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Coronavirus Pandemic

An at-home and electro-free COVID-19 rapid test based on colorimetric RT-LAMP

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Abstract

Introduction: In the fight against virus-caused pandemics like COVID-19, diagnostic tests based on RT-qPCR are essential, but they are sometimes limited by their dependence on expensive, specialized equipment and skilled personnel. Consequently, an alternative nucleic acid detection technique that overcomes these restrictions, called loop-mediated isothermal amplification following reverse transcription (RT-LAMP), has been broadly investigated. Nevertheless, the developed RT-LAMP assays for SARS-CoV-2 detection still require laboratory devices and electrical power, limiting their widespread use as rapid home tests. This work developed a flexible RT-LAMP assay that gets beyond the drawbacks of the available isothermal LAMP-based SARS-CoV-2 detection, establishing a simple and effective at-home diagnostic tool for COVID-19.

Methodology: A multiplex direct RT-LAMP assay, modified from the previously developed test was applied to simultaneously identify the two genes of SARS-CoV-2. We used a colorimetric readout, lyophilized reagents, and benchmarked an electro-free and micropipette-free method that enables sensitive and specific detection of SARS-CoV-2 in home settings.

Results: Forty-one nasopharyngeal swab samples were tested using the developed home-testing RT-LAMP (HT-LAMP) assay, showing 100% agreement with the RT-qPCR results.

Conclusions: This is the first electrically independent RT-LAMP assay successfully developed for SARS-CoV-2 detection in a home setting. Our HT-LAMP assay is thus an important development for diagnosing COVID-19 or any other infectious pandemic on a population scale.

Key words: COVID-19 diagnosis; home-testing kit; rapid test; electro-free; RT-LAMP.

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Introduction

In December 2019, a new coronavirus (SARS-CoV-2) emerged, causing a global pandemic of COVID-19, a deadly pneumonia disease that quickly spread worldwide, resulting in a global outbreak [1]. Since then, hundreds of molecular diagnostic tests have been developed to detect SARS-CoV-2. Specific host antibodies, viral proteins, and viral RNA are the primary diagnostic targets, each with its advantages and limitations in accurately detecting SARS-CoV-2 during its infectious period to more effectively prevent its spread [2,3]. The current gold standard for SARS-CoV-2 diagnosis requires professional experience in sampling, performing the reaction, and analyzing the outcomes. It also necessitates the use of specialized machines, chemical reagents, advanced sample collection procedures, and transportation procedures, which can be especially challenging when faced with recent unprecedently large testing volumes.

Quantitative real-time PCR following reverse transcription (RT-qPCR) assays for detecting SARS-CoV-2 have been critical in preventing and managing the COVID-19 outbreak [4]. RNA purification, reverse transcription, and quantitative PCR are all common procedures for detecting viral RNA in patients [4]. These procedures are time-consuming, require the use of multiple biochemical reagents, equipment ranging from basic to advanced, and qualified personnel, and can only be conducted exclusively at central laboratories. As a result, they are unable to handle the rapidly increasing demand for testing suspected and silently infected patients. Moreover, with the emergence of asymptomatic infections and their potential for transmission, the number of people who need to be screened is rapidly increasing. Apart from RT-qPCR, detection methods based on immunoglobulin (IgM/IgG) antibodies have been expected to identify COVID-19 patients rapidly and easily at the point-of-care [5,6]. However, IgM antibodies are produced between 4 and 10 days after infection, while the IgG response takes about 2 weeks [7]. As a result, low-abundance antibodies in the sample

will produce false-negative results in the early stages of illness. Therefore, there is a need for diagnostic tools that can precisely detect SARS-CoV-2 RNA and be easily used at home for the rapid evaluation of the patients' treatment impact and prognosis. Early diagnosis is also essential for preventing virus transmission in communities and controlling the spread of the disease. Identifying infected individuals early on and isolating them can halt the further spread of the virus, thereby reducing the burden on healthcare systems.

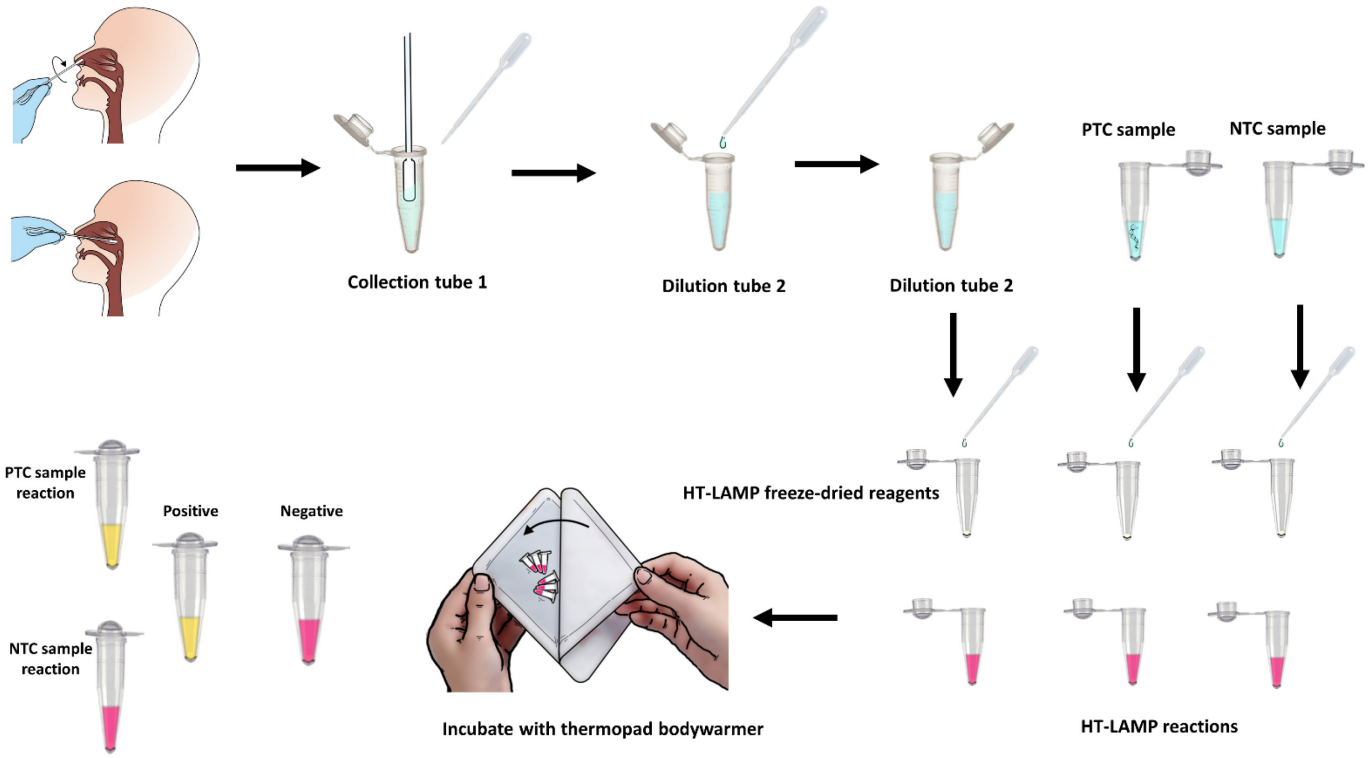
Different nucleic acid isothermal amplification approaches for SARS-CoV-2 RNA detection have been successfully developed [8,9]. Among them, colorimetric reverse-transcription loop-mediated isothermal amplification (RT-LAMP) appears to be the most attractive alternative diagnostic method because of its increased simplicity, quicker time to achieve a result, and lower cost. Nevertheless, they still require basic laboratory equipment and devices. Consequently, a rapid, low-cost test based on nucleic acid amplification that can quickly identify the presence of SARS-CoV-2 RNA at home is currently unavailable. In this study, we developed a COVID-19 home-testing RT-LAMP (HT-LAMP) kit that is rapid, reliable, sensitive, and electro-equipment-free. Our test employs a colorimetric readout to enable reliable SARS-CoV-2 detection while skipping the RNA extraction step. The COVID-19 HT-LAMP kit benefited from freeze-dried reagents and disposable body warmers, attempting to make COVID-19 molecular diagnosis more accessible and facilitating large-scale implementation even in settings with limited economic or infrastructural resources.

Methodology

SARS-CoV-2 Specimen Collection

Following the optimization of sample processing in a previous study [8], the SARS-CoV-2 Specimen Collection Kit is designed to collect patient samples specifically for the HT-LAMP assay. Sample Collection tube 1 and Dilution tube 2 contain 400 and

Figure 1. LAMP assay for SARS-CoV-2 detection in limited and home-settings.



A sterile sampling swab is used to collect nasal or nasopharyngeal samples. The swab is then dipped up and down and gently swirled into Sample Collection tube 1 for 10 times. The solution in tube 1 is then mixed by gently squeezing and releasing an eye dropper, and one drop is transferred to Sample Dilution tube 2. To mix the fluid in tube 2, gently squeeze and release the dropper several times. Next, transfer one drop of solution from tube 2 to a lyophilized LAMP reaction tube using a fresh dropper. Negative (NTC) and positive control (PTC) samples are similarly transferred into the corresponding control reaction tubes, by dropping them using fresh and separated droppers. The warmer is prepared by opening the package, removing the warmer, and shaking it a bit. Wait one to two minutes for the oxygen activation. Remove the protective foil and place the reaction tubes in the center of the adhesive part of the patch. Fold the patch in half carefully to completely cover the tubes. Wait for 60 minutes, then open the patch and examine the results by the color shift of the reactions. After a single usage, discard the body warmers as general garbage.

980 µL of nuclease-free water, respectively. Nasal or nasopharyngeal samples were collected with a sterile sampling swab, and the swab sample was dipped into Sample Collection tube 1. Gently swirl the swab in the tube and dip the swab up and down in the tube 10 times. Then, an eye dropper with a volume of 200 µL is used to mix the solution in tube 1 and transfer one drop with a volume of approximately 20 µL to the Dilution tube 2. Squeeze and release the dropper gently to mix the solution in tube 2. For samples collected in the viral transport medium (VTM) or the universal transfer medium (UTM), mix the sample with an eye dropper and transfer one drop to Tube 2.

HT-LAMP Assay

The HT-LAMP assay is a colorimetric multiplex RT-LAMP reaction that uses two sets of specific primers to simultaneously detect distinctive sequences of the N and ORF1ab genes of SARS-CoV-2, which were designed and optimized in a previous study [8]. The primers were synthesized by Phu Sa Biochem (Can Tho, Vietnam). The colorimetric multiplex RT-LAMP reaction was carried out as described previously [8]. Briefly, the reaction (20 µL) consists of 10 µL of WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA) (NEB, MA, USA), 1.6 µM each FIP/BIP primer, 0.2 µM each F3/B3 primer, and 0.4 µM each LoopF/LoopB primer. The HT-LAMP assay using lyophilized reagents was prepared and performed as in the earlier work [8]. The color shifting visualized the amplification products from red to yellow of the testing reaction, which is based on a pH-sensitive indicator, as instructed by the manufacturer. The assay includes a non-template sample that serves as a negative control (NTC) to confirm the absence of contamination. Positive control (PTC) samples are supplied to verify that the provided reactions are not faulty. A conventional eye dropper is used instead of an expensive micropipette to transfer a small amount of liquid, allowing for controlled dispensing during the assay in home-based testing.

The protocol of the HT-LAMP assay is shown in Figure 1. The collected specimens are first processed as described above. Next, using a new 200 µL dropper, transfer one drop of solution from tube 2 to a lyophilized LAMP reaction tube. The solution should now be bright pink. At the same time, activate the body warmer by tearing open the outer package. Cap the reaction tube tightly and place it in the center of the heating patch. Fold the patch so that it covers the entire reaction tube. Incubate the reaction for 60 min on the heating patch, then examine the results by color. Both controls (NTC and PTC) must be used to accurately display the expected color. The positive control should turn yellow/orange, and the negative control should remain pink/red. A positive sample will change from pink to yellow/orange, while a negative sample will remain pink/red.

Table 1. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Cycle threshold (Ct) values of SARS-CoV-2 positive samples.

Sample	RT-PCR Ct value
1	16.42
2	23.48
3	17.05
4	18.32
5	19.24
6	14.46
7	16.98
8	17.05
9	16.34
10	16.61
11	20.92
12	22.8
13	19.49
14	29.88
15	27.88
16	17.39
17	18.06
18	20.8
19	27.29
20	26.6
21	23.9
22	38.26
23	10.04
24	32.17
25	22.05

Evaluation of HT-LAMP Kit

Fifty-two nasopharyngeal and oropharyngeal swab specimens of suspected COVID-19 patients were used to evaluate the clinical performance of the COVID-19 HT-LAMP kit. The specimens were collected in VTM/UTM or nuclease-free water [8] and kept at -80 °C until analysis. Viral RNA from clinical samples was isolated using the QIAamp RNA mini kit (Qiagen, USA). The presence of SARS-CoV-2 (Table 1) was confirmed by the Luna® Universal One-Step RT-qPCR Kit using the F3/B3 primer set [10]. The collected specimens were processed as described above. The diluted samples in tube 2 were transferred by one drop (approximately 20 µL) to the lyophilized reaction tube using an eye dropper with a capacity of 200 µL. The color of the reactions determined the results after incubation on the heating pad for 60 minutes.

Results

Evaluation of operating temperature stability of the commercial thermopads

The evaluation aimed to determine the stability and consistency of the operating temperature of commercial thermopads to function as thermal incubators within the ideal range for LAMP reactions, between 60 and 65 °C. Here, four different commercial body warmers were tested to evaluate their heating capabilities. The results revealed that it took at least 15 minutes for the four different types of thermopads to reach their peak temperatures (Figure 2A). The weakest performer was type 3, which had the lowest maximum temperature and fastest heat dissipation rate. Type 2 and type 3 could reach temperatures of 55-60 °C between 20 and 30 minutes and sustain the heat for up to 70 minutes. Type 4 performed the best regarding maximum temperature reach, stability, and heat maintenance. Multiple tracking data also showed that type 4 thermopad provided the heating temperature and duration necessary for the LAMP reactions (Figure 2B).

When it comes to using pocket body warmers, incubation time is one of the critical parameters that must be defined. At the beginning of the heating process, the HT-LAMP reaction tubes were placed on the pads. After 50 minutes, the reactions showed the expected color shift from pink to yellow and products with better quality signals (Figure 3). Therefore, a longer incubation time of 60 minutes would ensure that all HT-LAMP reactions occur completely.

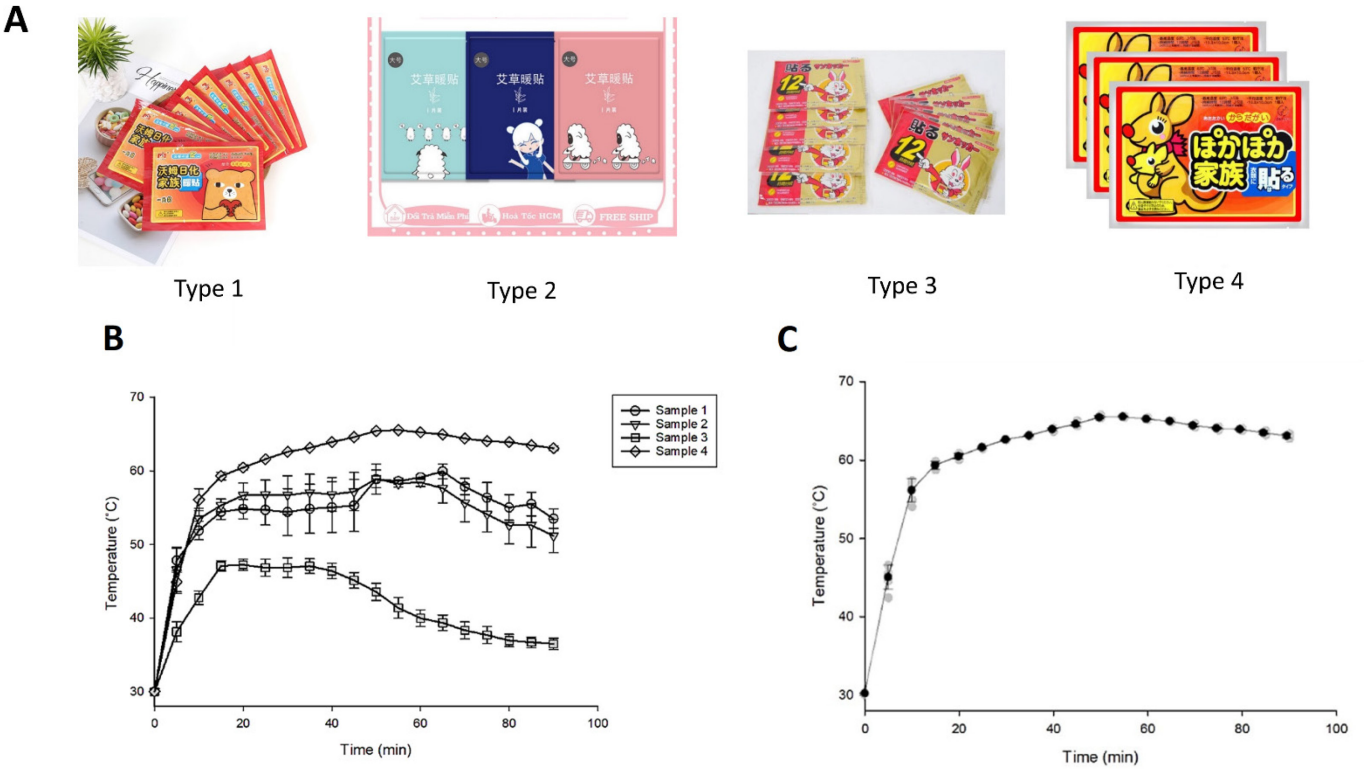
Limit of detection (LOD) of HT-LAMP Assay

Multiplex RT-LAMP was carried out to identify the LOD of the HT-LAMP assay. Extracted genomic-RNA of SARS-CoV-2 was quantified via a standard curve based on the Ct values of RT-qPCR as described in the previous study [11], and then serially diluted at the indicated concentration. The LOD_{95} value, the lowest concentration at which there is a 95 % probability of detecting the target, was found to be 52.365 (CI 20.046 – 136.416) copies per reaction (Table 2), equivalent to 2.62 copies per reaction µL.

Evaluation of HT-LAMP

Nasopharyngeal swabs were collected from 52 suspected COVID-19 patients. Of these, 25 samples

Figure 2. Temperature monitoring of the thermopads.



(A) Types of disposable bodywarmers tested. Type 1 is produced by Dongyang Dengpin Daily Chemical Co., Ltd. (China). Type 2 is made by Zhejiang-Yiwu Worm Supplies Co., Ltd (China). Type 3 is manufactured by Kobayashi Pharmaceutical Co., Ltd (Japan). Type 4 is from Iris Ohyama Inc. (Japan). (B) The temperature was recorded every 5 minutes. Using 4 different pads of each type, the experiment was conducted 5 times independently. (C) Following the seal's removal, the type 4 thermopad's temperature was measured every 5 minutes. Using 15 different pads, the experiment was conducted 15 times independently. The line represents the average values. Error bars indicate the standard deviation.

were positive and 27 samples were negative according to the RT-PCR results. All 52 samples were tested using the HT-LAMP assay to validate the home-testing kit performance. As a result, the HT-LAMP assay showed 100% sensitivity and specificity compared to RT-PCR (Table 3).

Discussion

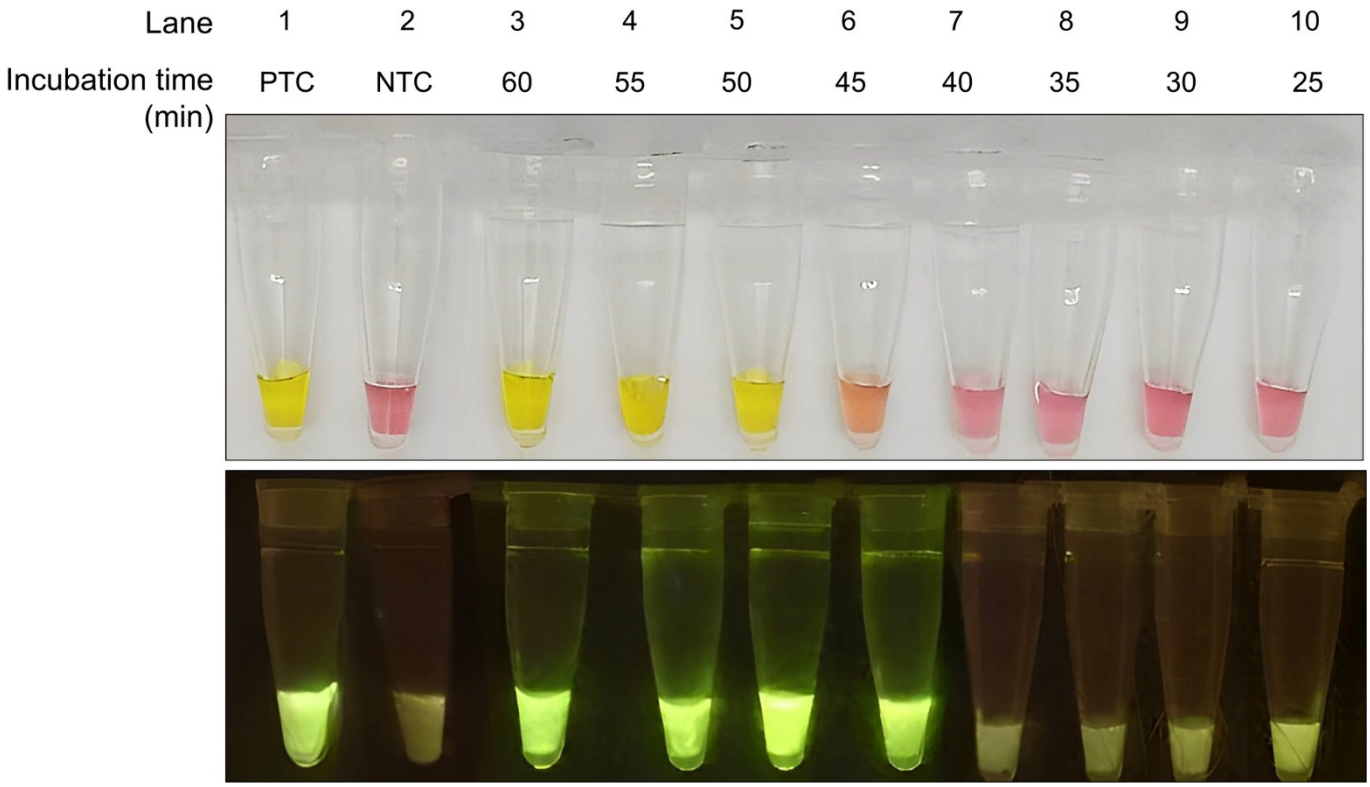
The current gold standard technique for detecting SARS-CoV-2 in specimen swabs is RT-PCR, which requires laboratory-based viral extraction techniques to isolate viral genetic material [4]. However, there is an urgent need for alternatives that can increase testing capability in limited settings and point-of-care settings, despite the current laboratory-based approach for SARS-CoV-2 is scalable and can be automated for high throughput. The current pandemic has drawn attention to the use of LAMP for COVID-19 diagnostics, which requires more effort and attention in primer selection than other nucleic acid amplification methods, but offers a simpler and more efficient diagnosis in return. To ensure specificity and avoid false positives, two or three sets of primers must be carefully designed and optimized to target the desired DNA sequence while do not bind to other genomic regions, making LAMP amplifies DNA with high specificity and reducing the likelihood of non-specific amplification. Additionally, the isothermal amplification process used in LAMP allows for the amplification of DNA at a constant temperature, which also decrease the probability of non-specific amplification due to temperature fluctuations. Moreover, the amplified products of LAMP can be easily detected using methods such as colorimetric or fluorescence detection. Besides, LAMP has the benefit of having high tolerance to impurities

Table 2. LOD evaluation of HT-LAMP assay.

Viral RNA copies (per reaction)	Ratio of positive tests to the total test number
2×10^4	3/3
2×10^3	3/3
2×10^2	3/3
20	3/3
10	1/3
5	0/3
2	0/3
LOD	52.365 (95%, CI 20.046-136.416)

The viral RNA was serially diluted in nuclease-free water to the indicated concentrations, and 20 µl of the diluted sample was added into the HT-LAMP reaction. PODLOD ver11 [12] was used to calculate the LOD95 value. LOD: Limit of detection; CI: confidence interval.

Figure 3. Optimization of the incubation time for HT-LAMP assay.



The LAMP reaction containing 102 copies of SARS-CoV-2 genomic-RNA template [8] was incubated in bodywarmers for 25 to 60 minutes. The color was captured by the personal mobile phone (upper panel) and the SYBR Green I fluorescent signal was visualized by a blue light transilluminator (lower panel). PTC stands for positive-template control; NTC stands for non-template control.

Table 3. Sensitivity and specificity of HT-LAMP assay.

RT - qPCR	HT-LAMP		Total
	Positive	Negative	
Positive	25	0	25
Negative	0	27	27
Total	25	27	52
Sensitivity: 100%; Specificity: 100%			

RT-qPCR: Quantitative reverse transcription polymerase chain reaction.

[13]. Consequently, the simplicity of the equipment and techniques involved in LAMP make it an attractive diagnostic method for various applications.

Significant advancements in colorimetric RT-LAMP assays have led to the development of more reliable, sensitive, and straightforward SARS-CoV-2 detection tests. However, the assay still requires specialized lab tools, such as temperature-controlled incubators or fine pipettes. Therefore, we investigated methods for adapting the RT-LAMP protocol completely to home settings. Due to the isothermal nature of RT-LAMP reactions, steady incubation temperatures of 60–65 °C are required. High-end instrumentation and the most basic arrangement, in which boiling and room temperature water are combined in a certain ratio and then kept insulated, can both be used to produce a temperature-controlled reaction environment. A commercially available sous-vide heater (water bath) has also been proposed previously for home-based testing [14]. However, this approach still demands an electrical device and a water-based setup, which are not always available and convenient for in-home testing. To establish the assay free of electrical devices, we used commercially available, inexpensive, convenient, disposable, and instrument-free thermopad body warmers. Thermopads generate heat through oxidation reactions. The heat is produced by the reaction of iron powder with oxygen in the air, causing it to rust and release heat. Activated carbon acts as a catalyst to speed up the oxidation reaction, while vermiculite and salt act as moisture absorbers to prevent premature oxidation. Finally, water is added to initiate the oxidation reaction and activate the mixture. Typically, the body warmer comes with a special textile adhesive. The heat pad warms up automatically as it comes into contact with oxygen and can provide natural heat for up to 6 or 12 hours.

In this study, we have showed that a specific brand of thermopads successfully delivered the required heating temperature and duration for the LAMP reactions. We have also confirmed that one of the most rapid, straightforward, affordable, and scalable protocols for detecting SARS-CoV-2 nucleic acid is not only highly specific but also reliable, as compared to RT-PCR detection. The LOD results indicated a robust performance of the colorimetric HT-LAMP assay across a broad range of genomic-RNA samples, meeting the clinical requirement of the viral load of SARS-CoV-2 in clinical samples [15]. In particular, a study conducted on more than 3303 patients who were confirmed positive for SARS-CoV-2 estimated that the viral load in swab samples was in the range of 10^5 to 10^8 copies per mL of sputum [15], which can be easily measured using our HT-LAMP assay. The data on primer specificity and comparison to real-time PCR demonstrate the reliability of the colorimetric LAMP assay developed. However, to further enhance the specificity of the colorimetric assay, it may be beneficial to utilize a probe-based system as previously described [16]. Therefore, additional investigation into this approach is recommended. Most importantly, our HT-LAMP protocol does not require an RNA purification step, and the method does not need complicated equipment other than pocket-size body warmers and eye droppers. From sampling to detection, the protocol takes about 65 minutes, only requires a few reagents, and can even be self-performed by non-professionals. Furthermore, the HT-LAMP assay results were compared to those of RT-PCR with a 100% correspondence rate. These characteristics indicated that the HT-LAMP assay could be used as an alternative tool for SARS-CoV-2 detection, allowing for a rapid and electro-equipment-free diagnosis in a home setting.

Rapid antigen detection tests (RADTs) are widely used for diagnosing SARS-CoV-2 infections in restricted settings, but they have limitations. The HT-LAMP test, developed here, offers several advantages over RADTs, which are crucial for disease control and management. First, LAMP has higher sensitivity compared to RADTs, with various studies reporting sensitivity up to 90-95%. Secondly, RADTs can also produce false-positive results due to cross-reactivity with other viral antigens or non-specific binding of the test antibodies. In contrast, LAMP is a molecular technique with significantly higher specificity due to the use of multiple primers. Third, RADTs may not detect SARS-CoV-2 at early stages when the viral load is low, while LAMP is capable of detecting the virus at very low titer. Lastly, RADTs may not detect new variants of SARS-CoV-2 that have mutations in the

target antigen. In contrast, LAMP is flexible and the primer sequences can be adjusted rapidly to recognize new variants. Additionally, the evaluation of new primer designs is much faster and easier than the development of RADTs against new variants, which is time-consuming and labor-intensive.

Conclusions

Our advancements over the current RT-LAMP procedures allow for reliable and affordable detection of SARS-CoV-2. The findings serve as the foundation for further clinical performance investigations. We recommend testing this protocol on a larger cohort of clinical specimens to further promote the use of HT-LAMP as a rapid home test for identifying COVID-19 patients. This SARS-CoV-2 detection technique can be used as a surveillance tool for sampling large populations after receiving additional validation. Indeed, its ease of use, product availability, and low cost make it convenient to continuously monitor suspect-infected individuals. Additionally, this method can be applied in various places, such as workplaces, nursing homes, medical facilities, and points of entry.

Last but not least, this technique can be easily modified for detecting any other pathogen, which will be crucial when new infectious diseases emerged. Access to diagnostic testing for everyone is essential for effectively fighting any outbreak, and developing an electro-equipment-free RT-LAMP assay will be a significant advancement to meet this requirement.

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Ethics approval

The hospital management approved the sample collection. The internal use of samples was agreed upon under each participating individual's medical and ethical rules. The Research Ethics Committee of Nguyen Tat Thanh University approved the study.

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