCAGCAT-ATCTTCCAGGAGCGAGATCC
BIP			39					GCGTCTT	CACCACCATGGAGA-CAGAGGGGGCAGAGATGA
F2	424	443	20	60.45	-3.75	-4.59	0.55	ATCTTCC	AGGAGCGAGATCC
F1c	464	483	20	64.41	-4.92	-5.29	0.6	GACGTA	CTCAGCGCCAGCAT
B2	557	574	18	59.53	-5.19	-4.15	0.61	CAGAGG	GGGCAGAGATGA
B1c	497	517	21	64.12	-6.42	-5.11	0.57	GCGTCTT	CACCACCATGGAGA
LF	444	463	20	61.06	-7.97	-4.86	0.55	CGCCCC	ACTTGATTTTGGAG
LB	518	535	18	62.22	-6.08	-4.56	0.61	AGGCTG	GGGCTCATTTGC
2	ID:7		dimer(minimum)dG=-1.11						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence	
F3	715	734	20	60.83	-5.2	-5.25	0.55	GGACTCA	TGACCACAGTCCA
B3	863	880	18	59.55	-5.7	-5.59	0.61	GCTTCC	CGTTCCAGCTCAG
FIP			38					CGGCCAT	CACGCCACAGTTT-CCATCACTGCCACCCAGA
BIP			40					CGGGGCT	CTCCAGAACATCATC-GATGACCTTGCCACAGC
F2	737	754	18	60.78	-4.91	-5.7	0.61	CCATCA	CTGCCACCCAGA
F1c	778	797	20	65.57	-7.53	-4.16	0.6	CGGCCAT	CACGCCACAGTTT
B2	844	861	18	59.97	-4.41	-5.9	0.61	GATGAC	CTTGCCACAGC
B1c	798	819	22	64.48	-7.97	-3.8	0.59	CGGGGCT	CTCCAGAACATCATC
3	ID:10		dimer(minimum)dG=-2.16						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence	
F3	907	924	18	59.35	-6.24	-4.91	0.56	ACTGCC	AACGTGTCAGTG
B3	1073	1090	18	59.52	-6.57	-5.7	0.61	CAGCGT	CAAAGGTGGAGG
FIP			38					ACGCCT	GCTTACCACCTTC-GACCTGACCTGCCGTCTA
BIP			39					AAGGGC	ATCCTGGGCTACACT-AGTGGGTGTCGCTGTTGA
F2	928	945	18	59.67	-5.35	-4.76	0.61	GACCTG	ACCTGCCGTCTA
F1c	975	994	20	64.74	-6.91	-4.85	0.6	ACGCCT	GCTTACCACCTTC
B2	1055	1072	18	60.52	-5.84	-4.58	0.56	AGTGGG	TGTCGCTGTTGA
B1c	1009	1029	21	65.49	-6.19	-4.13	0.57	AAGGGC	ATCCTGGGCTACACT
LB	1030	1048	19	62.85	-5.75	-4.94	0.63	GAGCAC	CAGGTGGTCTCCT
4	ID:13		dimer(minimum)dG=-2.33						
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence	
F3	1153	1170	18	59.57	-5.42	-5.84	0.61	GGCTAC	AGCAACAGGGTG
B3	1322	1340	19	59.91	-4.86	-5.8	0.53	TCAAGG	GGTCTACATGGCA
FIP			38					TTGTGCT	CTTGCTGGGGCTG-GGACCTCATGGCCACAT
BIP			40					ACCCTA	CTGCTGGGGAGTC-GTGAGGAGGGGAGATTCA
F2	1173	1190	18	60.94	-5.69	-5.05	0.61	GGACCT	CATGGCCACAT
F1c	1221	1240	20	65.13	-5.57	-6.69	0.6	TTGTGCT	CTTGCTGGGGCTG
B2	1299	1318	20	60.06	-5.35	-4.41	0.55	GTGAGG	AGGGGAGATTCA
B1c	1254	1273	20	65.43	-5.69	-5.2	0.65	ACCCTA	CTGCTGGGGAGTC
LF	1191	1210	20	61.26	-4.85	-6.54	0.6	GGTCTT	ACTCCTTGAGGCC

LB	1278	1297	20	65.68	-5.66	-6.01	0.65	CCACACTCAGTCCCCACCA
5	ID:3		dimer(minimum)dG=-2.12					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	463	480	18	59.63	-5.14	-4.08	0.61	GATGCTGGCGCTGAGTAC
B3	653	671	19	60.91	-4.42	-6.41	0.58	GCTAAGCAGTTGGTGGTGC
FIP			40					CTTTTGGCTCCCCCTGCAAAT-GGAGTCCACTGGCGTCTT
BIP			41					TCTGCTGATGCCCCATGTTC-GGAGGCATTGCTGATGATCT
F2	486	503	18	60.63	-5.2	-5.18	0.61	GGAGTCCACTGGCGTCTT
F1c	530	551	22	65.71	-3.77	-4.56	0.55	CTTTTGGCTCCCCCTGCAAAT
B2	632	651	20	59.47	-6.54	-4.15	0.5	GGAGGCATTGCTGATGATCT
B1c	571	591	21	64.66	-5.49	-4.06	0.57	TCTGCTGATGCCCCATGTTC
LF	504	522	19	61.2	-5.58	-5.66	0.58	AGCCTTCTCCATGGTGGTG
LB	592	612	21	60.54	-4.56	-4.4	0.52	GTCATGGGTGTGAACCATGAG

IFI44L LAMP Primer mixes								
1	ID:10		dimer(minimum)dG=-2.18					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	21	38	18	56.15	-4.9	-4.46	0.5	GACTGTGCATGGATGACA
B3	212	231	20	56.75	-4.41	-4.68	0.4	TGAAGTGTCTTCACTTTTGC
FIP			45					AGGTGTAATTGTTTACGGGAATTA-CCCCACATCTTAAAAGTTG
BIP			47					TCCATCTCTGAAGGACAGGATT-CAACATTTTAGAGTAGAGATTGTCA
F2	41	60	20	55.74	-6.45	-5	0.45	CCCCACATCTTAAAAGTTG
F1c	82	106	25	60.38	-5.39	-2.75	0.36	AGGTGTAATTGTTTACGGGAATTA
B2	187	211	25	56.72	-4.21	-4.58	0.32	CAACATTTTAGAGTAGAGATTGTCA
B1c	130	151	22	60.28	-4.76	-4.24	0.45	TCCATCTCTGAAGGACAGGATT
LB	152	175	24	60.93	-5.05	-3.59	0.46	CACTGTGTGGCTTATGTCTTAGAC
2	ID:70		dimer(minimum)dG=-1.16					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	258	276	19	55.27	-2.85	-4.58	0.47	CATATGTGGCCTTGCTTAC
B3	450	468	19	56.83	-5.8	-4.27	0.47	AGTGCAGAGAGGATGAGAA
FIP			44					CGGCTTTGAGAAGTCATAGATCTAC-GTGGATGATTGCAGTGAGG
BIP			43					ATGTCCATAAAATGCTAGGCATTCC-TTCATGGGGTCCAGTTCC
F2	281	299	19	57.5	-4.9	-5.35	0.53	GTGGATGATTGCAGTGAGG
F1c	327	351	25	60.62	-6.52	-3.47	0.44	CGGCTTTGAGAAGTCATAGATCTAC
B2	427	444	18	57.95	-4.07	-4.85	0.56	TTCATGGGGTCCAGTTCC
B1c	360	384	25	61.56	-4.9	-4.51	0.4	ATGTCCATAAAATGCTAGGCATTCC
LB	398	422	25	61.55	-4.07	-4.35	0.4	TTGATGGTTGGAAACTATGCTTCAG
ID:38			dimer(minimum)dG=-2.28					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	78	100	23	56.51	-3.24	-4.16	0.3	AGTTTAATTCCTGTAACCAATT
B3	271	292	22	56.97	-4.91	-4.58	0.41	GCAATCATCCACTTTAGTAAGC
FIP			45					ATAAGCCACACAGTGAATCCTG-CTGAGCATTCTACTTTATCACC

BIP			44					AATGTTGGCAAAAGTGAAGCAAG-GCCACATATGCTATACCACAG
F2	105	127	23	56.65	-5.59	-4.9	0.39	CTGAGCATTCTACTTTTATCACC
F1c	145	166	22	60.12	-3.97	-4.74	0.45	ATAAGCCACACAGTGAATCCTG
B2	247	267	21	57.81	-6.41	-5.5	0.48	GCCACATATGCTATACCACAG
B1c	205	227	23	60.73	-3.71	-5.24	0.39	AATGTTGGCAAAAGTGAAGCAAG
4	ID:135		dimer(minimum)dG=-2.16					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	506	525	20	56.05	-3.67	-4.62	0.45	GATTTCCTCTTGAGGAAAC
B3	672	690	19	55.96	-4.9	-4.41	0.47	GACTGTTCCAAGGACAGAA
FIP			40					TGGGCCAAATGTCAGAATCAAG-TGGTGCAATTGAGAGAGC
BIP			39					GTACTGGTGTGCCGCAATGA-GGCAAAACGAACAAAAGCA
F2	526	543	18	56.47	-6.41	-5.44	0.5	TGGTGCAATTGAGAGAGC
F1c	573	594	22	60.94	-7.2	-3.9	0.45	TGGGCCAAATGTCAGAATCAAG
B2	653	671	19	57.11	-5.52	-4.91	0.42	GGCAAAACGAACAAAAGCA
B1c	599	618	20	62.54	-4.23	-4.07	0.55	GTACTGGTGTGCCGCAATGA
LF	550	572	23	62.32	-5.4	-6.69	0.48	GCAACTTATCTCAAATGCAGGGC
LB	619	642	24	60.55	-4.76	-6.08	0.46	GAGTCAATCTCTATTGACAGCCTG
5	ID:1		dimer(minimum)dG=-2.33					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	10	27	18	57.53	-4.69	-5.05	0.56	AGAAGGAGCAGGACTGTG
B3	206	224	19	56.28	-5.26	-4.21	0.42	GCTTCACTTTTGCCAACAT
FIP			44					AGGTGTAATTGTTTACGGGAATTA-GGATGACATTCCTCCACATC
BIP			48					TTATCACCTCTCCATCTCTGAAGG-GTAGAGATTGTCAATAGAGTTGAT
F2	31	49	19	56.97	-4.76	-4.56	0.53	GGATGACATTCCTCCACATC
F1c	82	106	25	60.38	-5.39	-2.75	0.36	AGGTGTAATTGTTTACGGGAATTA
B2	176	199	24	55.58	-3.92	-4.06	0.33	GTAGAGATTGTCAATAGAGTTGAT
B1c	120	143	24	60.99	-3.15	-4.86	0.46	TTATCACCTCTCCATCTCTGAAGG
LF	53	77	25	60.7	-2.55	-4.5	0.4	GATATCTGTCTGGCATACAACCTTT
LB	144	165	22	61.5	-5.25	-4.93	0.45	ACAGGATTCAGTGTGTGGCTTA

<i>Fam89A</i> LAMP Primer mixes								
1	ID:22		dimer(minimum)dG=-2.08					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	605	624	20	60.58	-5.08	-5.37	0.5	AGGGATGTGACTGTTGGGAA
B3	803	821	19	59.26	-5.63	-4.27	0.58	ACTGCTGAGGACCTCTAGG
FIP			41					ACAACGGAGGTGCTTTCTTGCA-CCTTCTACTGGACACGCT
BIP			39					TAGGCAGGGAGTTCTCCGCG-GCAGAACTGCTGCCATCA
F2	627	645	19	60.44	-5.3	-6.57	0.58	CCTTCTACTGGACACGCT
F1c	667	688	22	65.01	-5.49	-5.41	0.5	ACAACGGAGGTGCTTTCTTGCA
B2	776	794	19	60.33	-5.26	-4.91	0.53	GCAGAACTGCTGCCATCA
B1c	734	753	20	65.57	-5.33	-7.71	0.65	TAGGCAGGGAGTTCTCCGCG
LB	757	774	18	62.07	-5.9	-5.49	0.61	GCATGGTGGCAGTCTGCT

2	ID:15		dimer(minimum)dG=-1.67					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	598	616	19	59.25	-4.86	-4.9	0.53	TCTTGGGAGGGATGTGACT
B3	776	794	19	60.33	-5.26	-4.91	0.53	GCAGAACTGCTGCCATCA
FIP			41					ACGGAGGTGCTTTCTTGCAAGA-GTTGGGAAGCCCTTCCTAC
BIP			38					GCACAGGGGATGAGCGCTAC-AAGCAGACTGCCACCATG
F2	617	635	19	59.16	-5.61	-4.43	0.58	GTTGGGAAGCCCTTCCTAC
F1c	664	685	22	64.53	-6.02	-5.26	0.5	ACGGAGGTGCTTTCTTGCAAGA
B2	758	775	18	59.27	-5.24	-5.05	0.56	AAGCAGACTGCCACCATG
B1c	702	721	20	64.85	-5.9	-5.75	0.65	GCACAGGGGATGAGCGCTAC
LF	636	656	21	60.73	-3.82	-5.42	0.48	CAAATGATGACAGCGTGCCA
LB	722	743	22	60.34	-4.16	-5.7	0.45	CAGTTTCATTGTAGGCAGGGA
3	ID:30		dimer(minimum)dG=-2.46					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	634	652	19	60.66	-5.25	-4.32	0.53	ACTGGACACGCTGTCATCA
B3	803	821	19	59.26	-5.63	-4.27	0.58	ACTGCTGAGGACCTCTAGG
FIP			41					CTGGTAGCGCTCATCCCCTGT-TCTTGCAAGAAAGCACCTCC
BIP			39					TAGGCAGGGAGTTCTCCGCG-GCAGAACTGCTGCCATCA
F2	664	683	20	60.07	-5.26	-5.69	0.5	TCTTGCAAGAAAGCACCTCC
F1c	704	724	21	65.85	-4.58	-5.84	0.62	CTGGTAGCGCTCATCCCCTGT
B2	776	794	19	60.33	-5.26	-4.91	0.53	GCAGAACTGCTGCCATCA
B1c	734	753	20	65.57	-5.33	-7.71	0.65	TAGGCAGGGAGTTCTCCGCG
LF	685	701	17	60.14	-6.47	-5.17	0.65	CCGAGGACCGTCCACAA
LB	754	772	19	60.43	-5.31	-4.74	0.58	GATGCATGGTGGCAGTCTG
4	ID:8		dimer(minimum)dG=-1.89					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	455	474	20	59.86	-6.52	-4.69	0.55	CGGCTTCTTCGATGAAGAGG
B3	642	660	19	59.32	-6.65	-6.57	0.53	GCAGCAAATGATGACAGCG
FIP			40					GAGACAGGCAGTGACAAAGTCCC-CAGAACTCCCTGCACGAC
BIP			40					CTCTCCAGCAGCGACTGGATTC-GGAAGGGCTTCCCAACAG
F2	492	509	18	59.15	-4.51	-5.84	0.61	CAGAACTCCCTGCACGAC
F1c	532	553	22	64.52	-4.76	-5.69	0.59	GAGACAGGCAGTGACAAAGTCCC
B2	615	632	18	59.31	-5.3	-4.66	0.61	GGAAGGGCTTCCCAACAG
B1c	558	579	22	64.58	-5.04	-4.36	0.59	CTCTCCAGCAGCGACTGGATTC
LB	580	600	21	60.91	-5.25	-5.69	0.52	TGGAGTCCATCTAGAGGGTCT
5	ID:2		dimer(minimum)dG=-2.17					
label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	368	387	20	59.26	-4.13	-5.57	0.5	ACTGTACAGCCTCTACGAGT
B3	569	588	20	60.55	-5.25	-5.68	0.55	TGGACTCCAGAATCCAGTCG
FIP			41					CGTTCTCCAGAGCGTAAGTGCA-CAGGAGTACAAGGGGGCAT
BIP			41					CAGAACTCCCTGCACGACAGG-GAGACAGGCAGTGACAAAGTC

F2	393	411	19	60.65	-5.19	-6.24	0.58	CAGGAGTACAAGGGGGCAT
F1c	435	456	22	64.38	-5.18	-5.8	0.55	CGTTCTCCAGAGCGTAAGTGCA
B2	534	553	20	59.7	-4.76	-4.51	0.55	GAGACAGGCAGTGACAAGTC
B1c	492	512	21	64.58	-4.51	-5.35	0.62	CAGAACTCCCTGCACGACAGG

LAMP primer sets developed and tested for each of the bacterial, viral and reference genes screened.

Table S5. Classification of the bacterial-viral signatures in Herberg and Pennisi.

IFI44L – FAM89A Herberg		IFI44L – EMR1 Herberg		IFI44L – EMR1 Pennisi	
(95% CI)		(95% CI)		(95% CI)	
AUC	80%		82.1%		97.2%
	(72.6 – 87.4)		(75.7 – 88.6)		(95 – 100)

The AUC of the Herberg 2016 JAMA signature in that cohort was 80.0% (72.6 – 87.4). The IFI44L – EMR1 signature showed an AUC of 82.1% (75.7 – 88.6), showing consistency and slight improvement [127].

The Pennisi IFI44L and EMR1 combination had a similar performance to the original two-transcript signature in Herberg (AUC in the training, test, and validation data sets: 93.4%, 97.4%, and 97.2%, respectively).

Table S6. LAMP primer sets developed by Dr. Rodriguez-Manzano team for the detection of the respiratory panel in DF.

Assay	Primer name	Protocol name	Sequence	Cf (uM) per test	Ci (uM)	Vi (uL)	Vf (uL)
CC	IC_B3	IC_B3	GTTTGCATGTAGATAGCGAAT	5.5	400	0.14	10
	IC_BIP	IC_BIP	AGCAATACATTACACATCAGACACATTTTATCCGTAATTTACGTCGCA	5.5	400	0.14	10
FluA	FluA	FluA-F3	GACTTGAARATGTCTTTGC	0.5	400	0.01	10
	FluA	FluA-B3	AGTTTAACTGCTYTRCCATGT	0.5	400	0.01	10
	FluA	FluA-LF	GTCTTGCTTTTAGCCA	2	400	0.05	10
	FluA	FluA-LB	CAGTGAGCGAGGACTG	2	400	0.05	10
	FluA	FluA-FIP	TTAGTCAGAGGTGACAGGATTGCGATCTTGAGGTTCTC	4	400	0.10	10
	FluA	FluA-BIP	TTGTGTTACGCTCACCCTGTTTGGACAAAGCGTCTACG	4	400	0.10	10
CoV	CoV	CoV1-F3	TTGTGATCAACTCCGCGAA	0.5	400	0.01	10
	CoV	CoV1-B3	TCAATTAATTTGTCATCTTCGTCCT	0.5	400	0.01	10
	CoV	CoV1-LF	CTGCACTTACACCGGAAAC	2	400	0.05	10
	CoV	CoV1-LB	ATGATAAGTAGCTGGTTTTGCT	2	400	0.05	10
	CoV	CoV1-FIP	TCAGTACTAGTGCCTGTGCCGTCAGCTGATGCACAAATCGTT	4	400	0.10	10
	CoV	CoV1-BIP	TGTCGTATACAGGCGCTTTTGACATCTTGAAGCGACAAATAGT	4	400	0.10	10
FluB1	FluB1	FluB1-F3	GCAACCAATGCCACYATA	0.5	400	0.01	10
	FluB1	FluB1-B3	AATTGCTTTTCTCTCTCAAGA	0.5	400	0.01	10
	FluB1	FluB1-LF	TGAAARYCTTTTCATAGCAC	2	400	0.05	10
	FluB1	FluB1-LB	CAAGAATAAAGACTCCACAAC	2	400	0.05	10
	FluB1	FluB1-FIP	TAGTCAAGGGCGYCTTTGCCACTTGAAGCAGGAATTTCTGGA	4	400	0.10	10
	FluB1	FluB1-BIP	CAAGACCGCTAMACAGACTAAACATCCTTTTAYTYTCAGGGTCAC	4	400	0.10	10
FluB2	FluB2	FluB2-F3	AGGGACATGAACAACAAAGA	0.5	400	0.01	10
	FluB3	FluB2-B3	CAAGTTTAGCAACAAGCCT	0.5	400	0.01	10
	FluB4	FluB2-LF	TYAAACGGAACTTCCTCTCTTTTC	2	400	0.05	10
	FluB5	FluB2-LB	GGATACAAGTCCTTATYAACCTGCG	2	400	0.05	10
	FluB6	FluB2-FIP	TMARGGACAATACATTACGCATATCGATAAAGGAGGAAGTAAACACTCA	4	400	0.10	10
	FluB7	FluB2-BIP	TAAAYGGAACATTCTCAACACCACCTCTGGTCATAGGCATTC	4	400	0.10	10
RSVA	RSVA	RSVA-F3	GGGGCAATATGGAAACATACGT	0.5	400	0.01	10
	RSVA	RSVA-B3	GAAGGTCATTGGGTGTG	0.5	400	0.01	10
	RSVA	RSVA-LF	TTGTATTGAACAGCAGCTGTGT	2	400	0.05	10
	RSVA	RSVA-LB	CATCCATGCCAGCAGATTTACTTATA	2	400	0.05	10
	RSVA	RSVA-FIP	AGGGTCATCGCTTTTTTCTAAGACATTTTTTTCACGAAGGCTCCACAT	4	400	0.10	10
	RSVA	RSVA-BIP	ATCACTTACAATATGGGTGCCCTTTGTATGTTGACATTAGCTAGTTCT	4	400	0.10	10
RSVB	RSVB	RSVB-F3	GATGGGGCAATATGGAAC	0.5	400	0.01	10
	RSVB	RSVB-B3	CCTTTGGGCGTAGAGATC	0.5	400	0.01	10
	RSVB	RSVB-LF	GTA CTGAACAGCTGCTGTGTAT	2	400	0.05	10
	RSVB	RSVB-LB	CAGACTTGCTCATAAAGAACTTGC	2	400	0.05	10
	RSVB	RSVB-FIP	GTTAGTGATGCAGGATCATCATCTTTTTTGAACAAGCTTCACGAAGG	4	400	0.10	10
	RSVB	RSVB-BIP	TATGGGTGCCATGTTCCAGTTTTTGCTTCACTAGTATGTTGATGCT	4	400	0.10	10
HRV	HRV	HRV_F3	TAGTYGGTCGATGAGGCT	0.5	400	0.01	10
	HRV	HRV_B3	CRGAATTRCTCVTTAYGACC	0.5	400	0.01	10
	HRV	HRV_LB	TTGTGANTCCTCCGGCCCCCT	2	400	0.05	10
	HRV	HRV_FIP	CCCCACGGYRACCGTGTGGCCGCCACGARGCT	4	400	0.10	10
	HRV	HRV_BIP	GGTGTGAAGACYRCATGTGCTGGGTTWRGGTTAGCCGCAT	4	400	0.10	10
Human	Human	Human-F3	GCGCGGCTACAGCTTCA	0.5	400	0.01	10
	Human	Human-B3	GGAAGAGTGCCTCAGGCG	0.5	400	0.01	10
	Human	Human-LF	AGCTTCTCCTTAATGTCACGC	2	400	0.05	10
	Human	Human-LB	CGAGCTGCCTGACGG	2	400	0.05	10
	Human	Human-FIP	TTGCTCGAAGTCCAGGGCGACGAGCGGGAATCGTGC	4	400	0.10	10
	Human	Human-BIP	TGCTTCCAGCTCCTCCCTGATTGCCAATGGTGATGACCT	4	400	0.10	10
IC	IC	IC_F3	ATTCATTGACCTCCAGC	0.5	400	0.01	10
	IC	IC_B3	GTTTGCATGTAGATAGCGAAT	0.5	400	0.01	10
	IC	IC_LF	CAAGATTAGGCAGATGCCTAAGAG	2	400	0.05	10
	IC	IC_FIP	AAGAACAGGCGCTGTTAGGATTGTTTATCATGATGAAACTTCGGTTC	4	400	0.10	10
	IC	IC_BIP	AGCAATACATTACACATCAGACACATTTTATCCGTAATTTACGTCGCA	4	400	0.10	10
Name	Specie	ID	Sequence	Ci (copies/uL)	Cf (copies/uL)	Vi (uL)	Vf (uL)
IC template	S. Scrufa	IC_template	ACGATTCTTGGCCTCCACTTTATCCTGCCATTCATACCGCCCTCG	1.00E+05	1.00E+03	0.20	20