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Investigation of real-time colorimetric LAMP method for the rapid detection of Influenza A and SARS-CoV-2

MASTER THESIS

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Abbreviations

LAMP: loop-mediated isothermal amplification method

qRT-PCR: quantitative real-time RT-PCR

qLAMP: quantitative LAMP

qcLAMP: quantitative colorimetric LAMP

RT-LAMP: Real-time LAMP

FIP: forward inner primer

BIP: backward inner primer

F3: forward outer primer

B3: backward outer primer

LF: loop forward primer

LB: loop backward primer

dsDNA: double-stranded DNA

CV: Crystal violet

LCV: Leuco crystal violet

HNB: Hydroxyl-Naphthol-Blue

PPase: pyrophosphatase enzyme

RNAase: Ribonuclease enzyme

POC: point-of-care

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

VTM: viral transport media

RNase P: human Ribonuclease P gene

NEB: New England Biolabs

Ct: cycle threshold

Abstract

The demand for efficient diagnostic test for detection of pathogens has been increasing lately. This need has led to the development of effective diagnostic tests, with the usage of nucleic acid molecular detection methods. Most of the methods that are used, require expensive and bench-top laboratory equipment. The ultimate goal of diagnostic tests is to be applied at point of care systems. Creating rapid, affordable and accurate diagnostic test is very important for the point of care. Loop-mediated isothermal amplification method (LAMP) can contribute to the development of rapid test for the detection of pathogens.

The objective of this study was the enhancement, the reduction of the detection time of the real-time quantitative colorimetric LAMP method and the application of it in clinical samples of Influenza A and SARS-CoV-2. To achieve the improvement of the method, two different parameters were evaluated: the colorimetric dye and the polymerase enzyme. Also, a portable new device (IRIS) was used for the development of real-time quantitative colorimetric LAMP.

The colorimetric dye that was used for the detection of LAMP products was the first element that was evaluated. Three different dyes were used for the detection, Hydroxyl-Naphthol-Blue (HNB), phenol red and crystal violet. Each dye change color in a different way when the DNA amplification takes place. More specifically, the color change of HNB dye depends on the concentration of the Mg^{2+} in the solution, phenol red dye changes color with the change of pH and of the crystal violet changes color when it binds to DNA products. Comparing the sensitivity in color changes of each dye, it was confirmed that they didn't have any difference in their performance as far as it concerns the sensitivity in color change. All the dyes could change color just as rapidly when there was the production of amplicons. The second parameter that was evaluated was the performance of the polymerase enzyme. Six different polymerase enzymes were used for the experiments, Bst 2.0, Bst 2.0 with master mix, Bst 3.0 from NEB, Bst 2.0 from Jena Bioscience, Bst from SBS Genetech Co and Bsm from Thermo Scientific. The best time-to-positive results in LAMP reaction was with Bst polymerase from SBS Genetech Co. This polymerase was able to reduce the time-to-positive results of the experiment at approximately 14 min, defining it the fastest amplification of all the other polymerases.

All the experiments were performed with the addition of Influenza A and SARS-CoV-2 as template. The template of Influenza A was DNA and of SARS-CoV-2 was RNA. The method was able to define the limit of detection of both targets. Real-time quantitative colorimetric LAMP was able to detect up to 1-10 copies per µL in the reaction. Moreover, during validation studies, the results showed 97.4% and 100% agreement with qRT-PCR for SARS-CoV-2 RNA detection extracted from positive and negative patients' samples (89), respectively. Furthermore, the method has the ability to operate with crude clinical samples like saliva. Finally, accomplishing the reduction of the detection time in real-time quantitative colorimetric LAMP, can provide a rapid and accurate solution for point-of care diagnostic methods.

Περίληψη

Τα τελευταία χρόνια ολοένα και αυξάνεται η ζήτηση για διαγνωστικά τεστ. Η ανάγκη αυτή οδήγησε στην δημιουργία αποτελεσματικών διαγνωστικών τεστ με τη χρήση μεθόδων μοριακής ανίχνευσης νουκλεϊκών οξέων. Οι περισσότερες από τις μεθόδους που χρησιμοποιούνται, απαιτούν ακριβό και κορυφαίο εργαστηριακό εξοπλισμό. Ο απώτερος στόχος των διαγνωστικών τεστ είναι να εφαρμοστούν στο σημείο (παροχής) φροντίδας. Η δημιουργία μιας ταχείας, προσιτής και ακριβής διαγνωστικής εξέτασης είναι πολύ σημαντική την εφαρμογή της στο σημείο (παροχής) φροντίδας. Η μέθοδος ισοθερμικής ενίσχυσης νουκλεϊκών οξέων μέσω δημιουργίας βρόχων (LAMP) μπορεί να συμβάλει στην ανάπτυξη γρήγορων τεστ για την ανίχνευση παθογόνων.

Ο στόχος αυτής της μελέτης ήταν η ενίσχυση, η μείωση του χρόνου ανίχνευσης της μεθόδου ποσοτικής χρωματομετρικής LAMP σε πραγματικό χρόνο και η εφαρμογή της σε κλινικά δείγματα των ιών InfluenzaA και SARS-CoV-2. Για να επιτευχθεί η βελτίωση της μεθόδου, αξιολογήθηκαν δύο διαφορετικές παράμετροι: η χρωματομετρική χρωστική και το ένζυμο πολυμεράση που χρησιμοποιείται στην αντίδραση. Επίσης, μια νέα φορητή συσκευή (IRIS) χρησιμοποιήθηκε για την πραγματοποίηση της ποσοτικής χρωματομετρικής αντίδρασης LAMP σε πραγματικό χρόνο.

Η χρωματομετρική χρωστική που χρησιμοποιήθηκε για την ανίχνευση προϊόντων LAMP ήταν το πρώτο στοιχείο που αξιολογήθηκε. Τρεις διαφορετικές χρωστικές χρησιμοποιήθηκαν για την ανίχνευση των προϊόντων, η HNB, phenol red και crystal violet. Κάθε χρωστική αλλάζει χρώμα με διαφορετικό τρόπο όταν γίνεται η ενίσχυση του DNA. Συγκεκριμένα, η αλλαγή χρώματος της χρωστικής HNB εξαρτάται από τη συγκέντρωση του Mg^{2+} στο διάλυμα, η χρωστική phenol red αλλάζει χρώμα βάση του pH της αντίδρασης, ενώ η χρωστική crystal violet αλλάζει χρώμα όταν συνδέεται με προϊόντα DNA. Συγκρίνοντας την ευαισθησία στις αλλαγές χρώματος κάθε χρωστικής, επιβεβαιώθηκε από τα πειράματα ότι δεν είχαν καμία διαφορά στην απόδοσή τους όσον αφορά την ευαισθησία στην αλλαγή χρώματος. Όλες οι χρωστικές μπορούσαν να αλλάξουν χρώμα εξίσου γρήγορα όταν γινόταν η ενίσχυση του DNA. Η δεύτερη παράμετρος που αξιολογήθηκε ήταν η απόδοση του ενζύμου πολυμεράση. Έξι διαφορετικά ένζυμα πολυμεράσης χρησιμοποιήθηκαν για τα πειράματα, Bst 2.0, Bst 2.0 με master mix, Bst 3.0 από NEB, Bst 2.0 από την Jena Bioscience, Bst από την SBS Genetech Co και Bsm από την Thermo Scientific. Η Bst πολυμεράση από την SBS Genetech Co. έδωσε τα καλύτερα αποτελέσματα καθώς κατάφερε να κάνει πιο γρήγορη την αντίδραση, μειώνοντας τον χρόνο ανίχνευσης του DNA στα περίπου 14 λεπτά, ορίζοντάς την πολυμεράση με την ταχύτερη δράση και ικανότητα ενίσχυσης του DNA μεταξύ όλων των άλλων πολυμερασών που χρησιμοποιήθηκαν στην αντίδραση.

Όλα τα πειράματα πραγματοποιήθηκαν με την προσθήκη Influenza A και SARS-CoV-2 ως πρότυπο. Το γενετικό υλικό που χρησιμοποιήθηκε της Influenza A ήταν DNA και του SARS-CoV-2 ήταν RNA. Η μέθοδος μπόρεσε να καθορίσει το όριο ανίχνευσης και

των δύο στόχων. Η LAMP ήταν σε θέση να ανιχνεύσει έως και 1-10 αντίγραφα ανά μL σε κάθε αντίδραση. Επιπλέον, τα αποτελέσματα των πειραμάτων έδειξαν 97,4% και 100% συμφωνία με τα αποτελέσματα όταν χρησιμοποιήθηκε qRT-PCR για ανίχνευση RNA από SARS-CoV-2 το οποίο προέρχεται από εκχύλιση από δείγματα θετικών και αρνητικών ασθενών (89) με COVID-19, αντίστοιχα. Επιπλέον, αποδείχθηκε ότι η μέθοδος έχει τη δυνατότητα να λειτουργεί με ακατέργαστα κλινικά δείγματα όπως το σάλιο. Τέλος, η επίτευξη της μείωσης του χρόνου ανίχνευσης της ποσοτικής χρωματομετρικής LAMP σε πραγματικό χρόνο, μπορεί να προσφέρει μια γρήγορη και ακριβή λύση στις τις διαγνωστικές μεθόδους στο σημείο (παροχής) φροντίδας.

1. Introduction

1.1 Loop-mediated isothermal amplification (LAMP)

Recently, loop-mediated isothermal amplification (LAMP) has gaining in popularity among other amplification techniques and more specific in scientific fields like clinical diagnostics, food testing and point of care studies. It is used as an alternative to the widely used Polymerase chain reaction (PCR)-based detection [1]. During the last decade and especially this year, due to the coronavirus disease 2019 (COVID-19) which is a contagious respiratory and vascular disease that caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2], LAMP is used widely in many studies and applications for the detection of pathogens like viruses and bacteria.

Loop-mediated isothermal amplification (LAMP) is a DNA synthesis method that depends on auto-cycling strand displacement DNA. It employs a DNA polymerase with high strand displacement activity and a set of four specially designed primers, two inner and two outer. These four primers are used to amplify six different regions on the target gene. An additional pair of primers that are called “loop primers” also participate in the reaction. The inner and outer primers increase the specificity of the reaction and the loop primers can later accelerate it [3]. The inner primers are called the forward inner primers (FIP) and the backward inner primers (BIP) and the two outer primers are called B3 and the F3, respectively [4]. The first step of the amplification includes the combined action of the two inner and the two outer primers. The primers consecutively bind to ssDNA and the polymerase continues the polymerization. This step leads to a structure that is called “dumbbell-like structure” which has one stem-loop in each end (Figure 1). After that, the loop primer continues the DNA synthesis by hybridized to the stem-loop region. The cycling amplification step and the elongation step follow next. The formations that are created, contain alternately inverted repeats of the target gene. Using the loop primers, the final products that are formed, are a mixture of stem-loop DNAs with different lengths and cauliflower-like structures (Figure 2). Although, the LAMP reaction can be performed only with four primers, (recognize six distinct recognition sequences) the usage of the loop primers can rise the sensitivity and the speed of the reaction.

The amplification occurs in less than an hour, reducing accordingly the time of the experiment. Moreover, the method requires a DNA polymerase like Bst and Bsm polymerase which is thermostable and has great tolerance at a specific temperature. In LAMP reaction, the polymerase is able to amplify a single copy up to 109 copies within 20-45 min [5, 6] which is considerably less than the required time for a similar PCR experiment. The main property of the LAMP reaction is that it needs isothermal conditions (60-65°C). This characteristic can provide the opportunity to avoid complex experimental equipment and furthermore gives the opportunity for point-of-care (POC) systems.

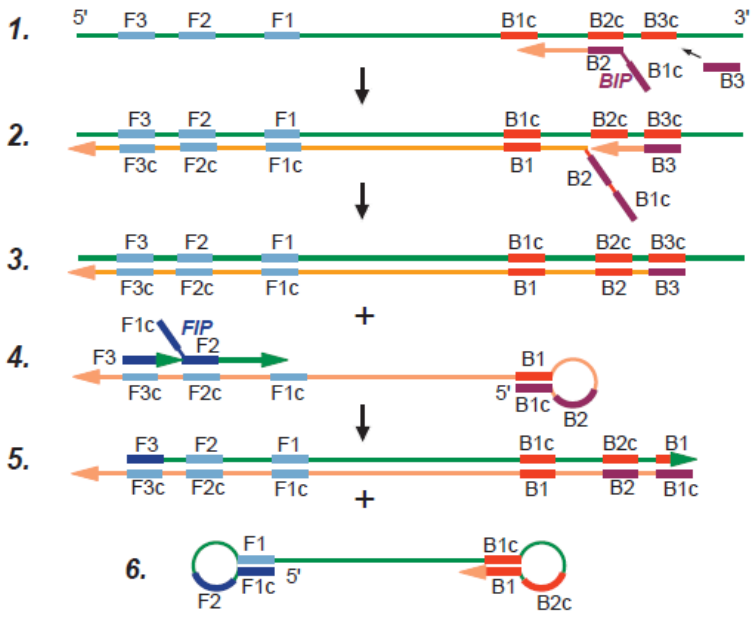
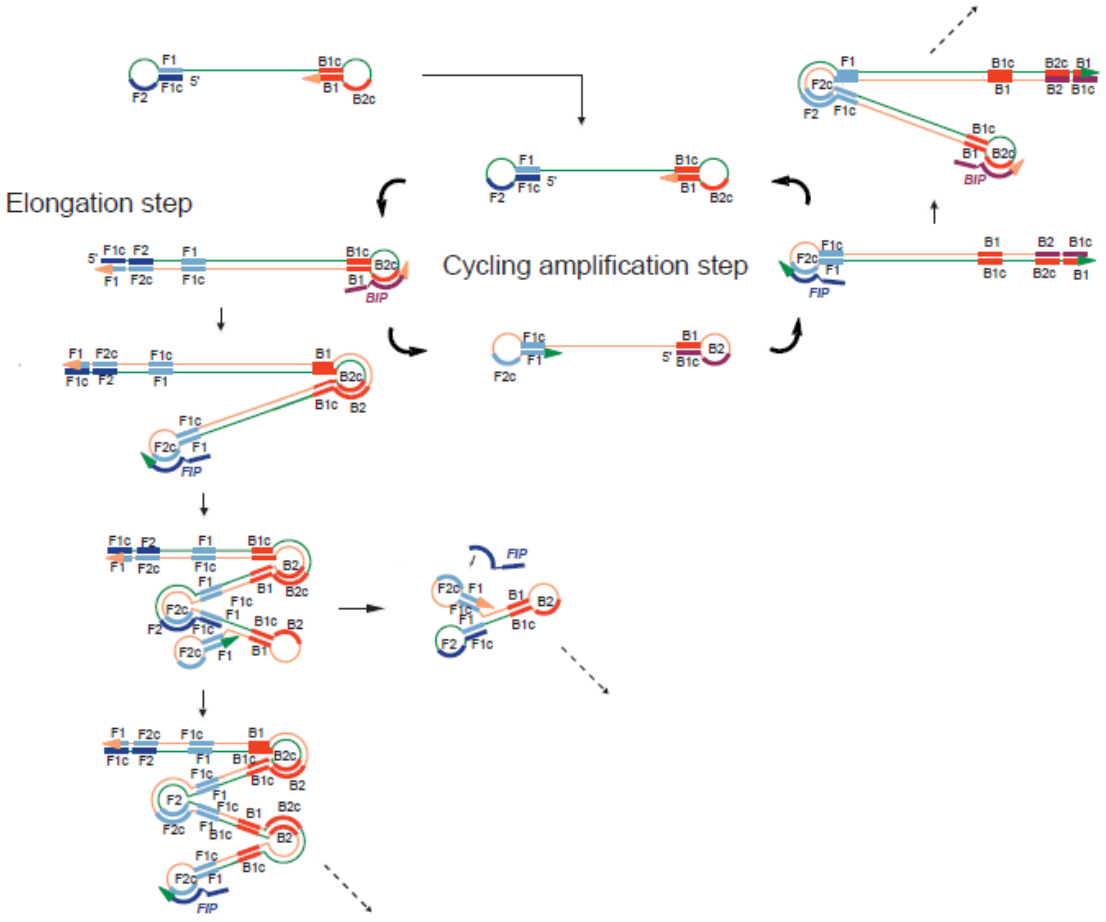


Figure 1: Schematic representation of the formation of the amplified structure with the stem-loop in each end. (1) Primer extension via BIP. (2, 3) Strand displacement extension by B3. (4, 5) In the displaced strand, the extension from FIP and then the displacement by F3. (6) The displaced single strand forms "dumbbell-like structure" [3].



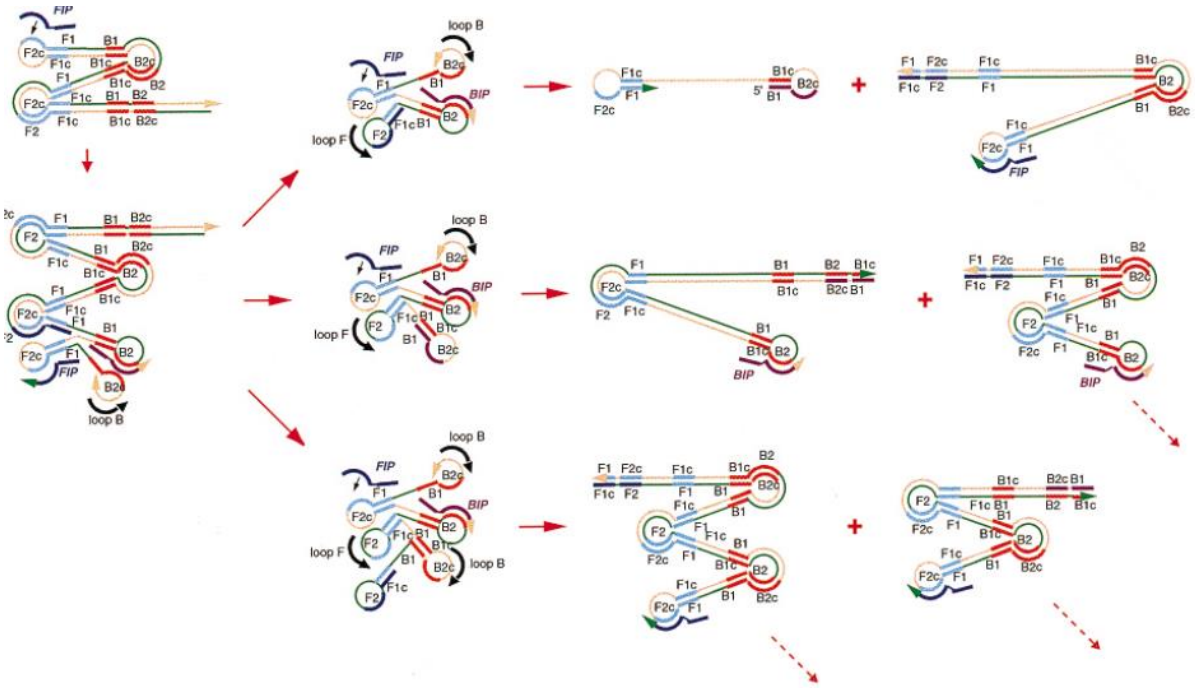


Figure 2: Schematic representation of the LAMP mechanism using loop primers and the formation of cauliflower-like structures [3].

1.2 Colorimetric dyes

Many laboratories prefer to run experiments that can offer real-time results/measurements. Specifically, real-time measurements can provide quantitative information about the product accumulation in the sample. Real-time LAMP reaction can be used for quantification.

There are many ways to validate a positive LAMP reaction. The detection of LAMP products can be accomplished with visualization methods by the naked eye or via simple photometric detection, with agarose gel electrophoresis or with pH-sensing mechanisms [5] (Figure 3). For instance, a photometer can detect the amplified product by measuring the turbidity caused by magnesium pyrophosphate precipitate in solution as a byproduct of the amplification [6].

Other common colorimetric detection methods for quantitative amplification include fluorescent dyes which can detect the LAMP products with UV radiation or by naked eye. There are several types of fluorescent dyes. The most common ones that are used for the detection are the following: specific probes [7], intercalating dyes, and calcein. Determined by the color range of their emission maxima, the most common green fluorescent dyes are SYBR Green I, EvaGreen, PicoGreen, and calcein, and the most common orange fluorescent dyes are SYTOX-orange and all SYTO dyes. Comparing the other dyes, only calcein does not intercalate to the DNA. The color changes due to the reduction of the free calcium ions in the solution when they associate with pyrophosphate ions that released from dNTPs during DNA amplification [8].

Other colorimetric methods include nanoparticle probes. The main characteristic of the nanoparticles, is that changing their size or the interaction between other substances, affects their color. More precisely, the color appears in the spectrum of visible light and it can be visually read out with unaided eyes to identify the positive or negative sensing process without any instrument. The most common used nanoparticles are the gold nanoparticles (AuNPs) [9] and the polydiacetylenes (PDAs) [10]. These two types of nanoparticles can change color after binding with recognition unit, and the aggregation that is caused from the binding with the amplified DNA, results in an apparent color change, which can be attributed to the size and distance dependent optical properties of these particles [11].

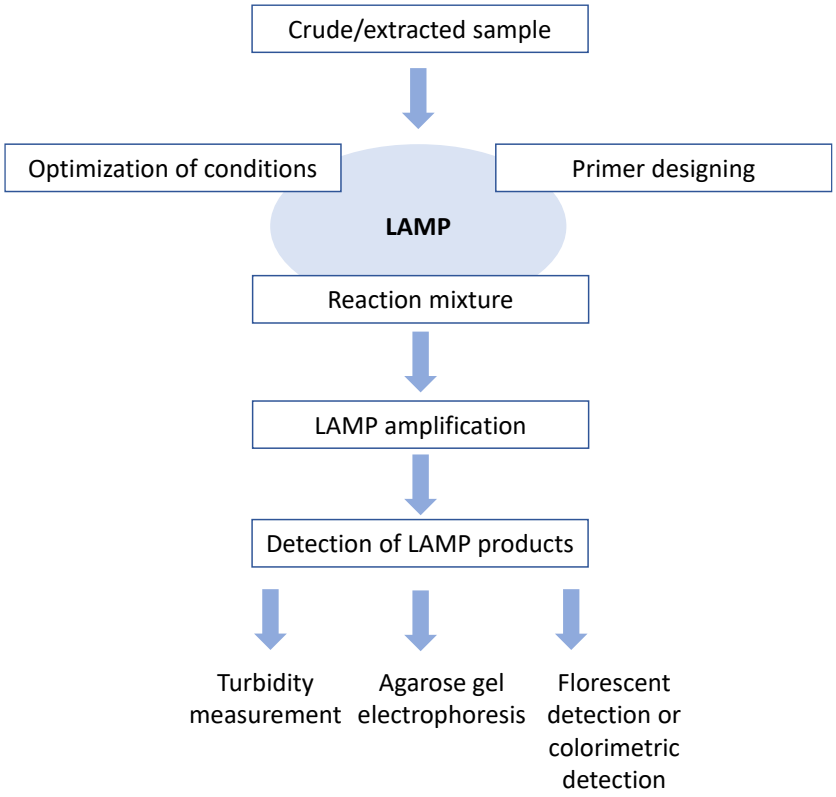


Figure 3: *Schematic representation of LAMP assay.*

This study focuses on organic colorimetric substances which change color rapidly when they are added to a LAMP reaction. The color change can be discriminated by naked eye or with a real-time colorimetric device. The colorimetric dyes that were used in all the experiments are HNB, phenol red and crystal violet.

1.2.1 HNB

HNB or Hydroxyl-Naphthol-Blue is an azo dye (Figure 4A). It is a metal colorimetric indicator for alkaline earth metal ion, rare earth metal ions, and uranium ions. In a LAMP assay, it is used as an indicator for magnesium Mg^{2+} and Ca^{2+}, and it acts as a titration process in the solution. The color of the solution changes from violet to sky blue when a positive reaction is indicated (Fig.4C). The concentration of the Mg^{2+}

changes as the large fragment of DNA polymerase synthesizes DNA under alkaline conditions (pH 8.8 at 25°C). More specifically, while the LAMP reaction takes place, pyrophosphates are generated as the primer is extended. Mg^{2+} ions have strong affinity for the derivable pyrophosphates. As a consequence, Mg^{2+} ions are subtracted by the released pyrophosphates in the initial Mg-HNB complexes and the $Mg_2P_2O_7$ complexes are precipitated. Under these circumstances, the initial HNB structure with Mg^{2+} ions changes, consequently changing the color of the solution from violet to sky blue [12]. Also, the pH of the solution can alter the color of HNB [13].

1.2.2 Phenol red

Phenol red or phenolsulfonphthalein is a triphenylmethane dye (Figure 4B). It is acid-base indicator and the structure gives rise to different equilibria according to pH. It can change color from yellow, when pH is below 6.8, to bright pink-fuchsia when pH is above 8.2. In solution with acidic conditions (low pH), the ketone group carrying an additional proton as there is protonation. Under more basic conditions, the proton from the ketone group is lost and this causes the solution to turn fuchsia (Fig.4D) [14]. Most of the times phenol red is more preferable for LAMP reaction because it can provide a clear distinction between positive and negative samples unlike many of the metal-sensitive indicators that can be difficult to distinguish by eye. In real-time LAMP reaction, this large color difference is very important for the sensitivity of the method, as it allows the discrimination of positive samples that have low concentration of amplified products by naked eye. Moreover, due to this color property, it is used more and more in colorimetric diagnostics test for pathogens, as it can simplify the detection process [15].

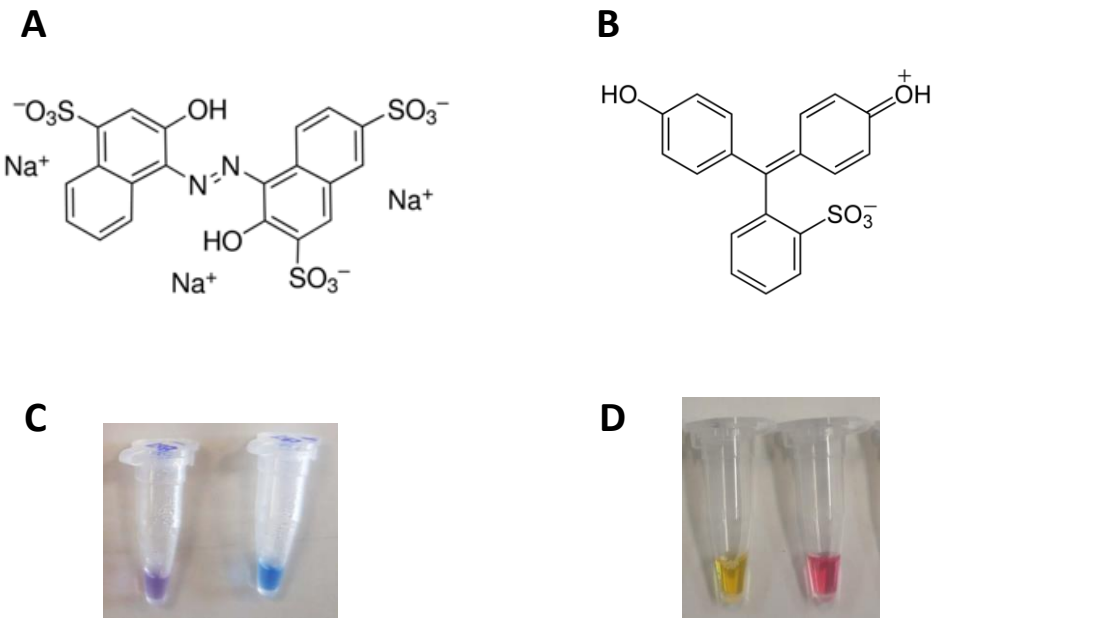


Figure 4: (A) HNB structure, (B) Phenol red structure, (C) Negative sample (violet) and positive sample (sky blue), (D) Negative sample (fuchsia) and positive sample (yellow), when HNB or phenol red dye is used, respectively.

1.2.3 Crystal violet

Crystal violet (CV) is another triphenylmethane dye. It is derivative from a tautomeric and defined by a pH-dependent equilibrium that exists between the triphenylmethyl cation and its quinoidal form [14]. The quinoid structure in aqueous solution exhibits with a violet color. Changing the structure of the molecule by adding to the center carbon a substituent, the conjugated system collapses and it is converted into the colorless leuco crystal violet (LCV) substance. As the equilibrium is reversible, LCV can be reconverted to colored CV in the presence of a color developer or in response to thermal stimulation (Figure 5). For instance, the colored CV can be converted into colorless LCV by sodium sulfite (Na_2SO_3) treatment. Adding excess amount of sodium sulfite to the solution, it push the balance toward the LCV side of the equilibrium by adding to the center carbon of the CV a sulfite ion [16].

Recently, many studies showed that LCV is a suitable dye for colorimetric detection method of LAMP reaction due to the simplicity and the excellent in visibility that contributes to the method. CV enables the attachment of the major groove of dsDNA to the central carbon. The complex that is created is stabilized by the electrostatic interaction of the negative electric charge of the phosphate group of dsDNA and the positive charge on the quinoid ring of CV. As a consequence of the creation of the complex CV-dsDNA, resistance against the nucleophilic attack of a sulfite ion is increased because of the steric hindrance and electrostatic interactions which appear. For that reason, the conversion of CV into LCV, and so the discoloration of the solution, is prevented. In this way, the presence of dsDNA in the LAMP reaction can turn the solution from colorless to violet [17]. Figure 5 presents the suggested mechanism of the colorimetric detection.

Furthermore, as many new methods are tested for enhancing the detection of the LAMP method, crystal violet offers a new colorimetric approach for creation diagnostics test for pathogens [18]. As CV interacts directly with the nucleic acids and does not depend on secondary parameters such as pH and ions concentrations, it is possible to reduce the required detection time of the LAMP products. This is a major incident for creating rapid colorimetric LAMP assays especially for point-of-care tests. Util now, many colorimetric protocols are using CV as a colorimetric dye. However, these methods are based at the end-point detection methods. Paper based detection methods with LC is also used in molecular diagnostic field [19]. In this study it was achieved the creation of a real-time LAMP method, using CV as a colorimetric dye.

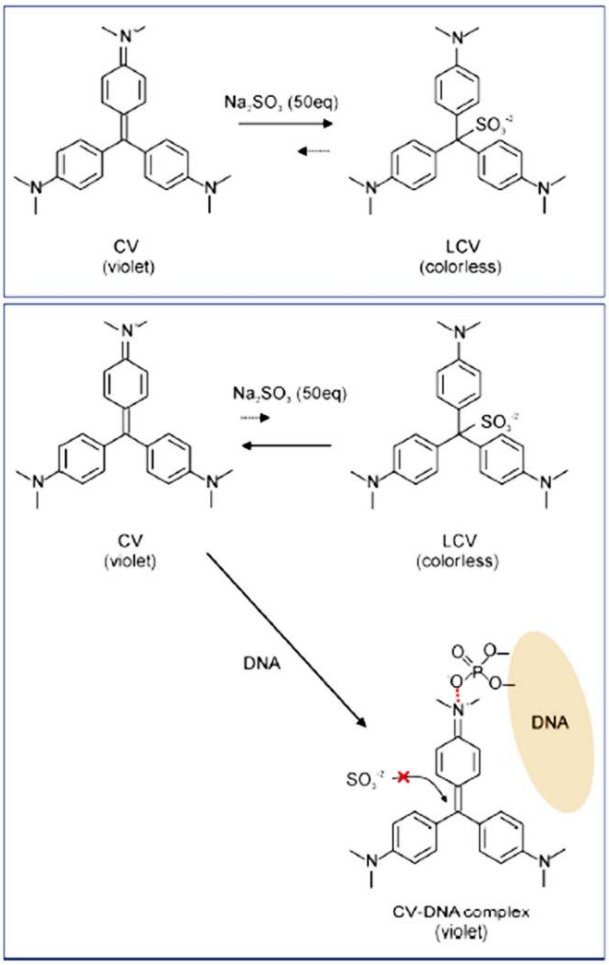


Figure 5: *Proposed dsDNA sensing mechanism of the colorimetric assay with crystal violet [17].*

1.3 Point-of-care diagnostic method with LAMP

Among the various isothermal amplification methods that have been developed for diagnostic tools, especially for in-field or point-of-care analyses including rolling circle amplification (RCA) [20], nucleic acid sequence-based amplification (NASBA) [21], the loop-mediated isothermal amplification (LAMP) method, has several major advantages such as high tolerance for inhibitors and the dependence on isothermal reaction conditions. Also, the sensitivity of the method is high as it is able to detect very low concentrations of gene targets and amplify them up to 10^9 times. Due to the above, it is useful not only for fast laboratory diagnostics but also for medical applications such as point-of-care (POC) testing where, often it is not possible to obtain purified nucleic acid samples [22]. Moreover, due to the fact that the amplification take place at isothermal conditions, enables the amplification of nucleic acids without the need for expensive laboratory equipment such as thermocyclers. LAMP requires only a heat source, like a heater or a water bath. In these settings point-of-care (POC) tests may provide an alternative and viable solution for rapid diagnosis. Many scientific teams have develop methods easy to handle like paper based diagnostic assays [23] and assays that are using home equipment and tools [24].

Portable devices are also have been developed, with or without external power sources, allowing the application of diagnostic test outside of laboratories [25, 26]. To simplify even more the POC methods, recently the creation of easy handling applications for smartphones and tabled devices are used in combination with POC tests [26, 27]. On the contrary, a disadvantage of POC tools is that the sensitivity can be reduced when the method is an end-point detection, due to contamination of the rest samples (especially negative samples) with LAMP products. However, this problem it can be easily avoided with real-time LAMP where the sample is never opened. Also, point-of-care test with LAMP shouldn't be a time-consuming process especially with diagnostics tools. It is necessary to reduce even more the detection time in POC methods, to less than 30 min, so it can offer diagnostic results in a shorter timeframe and also reduce the cost of the experiment. Real-time colorimetric LAMP can contribute to the time reduction, creating rapid point-of-care diagnostics. Finally, employing more complex equipment and specific reagents enable LAMP reaction to be performed in a quantitative real-time format: quantitative LAMP (qLAMP).

1.4 Clinical applications

Considering the simplicity, easy operation, no need for special equipment, superior sensitivity and speed, low contamination risk, and suitability for high-throughput DNA and RNA detection, POC tests are potentially attractive in diagnostic field. Therefore, colorimetric assays are suitable not only for laboratory research but also for clinical diagnoses of many infectious diseases. Additionally, reverse transcription loop-mediated isothermal amplification is a one-step nucleic acid amplification method to multiply specific sequences of RNA. This technique is used to diagnose infectious disease caused by RNA viruses This study was focused on the colorimetric detection of two RNA viruses, Influenza A and SARS-CoV-2.

1.4.1 Influenza A LAMP detection

Influenza A virus is an RNA virus. It can infect humans, birds, mammals and other animals and is classified into subtypes based on viral surface proteins, haemagglutinin (HA) and neuraminidase (NA). The first outbreak of human infection with a novel Influenza A virus was with InfluenzaH7N9. After that incident, the virus is spread worldwide. The most common causes of morbidity and mortality is based to the ability to evolve rapidly by reassortment and genetic drift. Although, quantitative reverse transcription PCR (qRT-PCR) diagnostic method for the detection of Influenza A has already been developed, loop-mediated isothermal amplification was able to simplify the detection of the virus.[28] The most common target gene that is used for the amplification is HA and NA gene. RT-LAMP is also a powerful tool for the detection of the virus as it provides rapid and very sensitive detection [29]

1.4.2 SARS-CoV-2 LAMP detection

In 2019, a novel coronavirus, with the name severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was emerged worldwide. SARS-CoV-2 is an RNA virus and like the other coronaviruses, it has four structural proteins, known as the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins; the N protein holds the RNA genome, and the S, E, and M proteins together create the viral envelope. Infected people can be diagnosed through computed tomographic scan of suspicious patient, and a confirmatory laboratory test which is performed using published quantitative RT-PCR (qRT-PCR) methods [30]. As the need for diagnostics tools is increasing rapidly, more and more scientific groups are developing rapid and sensitive LAMP assays for SARS-CoV-2 detection. Real-time colorimetric LAMP seems to show high sensitivity as far as the detection is concerned, including a fast turnaround time [2]. The target genes that are mostly used for the amplification are the N-gene, E-gene and the ORF1a gene[31].

1.4.3 Saliva test with RT-LAMP

Real-time LAMP (RT-LAMP) method can be applied to crude samples from patient's nasopharyngeal or oropharyngeal samples (swabs) or other samples such as tissue [29]. Collection of these samples can take a long time and sometimes it is a difficult procedure. It requires the transportation of the sample into viral transport media (VTM) or special equipment, the extraction of the RNA and finally the LAMP amplification. RT-LAMP method with crude samples can skip many of these steps.

Saliva-based testing also offers an improvement over the standard collection of nasopharyngeal swabs because people can collect their own samples with minimal discomfort, simply by spit into a sterile tube and mail it to a lab for processing. Using saliva samples can easily be combined with POC tests and simplify the whole detection procedure. Moreover, simplifying the collection of the sample from patients, is reducing the cost of the procedure, the specialized personnel and allows protective equipment that the medical should wear during sample collection to be saved for further and more important usage. Finally, real-time colorimetric LAMP can be performed after the collection of the saliva.

1.5 Aim of the study

This study aimed to develop a rapid method for real-time colorimetric LAMP reaction by evaluating different colorimetric dyes and polymerase enzymes obtained from different suppliers. Specifically, the dyes HNB, phenol red and crystal violet were applied and tested for the colorimetric detection of the LAMP reaction. The combination of the dye and the enzyme could enhance the nucleic acid amplification by reducing the time of the detection and at the same time increase the sensitivity of the method. The ultimate goal the of project is the application of the enhanced

real-time colorimetric LAMP to clinical samples for the detection of Influenza A and SARS-CoV-2 viruses.

2. Materials and Method

2.1 Preparation of crystal violet (CV) and leuco crystal violet (LCV)

The following chemicals were used for the preparation of leuco crystal violet (LCV) dye:

Crystal violet (CAS Number: 548-62-9), sodium sulfite (CAS Number: 7757-83-7), b-cyclodextrin (CAS Number 7585-39-9) were purchased from Sigma–Aldrich, Inorganic pyrophosphatase (PPase) was purchased from New England Biolabs (Catalog #: M0296S).

Different concentrations of sodium sulfite and crystal violet were used for the preparation of the LCV dye. The lower the concentration of crystal violet is added, the more colorless the solution appears. As the sodium sulfite is responsible for the convention of the crystal violet into the colorless leuco crystal violet, the increasing of the concentration turns the solution less purple. Moreover, β-cyclodextrin was added due to LCV has low solubility in water. A stock solution of 100 µL was prepared. The optimal mixture solution containing 0.5 mM crystal violet, 85 mM sodium sulfite, 28.4 mM β-cyclodextrin and 2.5 mM Tris-HCl pH 8.5. The leuco crystal violet dye remained at -20°C and protected from light until further use into LAMP reaction. In addition, the reaction is a reversible reaction and when the mixture solution was left at room temperature overnight, the color changed from colorless back to purple.

For the detection of LAMP products with crystal violet, 4 µL of the LCV dye mixture were added in each tube. The color of the solution before the amplification was light blue and it was transparent and after the amplification the color changed to violet. Total volume of the reaction was 25 µL.

Moreover, pyrophosphatase (PPase, 2,000 units/mL) was added to the reaction to help accelerate it. From 0.5 units to 4 units of PPase were added to the reaction.

2.2 Colorimetric detection with HNB and phenol red

Hydroxyl-Naphthol-Blue (HNB) purchased from Dojindo Molecular Technologies, Inc.. Phenol Red was already in solution and purchased as a WarmStart Colorimetric LAMP 2X Master mix DNA/RNA from New England BioLabs.

Two other dyes were used for the detection of LAMP products. Hydroxyl-Naphthol-Blue (HNB) is a metal indicator dye and was used as a colorimetric reagent. The color of the solution depends on the concentration of the magnesium ions (Mg^{2+}) that are free in the solution. The reduction of concentration of the Mg^{2+}, changes the color of the solution, from purple to light blue. In LAMP reaction, in each tube with total volume 25 µL, 0.4 µL HNB 10mM were added.

Phenol red (phenolsulfonphthalein) is a pH indicator dye. The color of the solution depends on the concentration of the hydronium ions (H_3O^+) or hydrogen ions (H^+). The more acidic conditions (low pH) the more yellow the solution appears. The color of

the solution before the amplification is bright pink and after the amplification the color changes to yellow. In a 25 µL reaction, 12.5 µL master mix, that contained phenol red were added to LAMP reaction.

2.3 Template DNA/RNA

Three different targets were used in this study, Influenza A, SARS-CoV-2 and human RNA target (RNAse P).

The double stranded DNA from Influenza A was provided form University College London Hospitals NHS. Two different samples were taken from patient, X096 and X297 with final concentration of 90 ng per µL and 100 ng per µL respectively. They were PCR product of 452 bp (X096) and 463 bp (X297) that correspond to the HA gene of Influenza A virus. Next generation sequencing was used to each sample. The sequencing of each sample after data analysis is shown below.

The sequencing of the sample X096 is the following. With bold/ underlined is the gene where the primers hybridize:

NNNNNNNNNNNNNNNNNNNNNNNcaatactagtagttctgctgtatacATTTACAACCGCAAA
TGCAGACACATTATGTATAGGTTATCATGCGAACAATTCAACAGACACT**GTAGACACAGTA**
CTAGAAAAGAATGTAACAGTAACACACTCTGTCAATCTTCTGGAAGACAAGCATAACGGA
AAACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGC
TGGATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGATCATGGTCCTACATTG
TGGAAACATCTAATTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCAATTATGAGG
AGCTAAGAGAGCAATTGAGCTCAGTGTCATCATTTGAAAGGTTTGAAATATTCCCCAAGA
CAAGTTCATGGCCTAATCATGACTCGGACAAAGGTGTAACGGCAGCATGTCCTCACGCTG
GAGCAAAAAGCTTCTACAAAAACTTGATATGGCTGGTTAAAAAAGGAAATTCATACCCAA
AGCTCAACCAAACCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTGTGGGGCATTCA
CCATCCACCTACTATTGCTGACCAACAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGT
GGGGACATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAACAAGACCCAAAGTGAG
GGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAACCGGGAGACAAAATAAC
ATTCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCATTCACAATGGAAAGAGATGCT
GGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACC
CGAGGGTGCTATAAACACCAGCCTCCCATTTCAGAATGTACATCCGATCACAATTGGGAAAT
GTCCAAAGTATGTAAAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTC
TATTCAATCTAGAGGCCTATTCGGGGCCATTGCTGGCTTCATTGAAGGGGGGTGGACAGGG
ATGGTAGATGGATGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCC
GATCTGAAGAGCACACAAAATGCCATTGATAAGATTACTAACAAAGTAAATTCTGTTATTGA
AAAGATGAATACACAGTTCACAGCAGTGGGTAAAGAGTTCAACCACCTTGAAAAAAGAATA
GAGAATCTAAATAAAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACT
GTTGGTTCTACTGGAAAACGAAAGAACTTTGGACTATCACGATTCAAATGTGAAGAACTTGT
ATGAAAAAGTAAGAAACCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTCG
AATTTTACCACAAATGCGACAACACATGCATGGAAAGTGTCAAGAATGGGACTTATGACTA
CCCAAAATACTCAGAGGAAGCAAAATTAAGCAGAGAAAAAATAGATGGAGTAAAGCTGGA

ATCAACAAGGATCTACCAGATTTTGGCGATCTATTCAACTGTTGCCAGTTCATTGGTACTGG
TAGTCTCCCTGGGGGCAATCAGCTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAGAATA
TGTATTTAACattaggatttcagaatcatgagaaaaacac

The sequencing of the sample X297 is the following. With bold/ underlined is the gene where the primers hybridize:

NNaaaacaaaagcaacaaaaatgaagacaatactagtagttctGCTGTATACATTTACAACCGCAAATGC
AGACACATTATGTATAGGTTATCATGCGAACAATTCAACAGACACT**GTAGACACAGTACTA**
GAAAAGAATGTAACAGTAACACACTCTGTCAATCTTCTGGAAGACAAGCATAACGGAAA
ACTATGCAAACTAAGAGGGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTG
GATCCTGGGAAATCCAGAGTGTGAATCACTCTCCACAGCAAGATCATGGTCCTACATTGTG
GAAACATCTAATTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCAATTATGAGGAG
CTAAGAGAGCAATTGAGCTCAGTGTCATCATTTGAAAGGTTTGAAATATTCCCCAAGACA
AGTTCATGGCCTAATCATGACTCGGACAAAGGTGTAACGGCAGCATGTCCTCACGCTGGA
GCAAAAAGCTTCTACAAAAACTTGATATGGCTGGTTAAAAAAGGAAATTCATACCCAAAG
CTCAACCAAACCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTGTGGGGCATTCACC
ATCCACCTACTATTGCTGACCAACAAAGTCTCTATCAGAATGCAGATGCATATGTTTTTGTGG
GGACATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAACAAGACCCAAAGTGAGGG
ATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAACCGGGAGACAAAATAACAT
TCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCATTCACAATGGAAAGAGATGCTGG
ATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAATACAACTTGTCAGACACCCGA
GGGTGCTATAAACACCAGCCTCCCATTTCAGAATGTACATCCGATCACAATTGGGAAATGTC
CAAAGTATGTAAAAAGCACAAAATTGAGACTGGCCACAGGATTGAGGAATGTCCCGTCTAT
TCAATCTAGAGGCCTATTCGGGGCCATTGCTGGCTTCATTGAAGGGGGGTGGACAGGGAT
GGTAGATGGATGGTACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGA
TCTGAAGAGCACACAAAATGCCATTGATAAGATTACTAACAAAGTAAATTCTGTTATTGAAA
AGATGAATACACAGTTCACAGCAGTGGGTAAAGAGTTCAACCACCTTGAAAAAAGAATAGA
GAATCTAAATAAAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCGAACTGT
TGGTTCTACTGGAAAACGAAAGAACTTTGGACTATCACGATTCAAATGTGAAGAACTTGTAT
GAAAAAGTAAGAAACCAGTTAAAAAACAATGCCAAGGAAATTGGAAACGGCTGCTTCGAA
TTTTACCACAAATGCGACAACACATGCATGGAAAGTGTCAAGAATGGGACTTATGACTACCC
AAAATACTCAGAGGAAGCAAAATTAAGCAGAGAAAAAATAGATGGAGTAAAGCTGGAATC
AACAAGGATCTACCAGATTTTGGCGATCTATTCAACTGTTGCCAGTTCATTGGTACTGGTAG
TCTCCCTGGGGGCAATCAGCTTCTGGATGTGCTCTAATGGGTCTCTACAGTGTAGAATATGT
ATTTAACATTAGGATTTCAGAATCATGAGAAAAACac

The RNA for SARS-CoV-2 was provided from the National SARS-CoV-2 Reference Laboratory, Hellenic Pasteur Institute. The viral RNA was extracted, and the targeted gene was the N-gene of the SARS-CoV-2 genome. Also, ORF1a gene was a second gene target for SARS-CoV-2.

For positive control, the human Ribonuclease P gene (RNase P) was used, which was already appear in the patients nasopharyngeal or oropharyngeal samples (swabs) after the extraction of RNA or in human saliva samples.

2.4 Primer design for LAMP

A set of six primers were used for each target gene (Influenza A, SARS-CoV-2, RNase P). All the primers purchased from Metabion (Germany). The primers were as shown in the table below (*Table 1*). The primers for Influenzavirus were designed using Primer Explorer v5 software (https://primerexplorer.jp/e/. Eiken Chemical Co. Ltd., Tokyo, Japan). The SARS-CoV-2 and the RNase P set of primers were purchased according to the work of Yinhua Zhang et. al. [31].

Table 1: Primer sequences of N-gene, ORF1a-A, HA and RNase P target.

Name	Sequence (5' → 3')
N-gene (SARS-CoV-2)	
N-gene F3	AACACAAGCTTTCGGCAG
N-gene B3	GAAATTTGGATCTTTGTCATCC
N-gene FIP	TGCGGCCAATGTTTGTAATCAGCCAAGGAAATTTTGGGGAC
N-gene BIP	CGCATTGGCATGGAAGTCACTTTGATGGCACCTGTGTAG
N-gene LF	LF TTCCTTGTCTGATTAGTTC
N-gene LB	ACCTTCGGGAACGTGGTT
ORF1a-A (SARS-CoV-2)	
ORF1a-A-F3	CTGCACCTCATGGTCATGTT
ORF1a-A-B3	AGCTCGTCGCCTAAGTCAA
ORF1a-A-FIP	GAGGGACAAGGACACCAAGTGTATGGTTGAGCTGGTAGCAGA
ORF1a-A-BIP	CCAGTGGCTTACCGCAAGGTTTTAGATCGGCGCCGTAAC
ORF1a-A-LF	CCGTACTGAATGCCTTCGAGT
ORF1a-A-LB	TTCGTAAGAACGGTAATAAAGGAGC
RNase P (Ribonuclease P)	
RNaseP POP7 F3	TTGATGAGCTGGAGCCA
RNaseP POP7 B3	CACCCTCAATGCAGAGTC
RNaseP POP7 FIP	GTGTGACCCTGAAGACTCGGTTTTAGCCACTGACTCGGATC
RNaseP POP7 BIP	CCTCCGTGATATGGCTCTTCGTTTTTTTCTTACATGGCTCTGGTC
RNaseP POP7 LF	ATGTGGATGGCTGAGTTGTT
HA (Influenza A)	
HA F3	AACAGTAACACACTCTGTCA
HA B3	CATTGTCTGAATTAGATGTTTCC
HA FIP	CCAAATGCAATGGGGCTACC-ATCTTCTGGAAGACAAGCA
HA BIP	TAACATTGCTGGCTGGATCCT-ACAATGTAGGACCATGATCT
HA LF	CCTCTTAGTTTGCATAGTTTTCCGT
HA LB	CCAGAGTGTGAATCACTCTCCAC

2.5 Sample collection

All samples with SARS-CoV-2 used for the experiments, were collected from respiratory specimens (swabs), either nasopharyngeal or oropharyngeal, from

patients infected or with suspicion of COVID-19 infection. This procedure was handled from specialist health personnel from several public and private hospitals of Greece. The swabs immediately placed into sterile tubes containing 3mL of viral transport media (VTM). Later they were transported to the National SARS-CoV-2 Reference Laboratory, Hellenic Pasteur Institute for analysis.

Saliva for healthy donors was used also for the experiments with SARS-CoV-2, which purchased Lee Biosolutions, USA.

In addition, a new innovated product was used for the collection of saliva for the experiments with SARS-CoV-2. The device is called SDNA-1000 and is a saliva collector device from SPECTRUM SOLUTIONS, LLC (Utah, USA). The device contained a tube, where the saliva was placed through drooling and a cup that contained a solution. After the collection of the saliva the two of them, saliva and solution, had to be mixed. The solution was added for the lysis of the cells and for preservation of the DNA and RNA that were released in the solution. The mixture contains 25-50 % Thiocyanic acid, compound with guanidine (1:1), 10-25 % Ethanol and 1-5 % Tris (hydroxymethyl) aminomethane. These chemicals are used mainly as chaotropic agents and as nucleic acid protector in the extraction of DNA and RNA from cells. Due to the presence of ethanol, the performance of the LAMP reaction was decreased significantly, as the activity of the polymerase enzyme was decreased. For this reason, the mixture that contained the saliva and the chemical solutions had to be diluted ten times before it was added into the LAMP reaction.

2.6 Extraction of Viral RNA and qRT-PCR assays for SARS-CoV-2

Total viral RNA was extracted from 200 µL of clinical samples in VTM with the NucliSens easyMAG automated system (BioMérieux, Marcy l'Etoile, France). Following the qPCR protocol by the WHO and the National Reference Center for Respiratory Viruses, Institut Pasteur, Paris, the amplification was performed with a 106 bp fragment. For confirmation, an assay using E-gene was performed from the Charité protocol was used by real-time RT-PCR [32]. All the extracted samples were stored at -80ºC. Also, all the experiments were performed in different days within 3 weeks and at independent runs.

For testing the limit of detection, three different referenced were used. First, SeraCare's SARS-CoV-2 AccuPlex solution 5000 copies/mL (Material number 0505-0126) was used, where 1 µL of the solution was added to the LAMP mix (directly or after treatment with heat at 80ºC for 10 min. The second reference that was used, was the 2019-nCoV_N Positive Control plasmid, at a concentration of 200.000 copies/µL (CAS Number 10006896) from Integrated DNA Technologies. Finally, for RNA reference, synthetic RNA the SARS-CoV-2 Standard (#COV019) at a concentration of 200.000 copies/µL and SARS-CoV-2 Negative (#COV000) from Exact Diagnostics, Bio-Rad (Texas, USA) were used.

2.7 DNA/RNA Polymerases

Six different polymerases were used in a series of experiments (5 Bst ones purchased from a different supplier and one Bsm), to test whether the method could involve in terms of speed. The time of each experiment was 30 min; and the performance of each polymerase was examined at this time by using varius dyes. The six polymerases that were used for LAMP reaction and their corresponding dyes appear at *Table 2*.

Table 2: All polymerases that were used for LAMP reaction.

Polymerase	Reverse transcription activity	Dye used for the detection	Company
Bst 2.0 polymerase	Yes	Phenol red	NEB
Bst 2.0 polymerase	Yes	HNB/Crystal violet	NEB
Bst DNA/RNA Polymerase	Yes	HNB/Crystal violet	SBS Genetech Co
Bst 3.0 polymerase	No/very little	HNB/Crystal violet	NEB
Saphir Bst2.0 Turbo Polymerase	No	HNB/Crystal violet	Jena Bioscience
Bsm DNA Polymerase	No/very little	HNB/Crystal violet	Thermo Scientific

2.8 LAMP reaction conditions and assay

The assay was performed in a total 25 µL reaction for every LAMP reaction a set of six primers, two outer primers, a forward outer primer (F3) and a backward outer primer (B3); two inner primers, a forward inner primer (FIP) and a backward inner primer (BIP), and two loop primers (LF and LB) were used. All primers that were used are shown in *Table 1*. Each LAMP reaction contained 10x LAMP primer mix and the concentration of the primers were 1.6 mM FIP and BIP, 0.2 mM F3 and B3, 0.6 mM of the LF and LB.

In each LAMP reaction, 2 µL of DNA or extracted RNA sample or 2 µL DNA from Influenza were added to the reaction. In addition, 1-5 µL of pure saliva or the mixture that contained saliva and the solution from SPECTRUM SOLUTIONS, were added to the reaction and mixed with the template. To prevent the disintegration of the RNA from the RNases A, B and C that appeared normally in saliva, RNase Inhibitor was used (RNase Inhibitor, Human Placenta, NEB).

Different enzymes were used each time and for that reason the concentration of the buffers were different. For every enzyme that was used for the experiments, the protocols shown in the *Tables 3*, below. Two hundred copies per µL commercially available RNA was used in all the experiments which they concerned the comparison of the activity of each polymerase.

Three polymerase, Bst 3.0, Saphir Bst2.0 Turbo Polymerase and Bsm DNA Polymerase didn't have or have less reverse transcriptase activity. In those cases, it was necessary

to add the enzyme of Reverse Transcriptase (15.000 units/mL, WarmStart® RTx Reverse Transcriptase from NEB) to enable cDNA synthesis from the RNA. This enzyme is capable to synthesize a complementary DNA strand initiating from a primer using RNA (cDNA synthesis) or single-stranded DNA as a template. For that reason, it was added to the LAMP reaction.

The time of each experiment was 30 min at 65°C.

Table 3: Collectively all protocols for the enzymes that were used for LAMP reaction.

A) ***Bst 2.0 polymerase*** from NEB with Phenol Red dye (WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA), NEB)

REAGENTS	VOLUME
WarmStart Colorimetric LAMP 2X Master Mix with Bst 2.0	12.5 µL
10X Primer Mix	2.5 µL
Nucleic acid sample (RNA or DNA)	2 µL
Nuclease-free water	Up to 25 µL
RNase Inhibitor (40.000 units/mL) only with saliva samples	1 µL
TOTAL VOLUME	**25 µL**

B) ***Bst 2.0 polymerase*** from NEB (Bst 2.0 WarmStart® DNA Polymerase, NEB)

REAGENTS	VOLUME
WarmStart LAMP Master Mix with Bst 2.0	12.5 µL
10X Primer Mix	2.5 µL
Nucleic acid sample (RNA or DNA)	2 µL
HNB dye 10 mM	0.4 µL
Nuclease-free water	Up to 25 µL
RNase Inhibitor (40.000 units/mL) only with saliva samples	1 µL
TOTAL VOLUME	**25 µL**

C) ***Bst DNA/RNA Polymerase*** from SBS Genetech Co. (Beijing SBS Genetech Co., Ltd.)

REAGENTS	VOLUME
Bst DNA/RNA Polymerase (8 units/µL)	1 µL
10X Primer Mix	2.5 µL
10X Isothermal Buffer (Mg^{2+} free)	2.5 µL
100 mM Mg^{2+}	1.75 µL
dNTP Mixture (10 mM each)	3 µL
Template (DNA/RNA/saliva etc.)	2 µL
HNB dye 10 mM	0.4 µL
Nuclease-free water	Up to 25 µL
RNase Inhibitor (40.000 units/mL) only with saliva samples	1 µL
TOTAL VOLUME	**25 µL**

D) ***Bst 3.0 polymerase*** from NEB (Bst 3.0 DNA Polymerase, NEB)

REAGENTS	VOLUME
Bst 3.0 Polymerase (8 units/µL)	1 µL
10X Primer Mix	2.5 µL
1X Isothermal Amplification Buffer II Pack	2.5 µL
dNTP Mixture (10 mM each)	3.5 µL
Template (DNA/RNA/saliva etc.)	2 µL
HNB dye 10 mM	0.4 µL
Nuclease-free water	Up to 25 µL
RNase Inhibitor (40.000 units/mL) only with saliva samples	1 µL
RTx Reverse Transcriptase (15.000 units/mL)	0.5 µL
TOTAL VOLUME	**25 µL**

E) ***Saphir Bst2.0 Turbo Polymerase*** from Jena Bioscience (Saphir Bst2.0 Turbo Polymerase, Germany)

REAGENTS	VOLUME
Saphir Bst2.0 Polymerase (8 units/µL)	1 µL
10X Primer Mix	2.5 µL
Saphir Bst2.0 Turbo Buffer	2.5 µL
dNTP Mixture (10 mM each)	3 µL
$MgCl_2$ Stock Solution	1
Template (DNA/RNA/saliva etc.)	2 µL
HNB dye 10 mM	0.4 µL
Nuclease-free water	Up to 25 µL
RNase Inhibitor (40.000 units/mL) only with saliva samples	1 µL
RTx Reverse Transcriptase (15.000 units/mL)	0.5 µL
TOTAL VOLUME	**25 µL**

F) Bsm DNA Polymerase from Thermo Scientific (Bsm DNA Polymerase, large fragment (8 U/µL), USA)

REAGENTS	VOLUME
Bsm Polymerase (8 units/µL)	1 µL
10X Primer Mix	2.5 µL
10X Bsm Buffer	2.5 µL
dNTP Mixture (10 mM each)	3.5 µL
$MgSO_4$ Stock Solution	1.25
Template (DNA/RNA/saliva etc.)	2 µL
HNB dye 10 mM	0.4 µL
Nuclease-free water	Up to 25 µL
RNase Inhibitor (40.000 units/mL) only with saliva samples	1 µL
RTx Reverse Transcriptase (15.000 units/mL)	1 µL
TOTAL VOLUME	**25 µL**

2.9 Specificity test

For the specificity test of LAMP reaction with SARS-CoV-2 samples bind tests were performed using other samples that contained different viruses. The samples were positive for influenza, CMV, RSV, adenovirus, and enterovirus. The LAMP reaction was performed with the protocol of Bst 2.0 polymerase from NEB with Phenol Red dye.

2.10 IRIS: a portable real-time quantitative colorimetric LAMP devise

A portable biomedical device was used for the performance of the real-time quantitative colorimetric LAMP (qcLAMP) experiments. The portable device is called IRIS and it was made for the use of point of care molecular diagnostics. In this study was used for the detection of SARS-CoV-2 and Influenza A. Figure 6A presents a schematic representation of the device and the whole procedure.

2.10.1 Design and Construction

IRIS is consisted of the main unit, the mini camera, the heater, the lights source and the tube-holder. The main unit of the device was designed and constructed by using digital manufacture based on 3D-printing. On the inside of the main unit are all the electronic components which are connected with the camera and the LED lights placed on both sides. On top, is the PCB resistive microheater which is connected to a temperature sensor. The holder is designed for standard 0.2 mL Eppendorf tubes. The 8-slot tube-holder is placed on top of the heater with the bottom of the tubes touching the heater (approximately 2 mm diameter of contact surface). In the meantime, the top cover applies pressure, around 2MPa, to determine the good contact with the heating element (Figure 6C). Both tube holder and cover top parts connected to each other and to the main unit with the aid of magnets. The camera and the LED light are facing the tubes. All the parts of the device are shown in Figure 6B. The device connects via Bluetooth to a smartphone or tablet and operates through an in-house developed Android application (Figure 6F).

2.10.2 Digital image analysis

The mini camera focuses on a specific area of the tube where the solution is located. It monitors in real-time the transition of the various color shades during a colorimetric LAMP reaction. Every 6 seconds the camera collects non-calibrated images and extracts values of the pixels form three different colors, red, blue and green (RGB). Each time, the specific dye that is used (pH, metal binding or DNA binding-based byes) contributes to a different color combination between green and blue or green and red pixels. There are three different combinations of colors for the three different dye that were used, phenol red the hydroxy napthol blue (HNB) and leuco crystal violet (LCV). These combinations are applied to the analysis and to the discrimination of the positive and the negative sample and employed as tree different formulas. For the HNB indicator the formula that is used is Green-Red pixel, for the phenol red indicator the formula is Green-Blue pixel and for the leuco crystal violet is Green-Red. The color

discrimination displayed in the Y-axis of a real-time plot as color index units (pixels) (Figure S7).

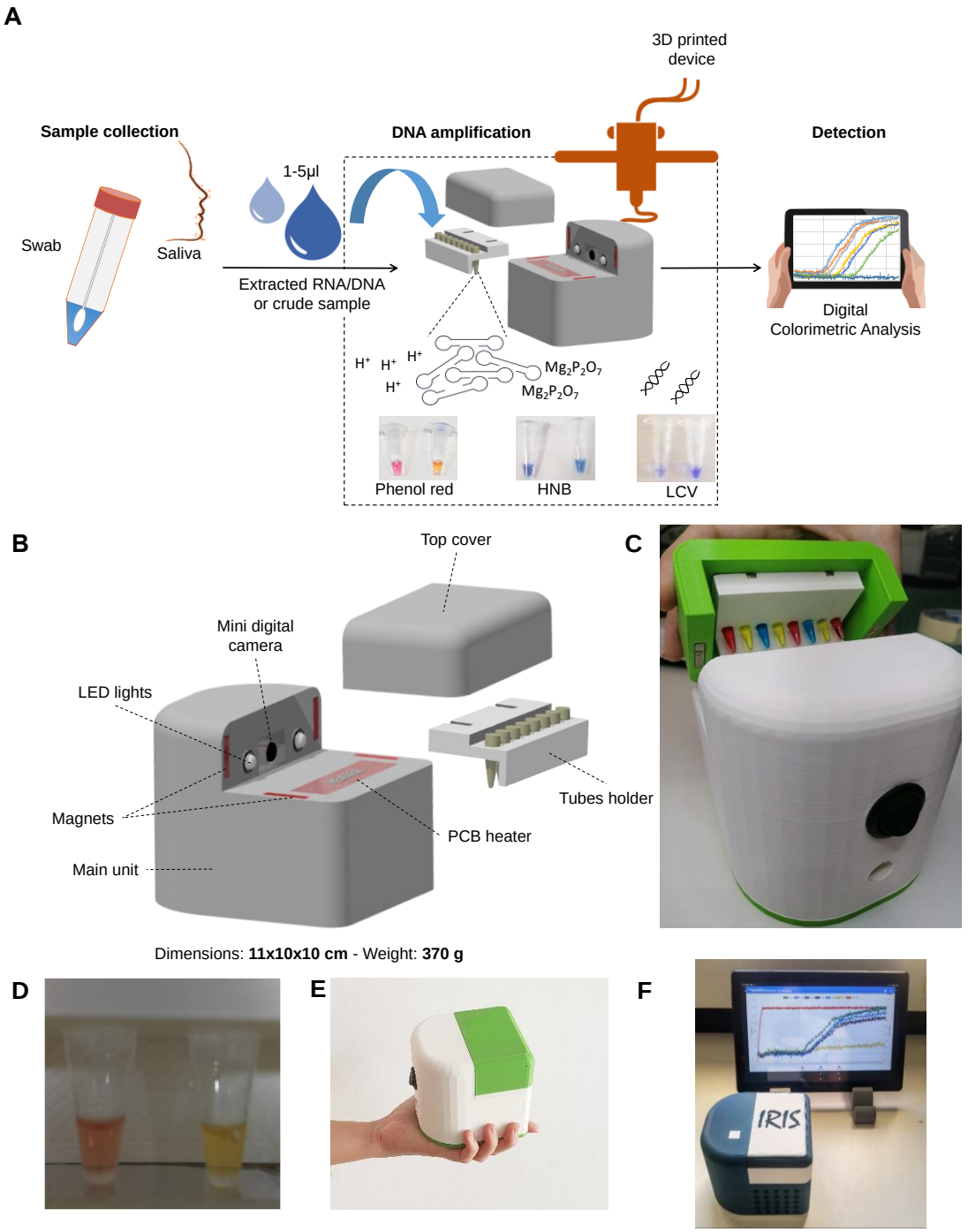


Figure 6: (A) Overview of the real-time quantitative colorimetric concept. (B) Schematic representation of the qcLAMP device and components. (C) Photograph of the device and tubes-holder. (d) Captured image of two Eppendorf tubes inside the chamber and during operation of the device. (e) Image of the handheld device for performing qcLAMP. (F) Device connected to a tablet (modified from Papadakis et al., 2020 [26]).

3. Results

3.1 Leuco crystal violet preparation

LCV was prepared using different concentrations of crystal violet and sodium sulfite. The first step of the preparation of LCV was the treatment of CV with sodium sulfite. In general, LCV is chemically unstable and two forms of the compound (LCV and CV) exist in dynamic equilibrium in the solution. Since the reaction of CV and sodium sulfite is reversible, with excess amount of sodium sulfite the balance of the equilibrium is pushed to the formation of LCV. Increasing the concentration of Na_2SO_3, the solution became more transparent but not completely. After several trials the concentration of Na_2SO_3 that had the best results was 85 mM (28.2 μL). Changing the final concentration of CV in the solution was also tried out. However, the calibration of the Na_2SO_3 concentration had better results in the solution before and after the amplification. The second step was the addition of β-cyclodextrin, to increase the solubility of the CV in the solution. The final concentration of β-cyclodextrin was 5 mM as it was suggested in bibliography [17]. In Figure 7, the seven different solutions that were prepared with different concentration of Na_2SO_3 can been seen. Tube with number 7 was evaluated as the best colorimetric dye for the qcLAMP experiments. In *Table 4* are all the concentrations that were used for the preparation of the LCV dye. The number of the tubes corresponds to the number of the tubes in Fig. 7.

Table 4: Seven different concentrations of Na_2SO_3 for the preparation of LCV. The final volume of each tube was 100 μL. With blue color is marked the solution with the final volumes of each compound that was used for qcLAMP.

Number of samples	Na_2SO_3 (stock concentration 300 mM)	Crystal violet (stock concentration 5 mM)	Tris-HCl, pH=8.5 (stock concentration 300 mM)	β-cyclodexrtin (stock concentration 8.8 mM)
1	13.3 μL	10 μL	5 μL	56.81 μL
2	16.3 μL	10 μL	5 μL	56.81 μL
3	18.6 μL	10 μL	5 μL	56.81 μL
4	20 μL	10 μL	5 μL	56.81 μL
5	80 μL	10 μL	10 μL	-
6	90 μL	10 μL	-	-
7	**28.2 μL**	**10 μL**	**5 μL**	**56.81 μL**

Figure 7: Different concentrations of Na_2SO_3 in LCV solution. Final volume was 100 μL. The number of each tube correspond to the number that appears in Table 1 above. The tube No 7 had the best performance as colorimetric dye in qcLAMP.

3.2 Real-time quantitative colorimetric LAMP for Influenza A with LCV colorimetric dye

With the aim of accelerating, the LAMP assay, pyrophosphatase enzyme (PPase 2.000 units/mL) was added to the reaction. Different amounts of the enzyme were used, from 0.5 units to 4 units. Influenza A was used as template from sample with concentration 100 ng/µL. To evaluate the ability of the PPase to reduce the estimated time of the detection with is approximately 15 min, different concentrations of the enzyme were compered. In Figure 8A real time data show the time-to-positive results for each concentration of PPase in the samples with 10^9 copies per µL. In the samples that had 2 units of PPase or didn't have at all PPase, the LCV dye changed color due to amplification at 14,1 min. On the other hand, 0.5 and 1 unit of PPase were able to accelerate the reaction at least 1 min.

Furthermore, while using Bst 2.0 polymerase from NEB, two different dilutions of Influenza A template and concentrations of PPase were compared. 10^9 and 10^3 copies per µL of template were added after the addition of 0.5 or 1 unit of PPase. In bar plot in Fig. 2B can be seen the time-to-positive results. In both concentrations of template, the fastest results were the samples with 0.5 unit of PPase. In the sample with 10^9 copies/µL, the color of the LCV changed at approximately 11.6 min and with 10^3 copies/µL changed at 18.8 min instead of 13 min and 19.4 min respectively when 1 unit of PPase was added. Real time graphs of each run with different template concentration, can be seen in Figure S1 in the appendices.

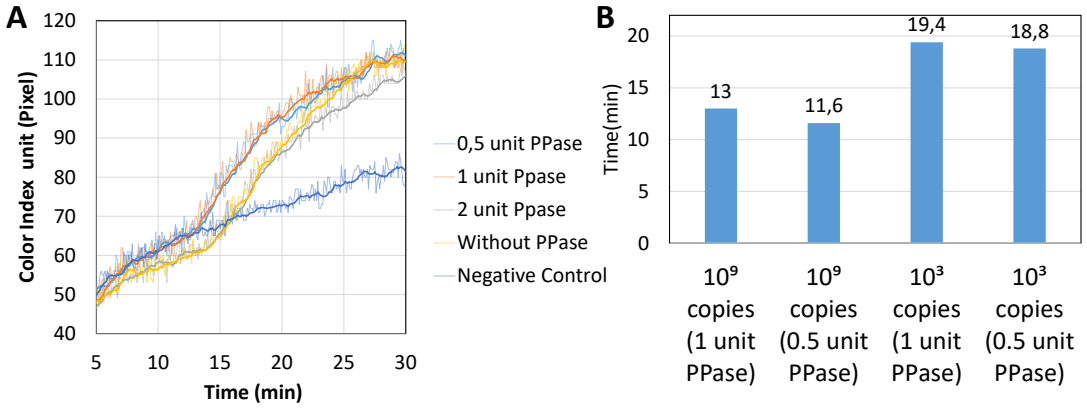


Figure 8: (A) Real time graph of sample with the addition of different concentration of PPase in the qcLAMP. The addition of 0.5 unit and 1 Unit of PPase had the best results (time-to-positive results). (B) Bar plot with the actual time-to-positive results of two different concentrations of template (10^9 and 10^3 copies) and two different concentrations of PPase enzyme.

3.3 Temperature optimization for RT-LAMP with LCV dye

Optimization of the RT-LAMP assay for detection of Influenza A with LCV colorimetric dye was determined using two different temperatures, 65°C and 60°C. For the LAMP reaction Bsm DNA Polymerase from Thermo Scientific and DNA from influenza A were used. Moreover, 0.5 unit of PPase enzyme were added to the solution to enhance the reaction. The results showed that Bsm polymerase with DNA from Influenza A had better amplification results at 65°C rather than 60°C. In particularly, the solution of the sample at 65°C started to change color at approximately 9.7 min, that it was 3 min faster than the sample at 60°C, which the solution started to change color at 11.7 min. In graph in Figure 9 can been seen the time-to-positive results of each temperature. Also, in Figure S2 in appendices, the real time graph shows the difference between the samples that contained PPase enzyme and the ones that didn't.

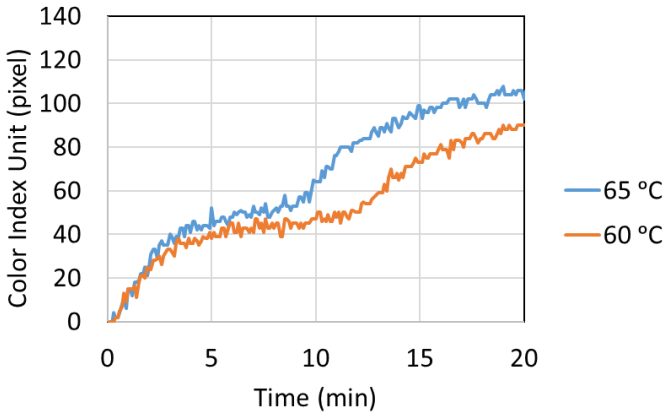


Figure 9: Real time graph of the same sample at different temperatures.

3.4 Bsm and Bst polymerase comparison with LCV dye

LCV colorimetric dye was used with two different polymerase enzymes for the comparison of the performance of each enzyme in terms of accelerating the reaction. Bst 2.0 polymerase from NEB and Bsm DNA Polymerase from Thermo Scientific were used. Also, Influenza A was used as a template. In each reaction the concentration that was used was 1000 copies/µL from sample X297 (100 ng/µL). In both samples, 1 unit PPase enzyme were added. Using low concentration of template, the time-to-positive results showed that each polymerase amplified the DNA in different speed. In sample with Bsm polymerase the amplification was faster than with Bst polymerase (Figure 10A). More specific, with Bsm polymerase at 14.5 min the color of the LCV dye changed to purple meaning that the amplification started earlier, and with Bst polymerase the color of the LCV changed at 17.5 min.

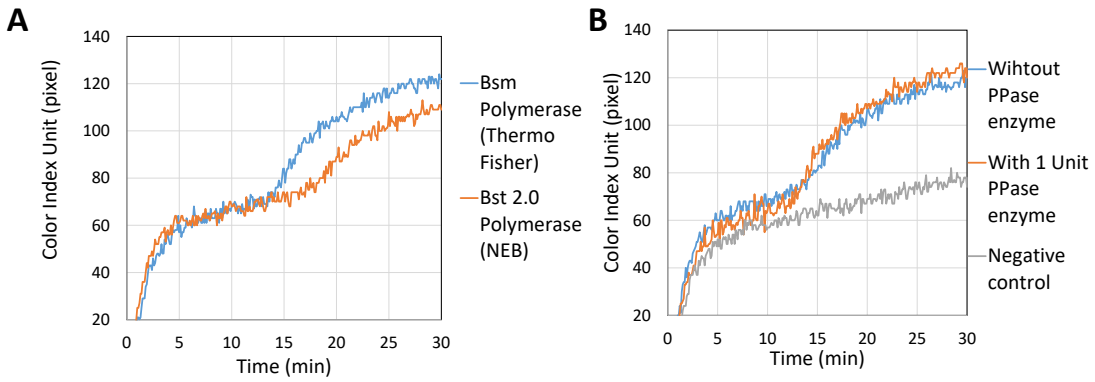


Figure 10: (A)Real time graph of samples with different polymerase enzyme and LCV colorimetric dye (1000 copies/µL). (B) Real time graph of the comparison between samples and the presence and absence of PPase enzyme. Bsm DNA Polymerase from Thermo Scientific was used.

Considering the fact that Bsm polymerase have the ability to amplify faster the DNA with LCV dye present in the solution, the addition of PPase in the solution was evaluated. In the solution 1 unit of PPase enzyme was added. The graph in Figure 10B showed that time-to-positive results differ from each other. In fact, in sample that didn't contain PPase enzyme the amplification was approximately 1.7 min slower than the one that contained PPase enzyme. In sample with PPase, in particular the time when the color of LCV started to change was at 13.1 min, whereas in the sample without PPase the color of LCV started to change at 14.8 min. The negative control sample didn't have any change in the color index. In all the experiments 1000 copies/µL from sample X297 (100 ng/µL) Influenza A were used as template.

Furthermore, wanting to know if Bst 2.0 polymerase will improve its performance with the addition of PPase enzyme, excess amount of PPase was used. The activity of the polymerase with PPase enzyme was evaluated with the addition of higher concentration of PPase. In this case 4 unit of PPase were added to the solution. As a template, Influenzas A was used with concertation 10^9 copies/µL. Figure 11 shows that the higher concertation of PPase enzyme didn't have any effect on qcLAMP. The reaction wasn't faster than the sample that didn't have PPase. The LCV dye started to change color at 13.6 min, 0.7 min later than the sample with no PPase.

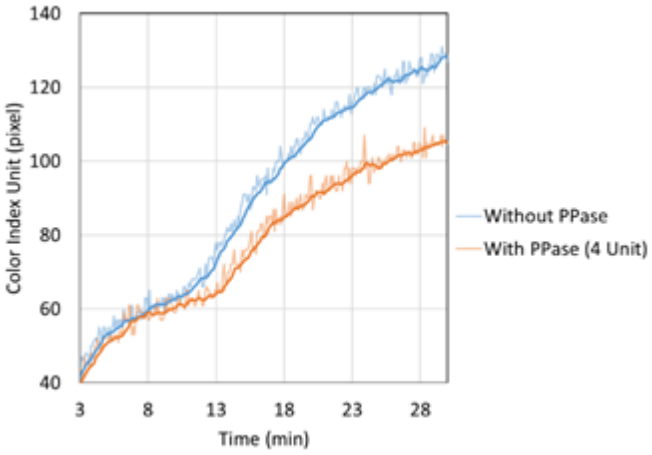


Figure 11: Real time graph of the comparison between samples that had PPase enzyme and ones that didn't. Bst 2.0 polymerase from NEB was used.

3.5 Saliva samples with LCV colorimetric dye

For the evaluation of the direct RT-LAMP assay in saliva samples with LCV dye, saliva was added to the LAMP solution. Two µL of saliva were added to the reaction. The saliva pool spiked with Influenza A virus. One µL of DNA with 10^9 copies per µL spiked into saliva, making a dilution 1:2. Bsm DNA Polymerase from Thermo Scientific was used for the amplification. Also, 0.5 unit of PPase were added to the solution. From the real-time data, it shows that LCV colorimetric dye didn't affected from the addition of the saliva (Fig. 12). The color of the solution started to change at 10 min. The negative control sample didn't change color during the experiment.

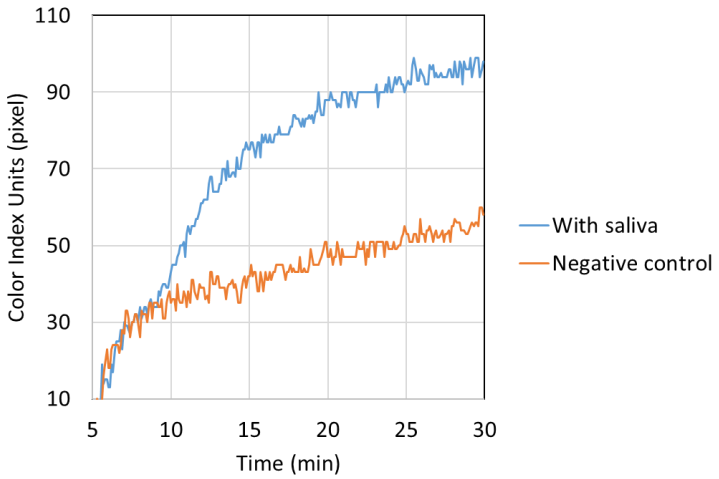


Figure 12: Real time graph of samples with LCV colorimetric dye with 2 µL of saliva.

3.6 Real-time quantitative colorimetric LAMP with Influenza A

Influenza A was used of the validation tests of the qcLAMP. A calibration curve was created using the range of 1 to 10^9 copies after several dilutions of the sample with concentration 10^{11} copies per µL. In Figure 13A the real time data are shown. Figure 13B shows the calibration curve of the same dilutions. The detection limit that was achieved is approximately 1 copy per reaction or 0.4 copies per µL input. the reaction with the highest template concentration showed a time-to-positive result at 11.8±0.2 min while the lowest at 20.0±2.4 min.

For the evaluation of the reproducibility of the method and of the device, 21 positive and 7 negative LAMP reactions were performed with the same initial target and with concentration of 10^9 copies per reaction. The standard deviation for the average time-to-positive results was only 36 sec. In all negative reaction there was not an increase of the color index. In the Figure S3 in the appendices can been seen the real data from the repeated experiments. In all the positive samples the color of the dye changed approximately at the same minute with average time at 10.2 min.

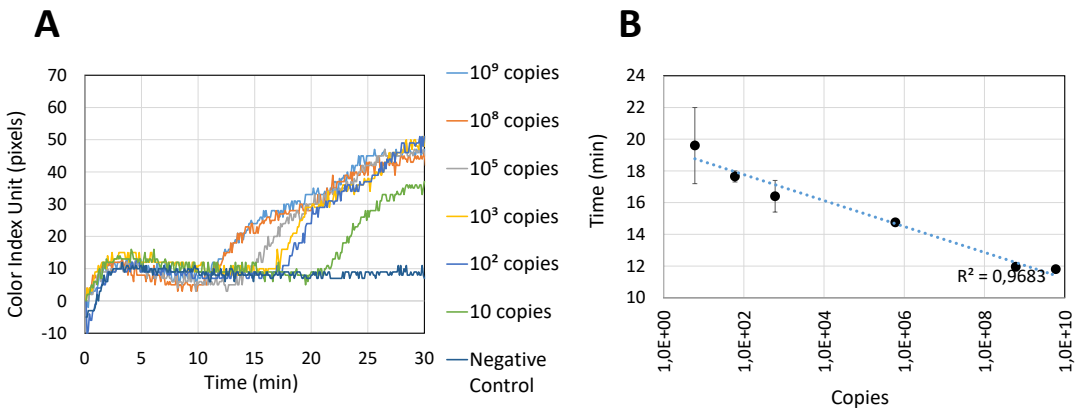


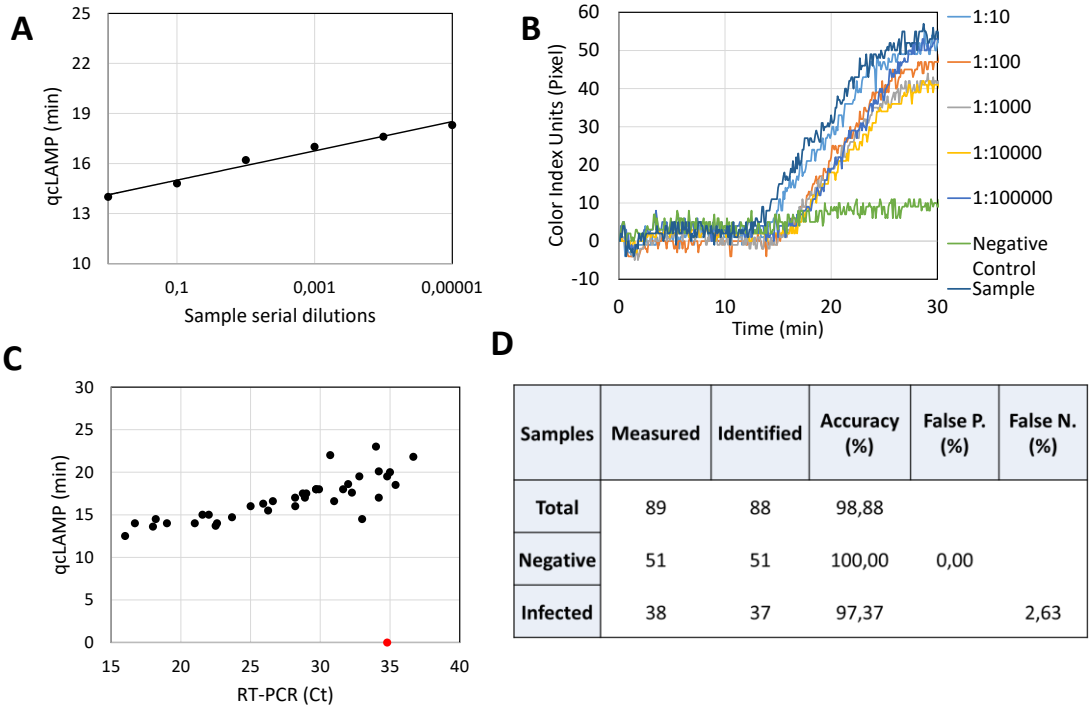
Figure 13: (A) Serial dilutions of an Influenza A sample. (B) Calibration curve for Influenza A detection using DNA template ranging from 1 to 10^9 copies per reaction.

3.7 Real-time quantitative colorimetric LAMP with SARS-CoV-2

After the evaluation tests on Influenza A, SARS-CoV-2 template was used for further evaluation of qcLAMP reaction with the device. The detection of the RNA was accomplished in one step LAMP reaction, with the reverse transcription and isothermal amplification using as a target the N gene. The samples that were used were clinical, from patients' nasopharyngeal or oropharyngeal swabs and were used after extraction procedure. Serial dilutions were performed from a SARS-CoV-2 positive sample which was extracted (Fig. 14B). The reported Ct value of the sample was 19 (qRT-PCR was performed for the he definition of the Ct value). Figure 14A show the linear correlation (R=0.99) between the RTC-LAMP time-to-positive results and the theoretical Ct values of this sample. In addition to the serial dilution using real sample from patients, serial dilutions performed using a commercially available kit from

Bio-Rad. The limit of the detection was 5 copies RNA per µL and can been seen in Figure S4.

Moreover, for the evaluation of qcLAMP method, 89 patient samples were used in total, of which 38 were positive samples (defined with qRT-PCR) and 51 were negative for SARS-CoV-2. The Ct value for positive samples (defined with qRT-PCR) ranging from 16 to 36.7. The sample with the Ct value that had the fastest time-to-positive result with qcLAMP, had Ct value 16 and the time was at 12.5 min, while the sample with the Ct value 36.7 had the slowest time-to-positive result at 23 min. Scatter plot in Figure 14C shows all the Ct values (from 16 to 36.7) for all the positive samples and the time that each sample had positive results. For all the positive samples only one of them was identified as negative the first time, which marked with red color in Fig. 14C. After second after the second iterative experiment the sample identified as positive.



Samples	Measured	Identified	Accuracy (%)	False P. (%)	False N. (%)
Total	89	88	98,88		
Negative	51	51	100,00	0,00	
Infected	38	37	97,37		2,63

Figure 14: (A) Linear correlation (R=0.99) between the qcLAMP time-to-positive results of the sample with theoretical Ct equal to 19 and on 10-fold serial dilutions of the viral RNA. (B) Serial dilutions of a SARS-COV-2 positive sample with a reported Ct value of 19 with qcLAMP method. (C) Scatter plot of the Ct values for 38 positive samples (ranging from 16 to 36.7) versus the qcLAMP time-to-positive (ranging from 12.5 to 23 min). (D) Summary table of SARS-CoV-2 samples using qcLAMP assay (38 COVID-19 positive and 51 negative).

3.8 Comparison of polymerase enzymes

In order to achieve the acceleration of the qcLAMP assay, six polymerases were used and evaluated for their performance. The validation was relied on the time-to-positive

results. The faster the amplification, the quicker the color of the dye changed. Moreover, each enzyme was evaluated for the accuracy and the reproducibility of the results. Eight positive samples with SARS-CoV-2 RNA 200 copies per µL and 8 negative samples were tested. The time-to-positive for each polymerase appear at the Figure 15A.

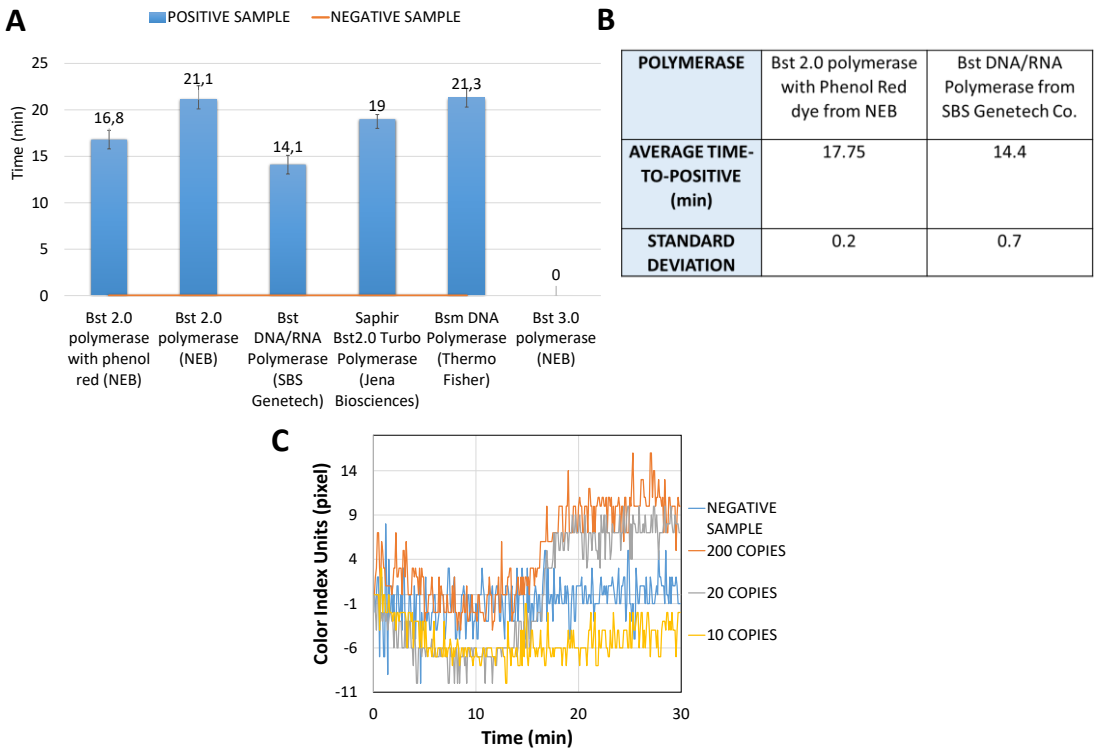
It is important to note that all the experiments with all Bst polymerases were performed at approximately 65°C and with HNB dye. Bsm polymerase was in another run due to the fact that needs low temperature to perform (approximately 60-63°C). Also, Bst 2.0 polymerase from NEB with Phenol Red dye (WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA), had already the dye inside the Master mix and for that reason different run was performed. The measurements of each experiment appear at Figure S5 in the appendices. The real data shows the different time in which the positive sample changed color using these two polymerases with RNA from SARS-CoV-2 as template.

3.9 Comparison of Bst DNA/RNA Polymerase from SBS Genetech with HNB dye and Bst 2.0 polymerase from NEB with Phenol Red dye

After the evaluation of the results, the two polymerases that had the best results depending on the time-to-positive results were the Bst 2.0 polymerase with Phenol Red dye from NEB and the Bst DNA/RNA Polymerase from SBS Genetech Co. These two enzymes had the most valid and repeatable results as far as it concerns the time that the dye in LAMP reaction changed due to the amplification. In table in Figure 15B shows the average time-to-positive results and the standard deviation of both of the two polymerases. These two different polymerases used different colorimetric dye and for that all the experiments were performed in different runs.

3.10 Limit of detection SARS-CoV-2 RNA with the usage of Bst DNA/RNA Polymerase from SBS Genetech Co.

The experiments with each polymerase revealed the best results in terms of how rapid amplification occurs. Bst DNA/RNA polymerase from SBS Genetech had the more accurate and fast results. The next step was the limit of detection of this polymerase in qcLAMP assay. Serial dilutions were performed of the commercially available kit from Bio-Rad of 200 copies/µL. Three different concentrations were tested 200, 20 and 10 copies/µL (Figure 15C). The time-to-positive results for the upper limit 200 copies/µL was 13.2 min and for the lower limit 20 copies/µL was 14.1 min.



POLYMERASE	Bst 2.0 polymerase with Phenol Red dye from NEB	Bst DNA/RNA Polymerase from SBS Genetech Co.
AVERAGE TIME-TO-POSITIVE (min)	17.75	14.4
STANDARD DEVIATION	0.2	0.7

Figure 15: (A) bar plot with the average time-to-positive results of each polymerase that was used. (B) Comparison of the two polymerases Bst 2.0 from NEB with Phenol Red and Bst DNA/RNA polymerase from SBS Genetech. (C) Limit of detection of Bst DNA/RNA polymerase from SBS Genetech.

3.11 Saliva samples evaluation with Bst DNA/RNA Polymerase from SBS Genetech Co.

Different conditions were evaluated when saliva samples were used. qcLAMP was tested for the sensitivity and the efficiency that can provide this method. The reaction was performed with 1 µL of saliva and with Bst DNA/RNA Polymerase with HNB colorimetric dye. Also, two different targets were used, the N gene and the RNaseP. For the N gene target SARS-CoV-2 from the commercially available kit from Bio-Rad was used and for the RNaseP target, human Ribonuclease P gene was used that was in the saliva sample. Because of the low amount of RNA copies into the reaction the RNase Inhibitor was used to prevent the degradation of the RNA from RNase enzyme.

The saliva samples naturally contain RNaseP as a template. Both of the different cases, (saliva samples with the addition of RNase Inhibitor and samples without the addition of RNase Inhibitor) changed color at approximately 21-22 min as the amplification took place (Figure 16). Also, to elucidate if the positive control samples with RNaseP template where amplified due to RNA or DNA that there is in the saliva, human genomic DNA (100 mg/mL from Clontech) was used as a reference. The samples with the human genomic DNA also changed color. On the other hand, when N gene was the target, the SARS-CoV-2 RNA that was added in the saliva sample was in lower

amount and RNases were able to degrade the template. In the sample which the RNase Inhibitor hadn't been added, the polymerase couldn't amplify the RNA and so the color of the dye didn't change. Adding the RNase Inhibitor in the saliva sample with RNA, RNases were prevented from degrade the template and for that the color of the solution changed to positive results. Important to be note that all the experiments with N gene target weren't with IRIS device and with real-time graphs.

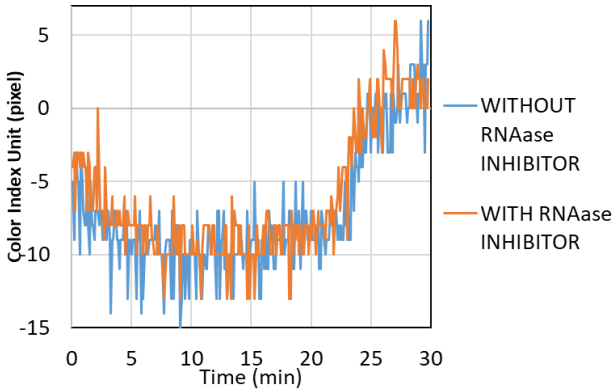


Figure 16: Real-time graph of samples with saliva and RNaseP template with and without RNase Inhibitor.

4. Discussion

In the aim of creating and using tools of point of care in molecular diagnostic field, colorimetric LAMP assay is probably one of the simplest and more sensitive methods that can be applied. The new device, IRIS, have helped providing quantitative information of real-time nucleic acid amplification. The advantage of the method is that is based on a contamination-free closed tube format, avoiding methods that need instruments like fluorescent detectors and microfluidic chips reduced the complexity of the method. Using IRIS device, the results of real-time colorimetric LAMP are similar to of real-time fluorescent LAMP (Fig. S6). Furthermore, considering the fact that the device is produced by a 3D-printer, the cost of the production significantly reduced.

Table 5: Comparison of different amplification methods: qcLAMP, qRT-PCR P, Sanger sequencing and ddPCR (modified from Papadakis et al., 2020 [26]).

Method	qcLAMP	qRT-PCR
TECHNOLOGY - ENGINEERING		
Instrumentation	3D printed device	Benchtop equipment
Manufacturability	Lab-based production in 12h	Commercial (Optical filters)
Cost	Low	High
Complexity	Low	High
Portability	Lightweight, battery operated	No
Connectivity	Smartphone/ cloud connection	PC based
Multiple samples	Yes (<10)	Yes (high throughput)
Quantitative	Yes	Yes

Improving the efficiency of the LAMP technique is essential for the development of rapid and sensitive diagnostic assays for pathogen detection and nucleic acid analysis. In this study, the main purpose was the development of new colorimetric substances that can detect amplicons not from the by-products of reaction, but from the main product of the reaction. Also, polymerase enzyme has the lead role in LAMP technique. The evaluation of the performance of six different enzymes, alongside with the optimization of the LAMP conditions, led to the reduction of the required time of the experiment while at the same time it maintained the sensitivity and specificity of the method.

Crystal violet is a suitable substance as a colorimetric dye, due to the fact that is able to react directly with dsDNA, during LAMP reaction. In previous work that have been developed for different groups, such as Miyamoto, *et al. 2015* [17], they suggested a colorimetric method using crystal violet for nucleic acid detection in LAMP. This visual detection method which they described, was an end-point detection method. However, optimizing the protocol for the crystal violet preparation, it was able to stabilize the leuco crystal violet in solution. The usage of 5 mM β-cyclodextrin in the

solution were added as a solubilizing agent. After the addition, the solution became more soluble in water and didn't create any purple precipitate in the solution. *Table 4* shows the different concentrations that were used for the LCV preparation. Increasing the suggested amount of sodium sulfite in crystal violet solution from 30 mM to 85 mM, made the solution more colorless, although it could easily change color, from colorless to blue-purple, when the amplification took place in LAMP reaction. With these alterations the solution that was made could be added a colorimetric agent in RT-LAMP assays.

Crystal violet had been already used in molecular diagnostics techniques [18, 23]. Nevertheless, for the detection the nucleic acids, the colorimetric visual detection with leuco crystal violet was end-point detection. LCV hasn't been used before in RT-LAMP experiments. Adding the LCV in the LAMP solution in a qcLAMP assay was a novel experimental procedure. The real time measurements for the negative control samples (without template) in Figure 8A and 10B shows that there is a drift in the graph during the experiment with IRIS device. As IRIS detects the color change in the solution, signifies that the color of LCV dye changed. Also, in the same graph, at first 5 minutes when the heater started to raise temperature, it is obvious that the color change rapidly from colorless to blue-purple. This color change responds to the increase of the temperature. A possible explanation of this color change is that the LCV is affected from the pH. In bibliography, it is suggested that the thermochromics' systems behavior like crystal violet is affected from acidity of the developer and the acidity of the so-solvent [33]. More specific, the rising of the temperature is decreasing the pH in the LAMP solution and so the solvent becomes more acidic. It has been shown that thermal treatment in dyes like crystal violet with a co-solvent increase the adsorption of the solution [34]. A second reason might be the steric factors of the color substance and developer-solvent. These can influence the formation of their complexes and thus the stoichiometry of color complexes depends on the structure of both components. The ring-closing mechanism is attributed to the solvation of the colored complex of a color former and a developer [35]. Taking into account these factors, they can explain the color change of LCV during the LAMP reaction.

Another reason the crystal violet was chosen as an intercalator dye was because it allows the addition of pyrophosphatase (PPase) enzyme. Using the other two colorimetric dyes (HNB and Phenol red) this combination could not be performed. Following the suggested protocol from Miyamoto, *et al. 2015* [17], it was proposed the addition of pyrophosphatase in the LAMP reaction. They suggested that adding pyrophosphatase in LAMP reaction, reduces the reaction time and increase the efficiency of the amplification. In another work, it is mentioned that pyrophosphatase enzyme is used in LAMP reaction to eliminate the effect of magnesium pyrophosphate and it helps LCV makes a distinctive analysis in both negative and positive control samples [19]. However, in Figure S2, it showed that the addition of the thermostable inorganic pyrophosphatase didn't affect dramatically the reaction time. The time-to-positive results were almost the same. Moreover, the addition of 0.5 unit of pyrophosphatase was able to reduce the reaction time approximately by one 1 min

(Fig.8A and 8B). The excess amount of pyrophosphatase (Fig.11 and 10B), didn't seem to reduce more the reaction time. Due to the fact that in this study the polymerases that were used (Bst 2.0 and Bsm) have improved the amplification reaction properties compared to Bst DNA Polymerase the reaction time is already reduced before the addition of pyrophosphatase enzyme. These next generation polymerase enzymes display improved amplification speed in LAMP reaction. One the hand, the presence of pyrophosphatase didn't have any affect as it was expected the coloration of the LCV.

Besides LCV, also SYBER Green I dye was used in RT-LAMP assay. Despite the fact that SYBER Green I can change color from orange to yellow-green discriminating the negative and the positive sample, still it wasn't able to be used in real-time experiments. SYBER Green I is an intercalator dye where this results in polymerase not being able to continue amplification [36]. The concentration that was required so that the color can be distinguished in the reaction was high enough that intercalate the amplification of the template (100x were added). On the other hand, when SYBER Green I was added to the reaction after the end of the amplification the color changed immediately. For that reason, is dye could only be used in end-point LAMP and not at RT-LAMP assays.

The qcLAMP methodology was used with two different targets in this study, for Influenza A and for SARS-CoV-2. For Influenza A the development of the assay was restricted only to the detection of DNA that originate from PCR product. It was used as a reference template for creating a calibration curve. The concentration rage that was used was 1 to 10^9 copies per µL. For SARS-CoV-2, the assay was developed with extracted RNA from patients' nasopharyngeal or oropharyngeal swabs. The N (nucleoprotein) gene was the target in LAMP reaction. There was also ORF1a (ORF1a polyprotein) gene [37] available as a target, however it needed more time for the optimization of the protocol and the temperature that is optimal for LAMP amplification. In Figure 14D shows the total number of samples that where amplified with qcLAMP method. 6 negative samples that were positive for Influenza, CMV, RSV, Adenovirus, and Enterovirus virus were successfully identified as negative. Moreover, 4 out of 51 negative samples that previously reported as positive for SARS-CoV-2, were negative when the amplified in IRIS device with LAMP. Since RNA is not a stable molecule, after multiple times of freezing at -80^{o}C and thawing, it was discomposed. To verify the result a second qRT-PCR performed proving the loss of the RNA. In addition, blind tests were performed with 18 samples (6 samples positive for SARS-CoV-2 and 12 samples of which 9 were negative for SARS-CoV-2 and 2 were positive for Influenza) and both negative and positive where successfully identified. Analyzing the results, it emerged that the sensitivity of the qcLAMP method is 97.4% and the specificity is 100%. Along with the N gene target, RNase P was used as a positive control. All the patient's samples were successfully identified as positive.

Different colorimetric systems were used with the two templates (Influenza A and SARS-CoV-2) for the LAMP reaction. To investigate the limit of detection of the assay,

serial dilutions from the started samples were measured. LAMP method was able to distinguish the different concentrations successfully each time. More specific, the colorimetric system with LCV and Bst 2.0 polymerase from NEB was able to differentiate the dilutions (10^9 and 10^3 copies per µL) of Influenza A sample. In both cases of template, with DNA from Influenza A and with extracted RNA from SARS-CoV-2, the colorimetric system that has Bst 2.0 polymerase from NEB and Phenol Red as a colorimetric dye, could distinguish all the different concentrations up to 1-10 copies per µL (Fig. 13A and 14B). Also, Bst DNA/RNA polymerase from SBS Genetech with HNB dye was able to amplify concentrations of SARS-CoV-2 RNA that is commercially available up to 20 copies per µL. The achieved detection limit for DNA from Influenza And SARS-CoV-2 RNA extracted from patients is 1-10 copies per µL input and for commercially available SARS-CoV-2 RNA is 20 copies per µL input.

The limit of detection is not so low in the case of commercially available RNA due to the fact that after freezing and thawing the quality of the RNA decreases. Another reason is that the reaction is not prepared in an RNase free environment. The RNases that were in the buffers of in the sample could cause degradation of the template. To prevent this degradation, RNase Inhibitor was added to the reaction. It has positive effect to LAMP amplification as the enzyme doesn't affect the polymerase activity and at the same time inactivate the RNases.

Additionally, all the experiments showed that the concentration of the clinical samples is related to the time-to-positive results. For both different targets, Influenza A and SARS-CoV-2 the sample with the higher concentration changed color faster from the sample that has the less amount of copies (Fig. 13 and S4). In *Table 6* it can be seen the comparison between the qcLAMP and qRT-PCR assays for the detection of SARS-CoV-2 and Influenza A.

Table 6: Comparison of the qcLAMP and qRT-PCR assays for SARS-CoV-2 and Influenza A (modified from Papadakis et al., 2020 [26]).

Method	**qcLAMP**	**qRT-PCR**
SARS-CoV-2 and Influenza A assay		
Detection steps	1	2
Temperature	65°C	50°C (RT) (95°C – 55°C) x 45 cycles
Assay time	30 min	80 min
Sample-to-result time	45 min	2 h 30 min
Target/control	N gene/ RNase P/HA	RdRp gene/ RNase P/HA
Preparatory steps	NA extraction	NA extraction
Analytical performance	5 copies/ reaction	Not available

The results of the DNA/RNA polymerase comparison indicate that among the commercially available polymerases that were tested for the LAMP reaction, Bst DNA/RNA polymerase from SBS Genetech with HNB dye has the highest potential for rapid amplification, making it the most suitable enzyme for attempting to detect the extracted RNA and DNA of pathogens in samples. In Figure S5 are presented all the time-to-positive results for each polymerase. Bst DNA/RNA polymerase from SBS Genetech has the biggest repeatability and yield. Figure 15A shows the comparison of each polymerase. Bst DNA/RNA polymerase from SBS Genetech with HNB dye had greatly better performance in terms of amplification speed. It was able to reduce the time of the experiment approximately 3.35 min that it is almost 18.9% decrease, of the time that needs the color to change, compared with Bst 2.0 polymerase from NEB. Also, the fidelity of, Bst DNA/RNA polymerase was high, as it could also amplify rapidly low concentration templates. Moreover, is shows great tolerance to crude samples like saliva which contains a variety of different enzymes (Fig.16). In addition, it works just as well with RNA and DNA samples due to the reverse transcriptase activity that it has.

The Bst 3.0 polymerase from NEB had the least good performance. Both reverse transcriptase and polymerase activity almost did not exist. It couldn't amplify any of the both targets (Influenza A and SARS-CoV-2). Only one time amplified the template that was added to the reaction, but it wasn't included to the final results as it was considered to be a false positive result. Also, Bsm polymerase form Thermo Scientific had strong strand displacement activity as far as the DNA amplification, but it didn't have any reverse transcriptase activity. For the amplification of the RNA template it was necessary the addition of Reverse Transcriptase enzyme. *Table 2* synopsizes the properties of each polymerase.

Finally, the ultimate goal after enhancing the LAMP assay, was its application with crude samples, especially with saliva samples. Is seems that the presence of saliva does not affect the performance of LAMP reaction. With the addition of 20% saliva the activity of polymerase not reduced. Additionally, Bst 2.0 polymerase from NEB with LCV could successfully amplify DNA from Influenza A that was spiked into saliva sample (Fig 12). Besides DNA, Bst 2.0 polymerase from NEB and Bst DNA/RNA polymerase from SBS Genetech with HNB dye were able to perform the LAMP reaction using RNA or DNA from the two viruses spiked into saliva sample as a template with the addition of 2 μL saliva. Nevertheless, the amplification of low concentration of RNA template required the addition of the RNase Inhibitor for the deterrence of the degradation of RNA from the RNAases that exist in saliva. On the other hand, the detection of the RNaseP target in the saliva sample was more effortless as the Ribonuclease P gene is available as RNA and as DNA in saliva. Its detection wasn't affected from the addition of RNase Inhibitor as the concentration was already high enough. Also, experiments with human genomic DNA confirmed that more experiments should be done to clarify if RNaseP is amplified through DNA or RNA in the saliva samples. At last the solution that is included to the saliva collector from

SPECTRUM SOLUTIONS, LLC which contains chemotropic and lysis substances, provoke easily the cell lysis and subsequently the performance of LAMP reaction.

5. Conclusion

In conclusion, the goal of this study was the development of more efficient colorimetric methods that can reduce the time of detection of the isothermal amplification method. More specifically, the method that was applied was loop-mediated isothermal amplification (LAMP) technique. It was used for the amplification of DNA and RNA targets from two different viruses, Influenza A and SARS-CoV-2. The analysis of the results leads to the following conclusions:

Overall, the usage of crystal violet in the form of leuco crystal violet in real-time LAMP reaction was a new and promising colorimetric dye for the detection of DNA targets. It was used with Influenzasamples and was able to detect the amplicons in a really short time (before 15 min). Nevertheless, the comparison of the three different colorimetric dyes seems that have no difference as far as the reduction of the time of the reaction. The selection of LCV was because of the ability to intercalate to the DNA. Taking into account this information it was considered that it would reduce the prerequisite reaction time. It wouldn't rely on other factors of the reaction like pH and Mg^{2+}. Besides the intercalation to DNA that LCV provokes the DNA, pyrophosphatase was added to the reaction for even more reduction of the time-to-positive results. However, the addition of the pyrophosphatase in the reaction didn't reduce furthermore the time of the detection. Nonetheless, HNB, Phenol red and LCV had the same correspondence as colorimetric dye. All of them can change color alike. The only difference is the contrast of the color change. Phenol red has the best color contrasts with naked eye, starting from the negative sample, fuchsia to the positive sample, yellow.

Between the six different polymerases that were used, the one that stood out due to the final time-to-positive results, when RNA was used as a template, was the Bst DNA/RNA polymerase from SBS Genetech. It was able to reduce the time of the detection approximately 18.9% in a 30 min reaction Bst 2.0 polymerase from NEB. It appears that the combination of HNB and Bst DNA/RNA polymerase from SBS Genetech has the best results considering the detection with LAMP reaction.

Furthermore, a new portable device, IRIS, was used for the execution of experiments. Testing the limit of detection in this method with this device, it delivers promising results as the limit of detection was up to 1-10 copies per µL input. Specifically, the limit of detection of DNA from Influenzawas 1-10 copies/µL and of SARS-CoV-2 commercially available RNA was 5 copies/µL. Moreover, real samples were used for this study from patients with COVID-19 disease. The samples that were used in LAMP method were extracted RNA with Ct values ranging from 16 to 36.8. The fastest time-to-positive result with qcLAMP was 12.5 min (Ct=16) and the slowest was 23 min (Ct=36.7).

In the end, qcLAMP technique was used with crude saliva samples. In case of LCV colorimetric dye, the addition of the saliva didn't affect the amplification and the color change. Also, the detection of RNA spiked in saliva samples was successful, however in low concentrations of template is almost mandatory the addition of RNase Inhibitor to eliminate the RNA degradation from the RNase that are into the saliva.

6. Future perspectives

Although, it is considered that the goal of the thesis has been accomplished, there are plenty of improvements that could be done in order to achieve better results. First of all, different colorimetric methods could be used in LAMP assay. For instance, gold nanoparticles (AuNPs) have colorimetric properties in specific conditions. They can change the color of the solution in the presence of nucleic acids. Also, other synthetic organic molecules like polydiacetylenes (PDAs), can change their color when they aggregate with DNA.

In addition, in future work, more research is needed to apply and test the LCV dye in LAMP reaction not only with DNA but also with RNA. LCV needs to be added to the reaction that contains the Bst DNA/RNA polymerase from SBS Genetech Co. (the polymerases with the better results in this study). Controlling and stabilizing the color drift of the LCV that it is appeared during the experiment is also very important for the better discrimination of the results, especially when low concentrations of template are detected.

Future approaches should include the collection of saliva samples from patients that are positive to Influenza A or SARS-CoV-2 and the usage of LAMP technique of the detection. More specific, using saliva samples could reduce the time and the effort of the experiment as further sample preparation, such as RNA extraction, is not necessary. Colorimetric LAMP technique can be used also for other pathogens in various cases. Overall, creating an effective assay for saliva samples with the whole virus is one of the most important issue for point of care diagnostic tools and devices.

7. References

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8. Appendices

1. LCV colorimetric dye with different concentration of PPase

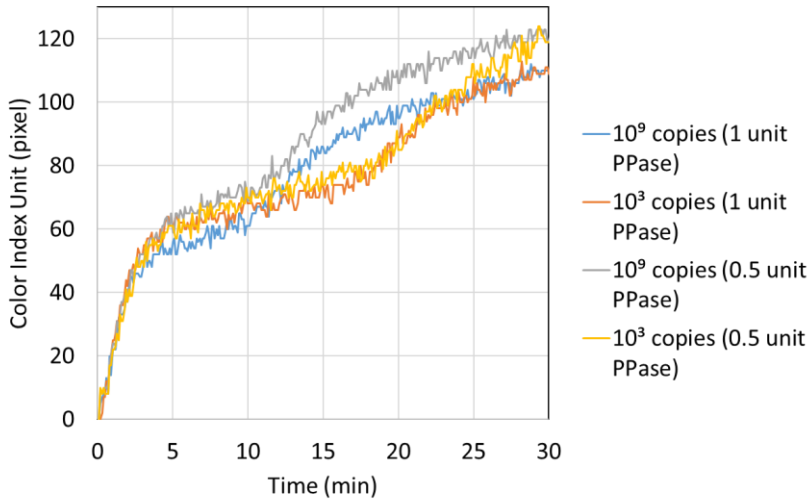


Figure S1: Comparison of two different concentrations of PPase with LCV dye qcLAMP assay.

2. Comparison of different temperatures for RT-LAMP with LCV dye

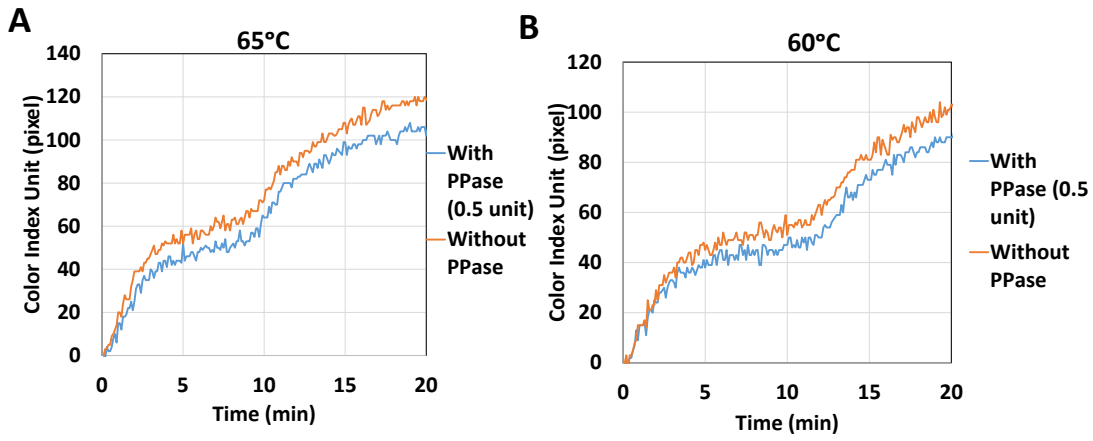
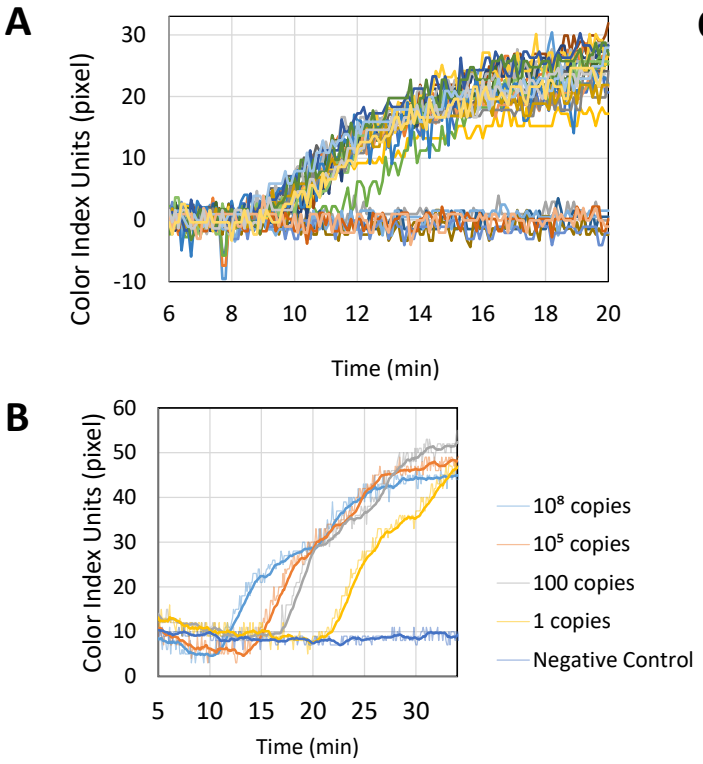


Figure S2: Different temperatures that was used for the same sample with Influenza A template with and without PPase enzyme.

3. Reproducibility of the qcLAMP assay with Influenza A samples



C

Number of samples	Time-to-positive (min)
1	9,7
2	10,2
3	10,9
4	10,1
5	11,7
6	10,2
7	9,4
8	10,5
9	10,4
10	10,1
11	9,2
12	10,6
13	10,6
14	10,2
15	9,9
16	10,2
17	9,6
18	9,9
19	9,7
20	10,5
21	10,7
AVERAGE TIME (min)	**10,2**
STANDARD DEVIATION	**0,6**

Figure S3: (A) Real-time data of total 21 of Influenza A samples (positive and negative controls) with 10^9 copies per µL. (B) Serial dilutions of an Influenza A sample with concentration 10^{11}. Limit of detection of Influenza A using qcLAMP reaction. (C) Table with all the Influenza A samples and the time-to-positive results of each one of them.

4. Limit of detection of the qcLAMP assay using SARS-CoV-2 RNA samples

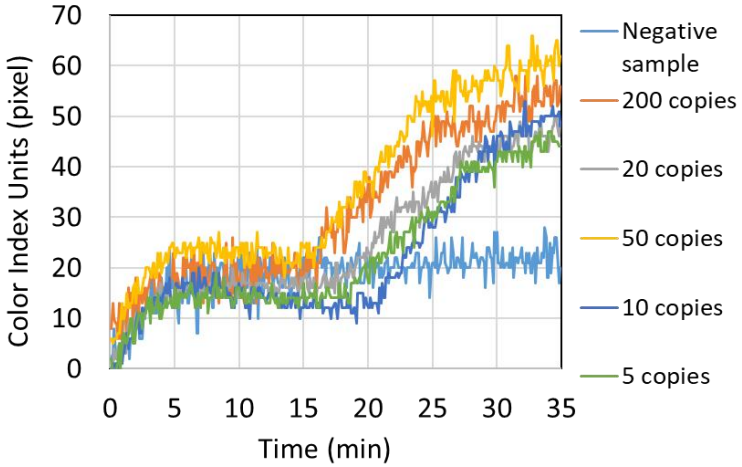


Figure S4: Serial dilutions of commercially available kit from Bio-Rad.

5. Comparison of six different polymerases

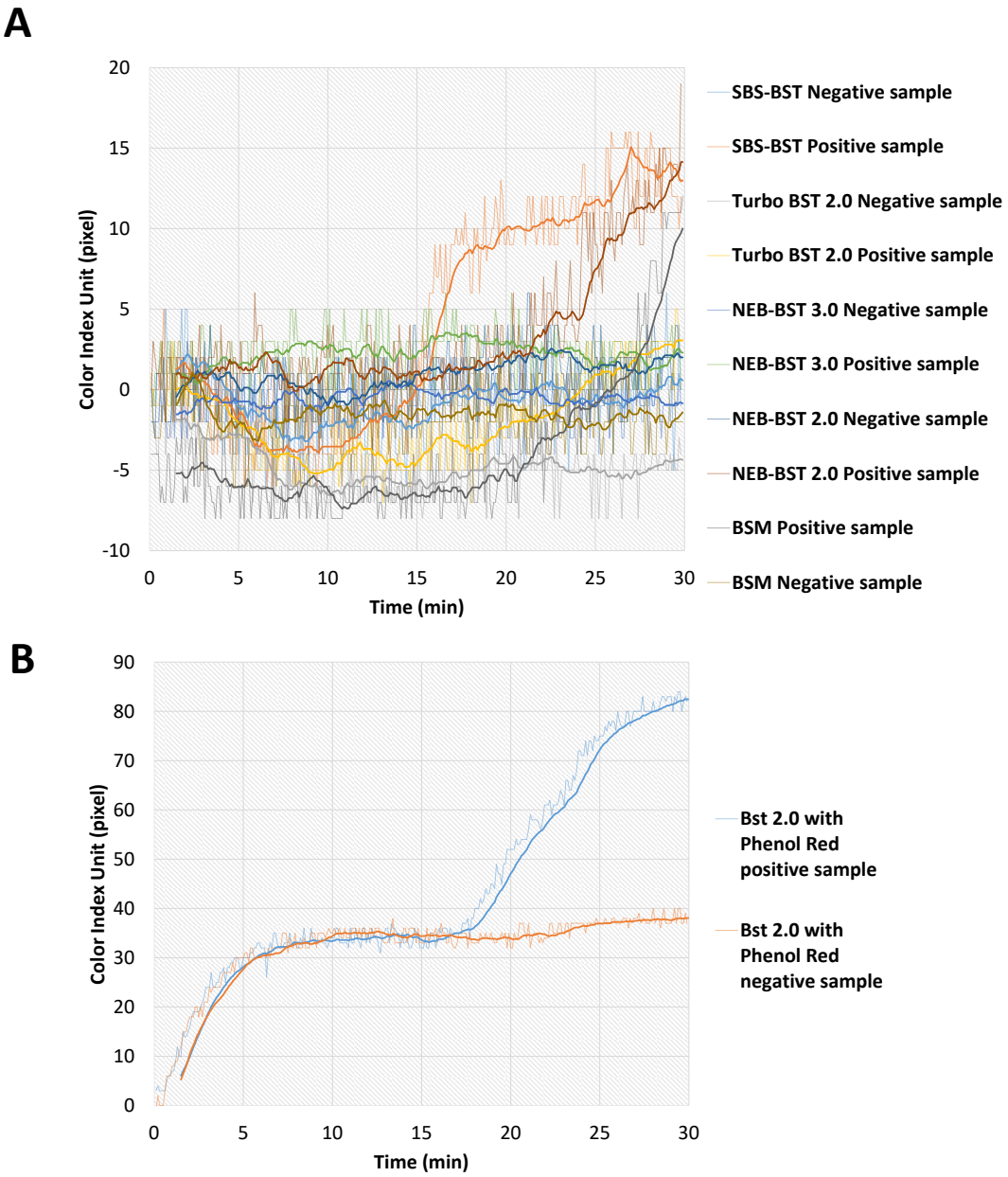


Figure S5: (A) Real time graphs from five different polymerases with qcLAMP assay and HNB dye. Commercially RNA from Bio-Rad was used with final concentration 200 copies per µL. (B) Real time graph from Bst 2.0 polymerase with qcLAMP assay and Phenol red dye. 200 copies per µL RNA were used from Bio-Rad.

6. Performance evaluation of colorimetric LAMP and fluorescent LAMP

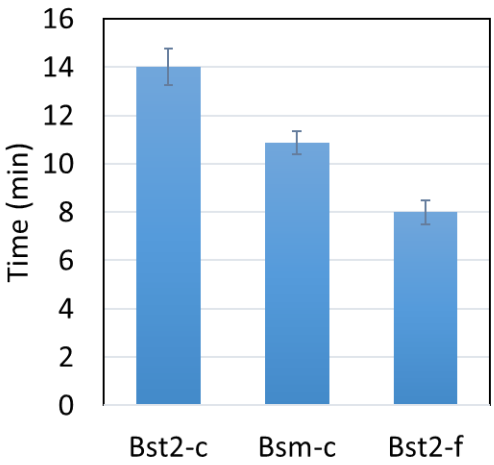


Figure S6: Comparison of the speed of detection of a LAMP reaction containing 10 bacteria as starting template using different combinations of 2 enzymes (Bst 2.0, Bsm), 2 colorimetric indicators (HNB, phenol red) and inside 2 real time systems (qcLAMP device, BIORAD). Bst2-c: Bst2 warm start polymerase mixed with either phenol red or HNB, tested with qcLAMP; Bsm c: Bsm polymerase (20 Units) with HNB, tested with qcLAMP; Bst2-f: Bst2 warm start with LAMP fluorescent dye tested in a real-time PCR machine (modified from Papadakis et al.2020 [26]).

7. Real-time graphs via the application

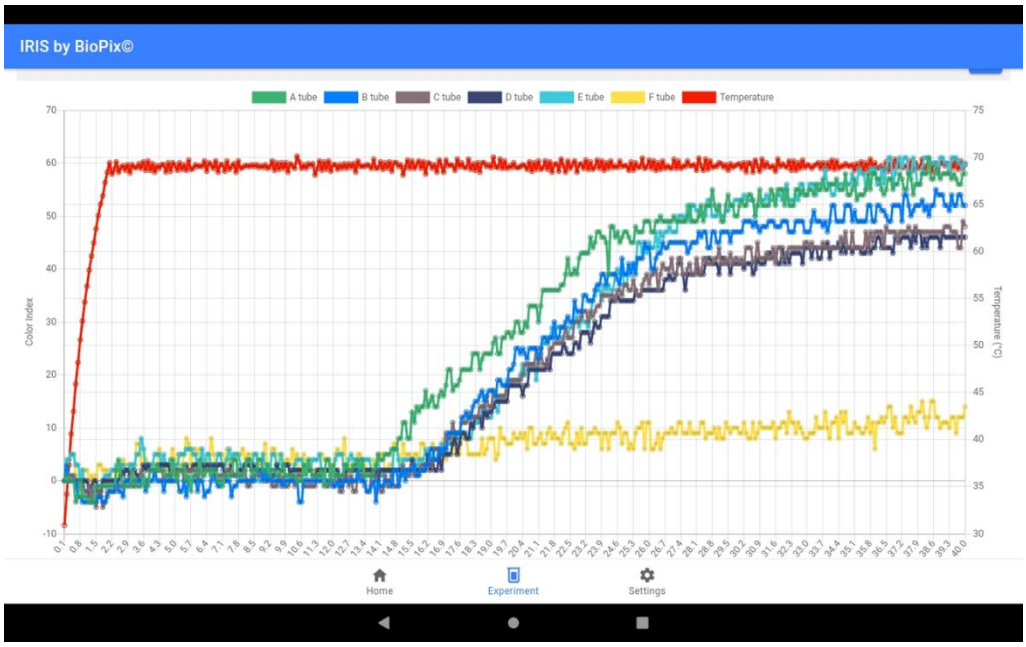


Figure S7: Real-time graphs as displayed in the application for 5 positive samples and one negative.