A comparative study of isothermal nucleic acid amplification methods for SARS-CoV-2 detection at point of care

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ABSTRACT

The COVID-19, caused by the novel coronavirus SARS-CoV-2, has broken out of control all over the globe and put the majority of the world under lockdown. There have been no specific antiviral medications for SARS-CoV-2 while vaccines are still under development. Thus, rapid diagnosis and necessary public health measures are currently key parts to contain the pandemic. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is the gold standard method for SARS-CoV-2 detection. However,

this method is not suitable for point-of-care (POC) diagnosis because of the time-consuming procedure, the requirements of biosafety conditions and expensive equipment. In this study, the colorimetric isothermal nucleic acid amplification tests (iNAATs) for SARS-CoV-2 based on loop-mediated isothermal amplification (LAMP), cross-priming amplification (CPA), and polymerase spiral reaction (PSR) were developed and compared. The three methods exhibited similar performance with the limit of detection (LOD) as low as just 1 copy per reaction when evaluated on the synthetic DNA fragments. The results can be read with naked eyes within 30 minutes without cross-reactivity to closely related coronaviruses. When tested with SARS-CoV-2 extracted genomic-RNA, LAMP outperformed both CPA and PSR assays. Moreover, the direct detection of SARS-CoV-2 in simulated patient samples (oropharyngeal and nasopharyngeal swabs) by colorimetric iNAATs was also successful. Further preparation of the lyophilized reagents for LAMP reactions revealed that the freeze-dried, ready-to-use kit maintained the sensitivity and LOD value of the liquid assays. These results strongly indicate that the colorimetric lyophilized LAMP test kit developed herein is highly suitable for detecting SARS-CoV-2 at POC.

Keywords: SARS-CoV-2; nucleic acid amplification test; LAMP; CPA; PSR; colorimetric; lyophilized kit; crude samples.

INTRODUCTION

Coronavirus is a large family of RNA viruses, including the human coronavirus 229E, OC43, NL63, and HKU1, which often lead to respiratory illnesses with mild cold symptoms [1, 2]. The two exceptions of human coronavirus that cause severe diseases including the fatal Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) [3] and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) [4] that caused two global outbreaks in the last two decades. Very recently, the mortal pneumonia disease caused by a novel a coronavirus called SARS-CoV-2, named "COVID-19" by the

World Health Organization (WHO), started in Wuhan, China and rapidly spread all over the globe [5]. In March 2020, WHO has classified the COVID-19 outbreak as "Global Pandemic" [6]. As of May 24th, 2020, COVID-19 has spread to over 210 countries and regions worldwide with more than 5.4 million confirmed cases and 344,000 casualties [7].

The diagnostic standard of SARS-CoV-2 involves clinical symptoms and molecular methods. For an accurate diagnosis, the molecular methods include metagenomic next-generation sequencing (mNGS) and quantitative reverse transcription PCR (qRT-PCR) are required. mNGS is the most precise technique, but it is extremely expensive, time-consuming, and reliant on highly skillful personnel. qRT-PCR is sensitive for the detection of SARS-CoV-2, which was introduced by WHO [8]. The test can be carried out using respiratory samples such as nasopharyngeal or oropharyngeal swabs, which is currently widely used as the gold standard method for SARS-Cov-2 diagnosis. Nevertheless, qRT-PCR requires high-cost equipment and results are only available within a few hours to 2 days, limiting its application at resource-limited settings.

Different virological and serological approaches for rapidly detecting SARS-CoV-2 at POC have been introduced. Virological diagnosis directly detects the viral nucleic acids via isothermal nucleic acid amplification tests (iNAATs) [9-12] and CRISPR□Cas12 based method [13]. Serological tests detect the rising titers of antibody between acute and convalescent stages of infection or detect IgM in primary infection [14-17]. However, serological diagnosis usually shows lower sensitivity especially in the early stage of infection [18]. Meanwhile, like PCR, iNAATs that amplify the viral nucleic acids at a constant temperature are expected to determine the presence of infectious viruses even in the patient without clinical symptoms. Loop-mediated isothermal amplification (LAMP) was introduced in 2000 and broadly utilized nowadays [19]. LAMP was shown to be rapid, specific, and remarkably sensitive compared to

conventional PCR [20, 21]. LAMP uses only one kind of DNA polymerase possessing the strand displacement activity and no modified/labeled DNA probes, simplifying the preparation procedure and significantly saving the cost [22]. Other isothermal DNA amplification methods developed later, which also depend on the use of a DNA polymerase strand displacement activity, include cross-priming amplification (CPA) [23, 24] and polymerase spiral reaction (PSR) [25]. While LAMP requires two to three primer pairs, PSR needs only one primer pair, and CPA uses multiple cross-linked primers (six to eight primers). CPA and PSR amplicon can also be visualized via detection methods that have been used for LAMP, such as using SyBr Green or a pH-sensitive indicator [26]. Both PSR and CPA methods have similar benefits to LAMP, including easy operation, low-cost equipment, and simple and fast readout of the results, which makes them ideal for resource-restricted settings. Various studies indicated that PSR and CPA performance regarding sensitivity and specificity was comparable to that of LAMP [27-31].

In this study, we developed and compared colorimetric LAMP, CPA, and PSR for SARS-CoV-2 utilizing a pH-sensitive dye for readout visualization. LAMP exhibited the best LOD value with respect to the identification of SARS-CoV-2 viral genomic-RNA. The ready-to-use lyophilized LAMP kit could detect SARS-CoV-2 genomic-RNA directly in clinical samples, which is suitable and convenient for POC diagnostics.

MATERIALS AND METHODS

Primer design

Primers for LAMP, CPA, and PSR assays targeting the *N* and *Orf1ab* sequences of SARS-CoV-2 (GenBank accession number MN908947) were designed using the free online software Primer Explorer V5 (https://primerexplorer.jp/e/). Primer selection was carried out as instructed (https://primerexplorer.jp/e/v4_manual/). Two sets of primer

pairs for LAMP (targeting the *N* and *Orf1ab* genes), two sets of primer pairs for PSR (targeting the *N* and *Orf1ab* genes), and one set of primer pairs for CPA (targeting the *Orf1ab* gene) were selected. Primers were synthesized by Phu Sa Biochem (Can Tho, Vietnam) and their sequences are listed in Table 1.

Synthesized DNA template preparation

The sequences from 282740 - 28516 of the *N* gene and from 2853 - 2452 of the *Orf1ab* gene of SARS-CoV-2 (GenBank MN908947) were selected to serve as the control templates for iNAATs. The *N* and *Orf1ab* sequences were obtained from Phu Sa Biochem, (Can Tho, Vietnam). The DNA templates of MERS-CoV, SARS-CoV, and bat SARS-like-CoV were prepared similarly. The sequences of synthesized DNA templates are listed in Table 1.

Primer specificity analysis

The reference genomes of SARS-CoV-2 and related species were downloaded from NCBI (https://www.ncbi.nlm.nih.gov). The primer sequences were aligned to genomes of different coronaviruses to calculate the number of mismatches using Geneious Prime 2020.0.3 (https://www.geneious.com). The percentage of mismatch was calculated by dividing the total number of different bases between primers and genome sequences to the total length of primers. The software FastPCR available at http://primerdigital.com/fastpcr.html was used for *in silico* PCR analysis.

Viral RNA preparation

The genomic RNA of SASR-CoV-2 was prepared in a biosafety level 3 laboratory in Pasteur Institute, Ho Chi Minh City, Vietnam. Vero E6 cells were infected with SARS-CoV-2 isolated from the oropharyngeal swab samples of COVID-19 patients in Vietnam

[32, 33]. After 72 h of infection, the virus culture was collected and heated at 65 °C for 1 h. Viral genomic-RNA was isolated using the QIAamp viral RNA extraction Kit (Qiagen, Hilden, German) following the manufacturer's instruction. The copy number of extracted genomic RNAs of SASR-CoV-2 was calculated using the standard curve based on qRT-PCR Ct-value as described in the previous study [33].

Collection and preparation of clinical specimens

Nasopharyngeal and oropharyngeal swab specimens from volunteer nurses and doctors were collected at a local hospital (Ho Chi Minh City, Vietnam). The volunteers had been already diagnosed negative with SARS-CoV-2 by qRT-PCR. All participants gave their informed consent to participate. Oropharyngeal and nasopharyngeal specimens were collected using a sterile flocked plastic swab which was then soaked into 400 µl of nuclease-free water. Fresh samples were kept on ice until analysis or frozen for subsequent assays.

To prepare the simulated clinical specimens, various concentrations of SARS-CoV-2 synthesized DNA or extracted viral genomic-RNA were spiked into the nasopharyngeal and oropharyngeal swab collected. Next, the simulated swab samples containing the spiked synthetic-DNA were 10-fold diluted in the nuclease-free water and 1 µl of the diluted sample was added to the iNAAT reactions. The simulated swab samples containing the viral RNA were 50-fold diluted in the nuclease-free water and 5 µl of the diluted sample were added to the LAMP reactions. Non-spiked specimens were used as negative samples.

Ethical statement

The sample collection was approved by Hospital Management. The internal use of samples was agreed under the medical and ethical rules of each participating individuals.

PCR assays

The PCR assays were carried out in a 20 µl reaction volume containing 0.2 µM each of primers F3 and B3 (LAMP), 1 µl of DNA template, 0.2 µl of MyTaq DNA polymerase (Bioline, London, UK) and 4 µl of 5X MyTaq reaction buffer (Bioline, London, UK). The amplification products were analyzed by electrophoresis using a 2% agarose gel.

Colorimetric iNAATs

WarmStart® Colorimetric LAMP 2X Master Mix (DNA & RNA) was purchased from NEB (MA, USA). The iNAAT reaction volume was 15 µl consisting of 1 or 5 µl of template sample and 7.5 µl of Colorimetric Mastermix. The LAMP reaction contains 0.8 µM each inner primer (FIP and BIP), 0.1 µM each outer primer (F3 and B3), 0.2 µM each loop primer (FLoop and Bloop). The CPA reaction contains 0.5 µM cross primer 1s, 0.3 µM each of primers 3a and 2a, 0.05 µM each of displacement primers 4s and 5a. The PSR reaction contains 1.6 µM each primer (PSR-F and PSR-R). The iNAAT reactions were run in BioSan Dry block thermostat Bio TDB-100 for 30 to 45 minutes (min) at 60 °C for LAMP and 63 °C for CPA and PSR reactions. The amplification products were detected by the color shifting from red to yellow of the test reaction, which is based on the use of phenol red, a pH-sensitive indicator as instructed by the manufacturer. The products were also analyzed by electrophoresis on a 2% agarose gel when necessary.

Optimization of Colorimetric iNAATs

One nanogram of the synthesized DNA template was used to perform the optimization experiment. Regarding the identification of optimal incubation time, the iNAAT reactions were incubated from 5 to 70 min at 60 °C for LAMP and 63 °C for CPA and PSR reactions. As for temperature optimization, the reaction mixtures were incubated at different temperatures from 50 °C to 70 °C for 30 min.

Evaluation of LOD of Colorimetric iNAATs

The iNAAT reactions were carried out in the duration and at the temperature optimized using synthetic-DNA templates. The copy number of the synthetic DNA template was calculated using Endmemo program (http://endmemo.com/bio/dnacopynum.php). The synthesized DNA template was serially diluted to various concentrations and 1 µl of the diluted DNA sample was added to the iNAAT reactions. The reactions were then incubated for 30 – 45 min at different temperatures according to each method. Extracted genomic-RNA of SARS-CoV-2 was quantified via a standard curve based on Ct-value and then serially diluted. Five µl of the diluted RNA samples were added to the iNAAT reactions. The reactions were then incubated for 45 min at the proper temperature.

RESULTS

Optimization of SARS-CoV-2 colorimetric iNAATs with the synthesized DNA templates

The synthesized sequences of the *Orf1ab* and *N* gene of SARS-CoV-2 were utilized as the template to perform the LAMP, CPA, and PSR reactions in the presence of the pH-sensitive indicator phenol red. The results indicated that the color change from red to yellow of the iNAAT reactions corresponding to the amplified products generated only when the relevant DNA templates were present (Fig. 1).

Next, the optimal temperature and required time of iNAAT reactions for the detection of SARS-CoV-2 were defined. For LAMP reactions, 15 min was the minimum time required for the readout of positive amplification judged by eye (Fig. 2A, left panel). Interestingly, *Orf1ab*-targeting primers performed the amplification process faster than the *N*-targeting primers as the product could be observed just after 10 min of incubation

based on the gel electrophoresis result (data not shown). LAMP amplicons were produced from 52 to 70 □, however, the clearest color changes were observed at 60 °C and higher temperature (Fig. 2A, right panel). Therefore, to guarantee the outcome signal, the conditions of LAMP reaction were set at 60 °C for 30 min. Regarding CPA and PSR reactions, amplified products could be observed the best after 30 min (Fig. 2B and C, left panels). Meanwhile, 61 and 63 °C were the minimal temperatures for CPA and PSR amplified products, respectively, to be visualized by the color change (Fig. 2B and C, right panels). Thus, 30 min and 63 °C conditions were selected to perform CPA and PSR reactions.

Specificity of SARS-CoV-2 colorimetric iNAATs

The sequences of primers used for iNAATs were aligned to genome sequences of different strains of coronaviruses including 13 SARS-CoV-2 strains, MERS-CoV, SARS-CoV, human coronavirus strains related to the common cold (HKU1, OC43, NL63, and 229E), bat SARS-like-CoV, Murine hepatitis virus (*Murine coronavirus*), and *Betacoronavirus England* 1. The results showed that 0% mismatch with all tested SARS-CoV-2 strains (MN938384, MN975262, MN985325, MN988668, MN988669, MN988713, MN994467, MN994468, MN997409, MT007544, MT121215, MT123292 and NC045512) was observed, suggesting that the developed iNAATs could detect different strains of SARS-CoV-2 (Table 2). In contrast, except for bat SARS-like-CoV 2015 and 2017 strains, most of the other coronaviruses gave nucleotide mismatch higher than 20% with our designed primers. Thus, it is likely that the designed primer sets would not amplify those sequences, ensuring the specificity of iNAATs for SARS-CoV-2 (Table 2). *in silico* PCR results also support the high specificity of primer sets used (SI 1 file). Further data demonstrated that the iNAATs primer sets used selectively detected the presence of SARS-CoV-2 DNA while no cross-reactivity was observed with DNA of

SARS-CoV, MERS-CoV, and bat SARS-like-CoV (Fig. 3), confirming the absolute specificity of the assay.

Limit of detection of Colorimetric iNAATs for synthesized SARS-CoV-2 DNAs

The LOD value of iNAAT reactions was evaluated using a serial-dilution of the synthesized DNA templates in nuclease-free water. As shown in Fig. 4A and B, roughly a single copy of the synthesized targeted gene per reaction was the lowest amount that LAMP and CPA assays could detect. As for PSR, LOD of the primer set targeting the *Orf1ab* sequence was 10^3 copies/reaction while *N*-primer set succeeded to detect a single copy of the synthesized *N*-sequence (Fig. 4C). The obtained results indicated that primer pairs designed for LAMP, CPA and PSR targeting *N*-sequence performed outstandingly and highly promising for a real diagnosis. Thus, the PSR primer set targeting the *Orf1ab* sequence was herein eliminated from further investigation.

Performance of SARS-CoV-2 colorimetric iNAATs with simulated clinical specimens

The presence of non-target DNA in samples was shown to not interfere with the sensitivity of LAMP [19, 21, 34]. Besides, the activity of an isothermal polymerase utilized in LAMP, CPA and PSR is more tolerant to various PCR inhibitors such as trace quantities of whole-blood, hemin, urine or stools [20, 35, 36]. Here, we used the nasopharyngeal and oropharyngeal swab specimens as crude samples to test the performance of iNAATs. Synthesized DNAs of SARS-CoV-2 were spiked into the crude samples to mimic the clinical specimens. Considering that the crude samples contain a variety of components that could interfere with the amplification reaction, dilution is required for considerably better outcomes. Nasopharyngeal and oropharyngeal swabs after collected are often preserved in a special solution called viral transport medium.

However, according to our previous studies, this medium strongly affected the reaction color; up to 100 to 160-fold dilution factor was required to allow the color change to be clearly observed (data not shown). It is no doubt that excessive dilution would remarkably decrease the concentration of infectious virus and thus, reducing the chance for the assay to detect the virus presence in clinical samples. With the aim of performing the rapid test immediately at POC, the use of the viral transport medium could be negligible. Therefore, we used nuclease-free water instead of the viral transport medium to store the collected nasopharyngeal and oropharyngeal swabs.

As expected, 1 µl of undiluted crude samples still interfered with the color change of the iNAAT reactions. Accordingly, from 5 to 10-fold dilution depending on the type of samples and assay used was required to clearly establish the colorimetric reactions between positive and negative signals (data not shown). Therefore, a 10-fold dilution of nasopharyngeal and oropharyngeal samples was selected to examine the simulated clinical specimens prepared. The results showed that all nasopharyngeal and oropharyngeal swab samples without spiked DNA gave negative signals, indicating the low rate of false-positive levels of our iNAATs (Fig. 5). DNA templates were spiked into the nasopharyngeal and oropharyngeal swab samples to simulate the clinical specimens containing SARS-CoV-2. The spiked samples were 10-fold diluted for iNAATs. As expected, the developed iNAATs successfully detected all of the positive simulated samples (Fig. 5), indicating the 100% sensitivity of the assays. The *N*-gene targeting LAMP and *Orf1ab*-gene targeting CPR reactions required 45 min to establish the clearest outcome, while *Orf1ab*-gene targeting LAMP and *N*-gene targeting PSR reactions required 30 minutes to produce the best-visualized results (Fig. 5).

Performance of colorimetric iNAATs on extracted SARS-CoV-2 genomic RNA

SARS-CoV-2 was isolated from the clinical positive COVID-19 samples and cultured at Pasteur Institute (Ho Chi Minh City, Vietnam). The viral genomic RNA was extracted and quantitated using the standard curve based on qRT-PCR Ct-value [33]. The LODs of the colorimetric iNAATs on this extracted SARS-CoV-2 genomic RNA was evaluated. Results revealed that the LODs of LAMP were around 21.57 (*Orf1ab*) and 43.14 (*N*) viral-RNA copies/reaction (Table 3). The obtained values were outstanding compared to 431.47 and 862.9 copies/reaction of CPA and PSR, respectively (Table 3). Therefore, the LAMP assays were used for further evaluation with simulated clinical samples containing the different amounts of spiked viral-RNAs. The results indicated that the LAMP assays could directly detect SARS-CoV-2 genomic-RNA in crude samples without the requirement of RNA extraction (Fig. 6).

Performance of SARS-CoV-2 colorimetric LAMP using lyophilized reagents

For better on-site testing, lyophilized reagents which are ready-to-use without strict storage conditions at low temperature would be highly advantageous. Thus, we made an attempt to verify the LAMP kit performance using the dried reagents. Accordingly, the reaction mixture containing the enzyme, primers, and dye was first lyophilized. LODs of the lyophilized LAMP kits for the simulated nasopharyngeal and oropharyngeal swab samples containing the spiked synthetic DNA template were examined. Consistently, the lyophilized kit targeting *Orf1ab*-sequence exhibited the faster amplification process than the one targeting *N*-sequence. As for the *Orf1ab*-sequence set, results could be read after 30 min with the LOD value of 1 DNA copy/reaction, which is equivalent to 10 DNA copies/µl in simulated specimens (Fig. 7A, upper panel). In contrast, the *N*-sequence set expressed the slower reaction, which required 45 min for the color change to be clearly

observed. The *N*-sequence set also exhibited a LOD value of 1 DNA copy/reaction corresponding to 10 DNA copies/µl in simulated samples (Fig. 7A, lower panel).

Subsequently, the LOD values of lyophilized LAMP kit for SARS-CoV-2 genomic RNA were also evaluated. Both primer sets targeting *Orf1ab*- and *N*-sequence could identify approximately 43.14 copies of viral RNA per reaction (Table 3). Note that the LOD values defined correspond to a Ct value of roughly 36.5 when tested by qRT-PCR for *E* gene [33]. This high Ct value (>35) strongly indicates that our lyophilized LAMP kit could identify the specimens with low-level infection of SARS-CoV-2 found during early infection or asymptomatic carriage.

Importantly, all mimicked samples containing different amount of synthesized DNA or viral RNA were detected by our lyophilized LAMP kit, indicating the 100% sensitivity of the dried-reagent LAMP assay (Fig. 7B and C). Accordingly, all nasopharyngeal and oropharyngeal specimens without spiked DNA or viral RNA generated negative results, ensuring the low false-positive occurrence of the assay (Fig. 7B and C). It is worth noting that the direct addition of unprocessed clinical specimens to the reaction is greatly convenient for diagnosis at POC, markedly reducing the time required for sample preparation and thus, simplifying operation procedure. When all those features are taken into account, the lyophilized LAMP kit is highly suitable for POC diagnosis.

DISCUSSION

The pneumonia outbreak COVID-19 has been announced by WHO as the global pandemic. Even though each country has its own strategy to prevent the spread of the disease, identifying the person who contracts the SARS-CoV-2 is one of the most essential actions. While the gold standard for identifying the patients is real-time RT-PCR (qRT-PCR), which shows remarkably high accuracy, sensitivity and specificity, it is not portable and only specific high-biosafety laboratories are able to perform the test.

Also, the time required for each qRT-PCR test is at least 2 hours, if not including the time needed for transferring the sample to the labs, thus slowing down the quarantine process of the infected patient.

POC such as airports is the hot spots for spreading COVID-19 globally. However, the prevention measures at the airports including body heat monitoring and checking personal travel history, as well as passenger symptoms, have been proven to be insufficient as some asymptomatic people are infectious [37-40]. Self-isolation and group quarantine of suspected cases can temporarily limit the transmission, but it will be more difficult to control when the number of suspects is high. The qRT-PCR tests only serve to verify a small number of cases while the actions cannot be made immediately. Therefore, POC diagnostic detection methods can help the authority to effectively monitor the spreading of the virus.

The three isothermal amplification methods including LAMP, CPA and PSR all require the DNA polymerase strand displacement activity. Among them, LAMP was first introduced and has been widely utilized [41-43]. Meanwhile, CPA and PSR were later developed and fewer studies evaluating these two methods have been published. In this work, for the first time, the three methods were applied to directly detect SARS-CoV-2 to compare their effectiveness. Surprisingly, despite showing similar performance regarding the identification of the synthetic DNA template of SARS-CoV-2, LAMP was superior to CPA and PSR in the detection of genomic RNA of SARS-CoV-2. Accordingly, the colorimetric lyophilized LAMP kit developed in this study possesses the following features: (i) fast detection of SARS-CoV-2 RNA directly from the nasopharyngeal and oropharyngeal specimens within 45 minutes; (ii) high sensitivity (roughly 43 copies of the viral RNA in the reaction is sufficient for detection); (iii) naked eye readout of results; (iv) possible detection of the virus in the early stage of infection; (v) does not require special laboratory equipment, only a popular thermal incubator is needed; (vi) is

amenable to high throughput testing; (vii) portable to use in any place and does not require specialized personnel to do the test; and (viii) particularly useful for resource-limited settings.

One of the most distinctive features of the SARS-CoV-2 colorimetric LAMP kit developed herein is the direct use of unprocessed clinical samples. The only step required to prepare samples before adding to the reaction is sample dilution in nuclease water. Without the requirement for viral RNA extraction, the developed LAMP kit is very suitable for on-site diagnosis, not only because it is much less time-consuming and laborious but also does not depend on RNA extraction kits which have been at shortage due to overwhelming global demand. We, therefore, propose the testing procedure as illustrated in Figure 8. We anticipate that our LAMP test kit can contribute significantly to a timely screening of COVID-19.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENT

This work was funded by NTT Hi-Tech Institute, Nguyen Tat Thanh University, and the Pasteur Institute in Ho Chi Minh City. The authors are grateful for the support of the staff at both institutes.

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Table 1. Primers and synthesized DNA sequences used in this study

Nº	Name	Nucleotide sequence (5′ - 3′)
iNAAT primers		
1	LAMP-Orf-F3	GATTTAGATGAGTGGAGTATGG
2	LAMP-Orf-B3	GCACCAAATTCCAAAGGTT
3	LAMP-Orf-FIP	CTGGAGGGTAGAAAGAACAATACATCATACTACTTATTTGATGAGTCTGG
4	LAMP-Orf-BIP	GAGGATGAAGAAGAAGGTGATTGTTTGGTAATCATCTTCAGTACCATA
5	LAMP-Orf-LoopF	GTGAAGCCAATTTAAACTC
6	LAMP-Orf-LoopR	AGTTTGAGCCATCAACTCAATAT
7	LAMP-N-F3	TGGACCCCAAAATCAGCG
8	LAMP-N-B3	GCCTTGTCCTCGAGGGAAT
9	LAMP-N-FIP	CCACTGCGTTCTCCATTCTGGTAAATGCACCCCGCATTACG
10	LAMP-N-BIP	CGCGATCAAAACAACGTCGGCCCTTGCCATGTTGAGTGAGA
11	LAMP-N-LoopF	TTGAATCTGAGGGTCCACCAAA
12	LAMP-N-LoopR	GTTTACCCAATAATACTGCGTCTTG
13	PSR-Orf-F	ACGATTCGTACATAGAAGTATAGAGAAGATTGGTTAGATGATGATAGTCAA
14	PSR-Orf-R	GATATGAAGATACATGCTTAGCATTCCATCTCTAATTGAGGTTGAACC
15	PSR-N-F	ACGATTCGTACATAGAAGTATAGTGATAATGGACCCCAAAATCAGCG
16	PSR-N-R	GATATGAAGATACATGCTTAGCAAACGCCTTGTCCTCGAGGGAAT
17	CPA-2a-Orf-B2	TTGGTAATCATCTTCAGTACCATA
18	CPA-3a-Orf-B1	ACAATCACCTTCTTCTTCATCCTC
19	CPA-Orf-1s	TTGGTAATCATCTTCAGTACCATACTGGAGGGTAGAAAGAACAATACAT
20	CPA-4s-Orf-F3	GATTTAGATGAGTGGAGTATGG
21	CPA-5a-Orf-B3	GCACCAAATTCCAAAGGTT
Synthesized DNA templates		
22	nCoV-Orf1ab (600 bp)	AACTCGGTACAGAAGTAAATGAGTTCGCCTGTGTTGTGGCAGATGCTGTCATAAAAACTTTGCAACCAGTATCTGAATTACTTA

		CACCACTGGGCATTGATTTAGATGAGTGGAGTATGGCTACAT ACTACTTATTTGATGAGTCTGGTGAGTTTAAATTGGCTTCACA TATGTATTGTTCTTTCTACCCTCCAGATGAGGATGAAGAAGA AGGTGATTGTGAAGAAGAAGAGTTTGAGCCATCAACTCAAT ATGAGTATGGTACTGAAGATGATTACCAAGGTAAACCTTTGG AATTTGGTGCCACTTCTGCTGCTCTTCAACCTGAAGAAGAGC AAGAAGAAGATTGGTTAGATGATGATAGTCAACAAACTGTT GGTCAACAAGACGGCAGTGAGGACAATCAGACAACTACTAT TCAAACAATTGTTGAGGTTCAACCTCAATTAGAGATGGAACT TACACCAGTTGTTCAGACTATTGAAGTGAATAGTTTTAGTGG TTATTTAAAACTTACTGACAATGTATACATTAAAAATGCAGA CATTGTGGAAGAAGCTAAAAAGGTAAAACCAACAGTGGTTG TTAATGCAGCCAATG
23	nCoV-N (243 bp)	ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCG CATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCA GAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGC CCCAAGGTTTACCCAATAATACTGCGTCTTGGTTCACCGCTCT CACTCAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGAC AAGGCGTTCCAATTAACACCAATAGCAGTCCAGAT
24	Bat SARS-Orf1ab (600 bp)	ACTTTTGTTGCGTGCACACCCACCGGGGAAGTTGTCCCACAA TTGCTCCAATATATGTTTGTGCCACCTGGAGCCCCTAAGCCTG ATTCTAGGGAATCCCTTGCATGGCAAACCGCCACTAACCCCT CGGTTTTTGTCAAGCTGTCAGACCCTCCATCGCAGGTTTCAGT GCCATTCATGTCACCTGCGAGTGCTTATCAATGGTTTTATGAC GGATATCCCACATTCGGAGAACACAAACAGGAGAAAGATCT TGAATATGGGGCATGTCCTAATAACATGATGGGCACGTTCTC AGTGCGGACTGTGGGGACCTCCAAGTCCAAGTACCCTTTAGT GGTTAGGATTTACATGAGAATGAAGCACGTCAGGGCGTGGA TACCTCGCCCGATGCGTAACCAGAACTACCTATTCAAAGCCA ACCCAAATTATGCTGGCAACTCCATTAAGCCAACTGGTGCCA GTCGCACAGCGATCACCACTCTTGGGAAATTTGGACA
25	Bat SARS-N (300 bp)	GTTGTTTTAGATTTCATCTAAACGAACAAACTAAAATGTCTG ATAATGGACCCCAAAACCAACGAAATGCACCCCGCATTACGT TTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGAATGGAG AACGCAGTGGAGCACGACCAAAACAACGTCGGCCTCAAGGT TTACCCAATAATACTGCGTCTTGGTTCACCGCTCTCACTCAAC ATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGTTC CGATTAATACCAATAGCAGTCCAGATGACCAAATTGGCTACT ACCGAA
26	MERS-Orf1ab (229 bp)	GATTTAGACGATTTTATTGACGCACCATGCTATTGCTTTAACG CTGAGGGTGATGCATCCTGGTCTTCTACTATGATCTTCTCTCT TCACCCCGTCGAGTGTGACGAGGAGTGTTCTGAAGTAGAGGC TTCAGATTTAGAAGAAGGTGAATCAGAGTGCATTTCTGAGAC TTCAACTGAACAAGTTGACGTTTCTCATGAGACTTCTGACGA

		CGAGTGGGCTGCTGCAG
27	MERS-N (185 bp)	TGGCATCCCCTGCTGCACCTCGTGCTGTTTCCTTTGCCGATAA CAATGATATAACAAATACAAACCTATCTCGAGGTAGAGGAC GTAATCCAAAACCACGAGCTGCACCAAATAACACTGTCTCTT GGTACACTGGGCTTACCCAACACGGGAAAGTCCCTCTTACCT TTCCACCTGGGCAGGGT
28	SARS-Orf1ab (209 bp)	GATCTTGATGAGTGGAGTGTAGCTACATTCTACTTATTTGAT GATGCTGGTGAAGAAAACTTTTCATCACGTATGTATTGTTCCT TTTACCCTCCAGATGAGGAAGAAGAGGACGATGCAGAGTGT GAGGAAGAAGAAATTGATGAAACCTGTGAACATGAGTACGG TACAGAGGATGATTATCAAGGTCTCCCTCTGGAATTTGGTGC
29	SARS-N (205 bp)	TGGACCCCAATCAAACCAACGTAGTGCCCCCCGCATTACATT TGGTGGACCCACAGATTCAACTGACAATAACCAGAATGGAG GACGCAATGGGGCAAGGCCAAAACAGCGCCGACCCCAAGGT TTACCCAATAATACTGCGTCTTGGTTCACAGCTCTCACTCAGC ATGGCAAGGAGGAACTTAGATTCCCTCGAGGCCAGGGC

Table 2. The percent mismatch of newly designed primers between SARS-CoV-2 and related taxa

Name	Accession number	LAMP-*Orf1ab*	LAMP-*N*	CPA-*Orf1ab*	PSR-*Orf1ab*	PSR-*N*
Murine hepatitis virus	NC001846	25.97	31.93	21.74	30.19	34.78
Betacoronavirus England 1	NC038294	29.83	29.52	25.36	35.85	30.43
Human Coronavirus 229E	NC002645	31.49	31.93	26.09	33.96	39.13
Human Coronavirus NL63	NC005831	28.73	31.33	25.36	32.08	34.78
Human Coronavirus OC43	NC006213	28.73	30.72	23.91	32.08	36.96
Human Coronavirus HKU1	NC006657	27.62	22.29	29.71	37.74	17.39
Middle East Respiratory CoV	NC019843	29.83	29.52	25.36	35.85	30.43
Civet SARS CoV SZ16/2003	AY304488	19.34	12.65	13.77	35.85	26.09
SARS CoV ZS-C	AY395003	19.34	12.65	13.77	35.85	26.09
SARS CoV MA15	FJ882957	19.34	12.65	13.77	35.85	26.09

SARS CoV	NC004718	19.34	12.65	13.77	35.85	17.39
Bat SARS CoV RM1/2004	KY417144	21.55	13.86	16.67	32.08	15.22
Bat SARS-like CoV 2015	MG772933	8.84	12.05	7.25	20.75	10.87
Bat SARS-like CoV 2017	MG772934	8.84	11.45	7.97	26.42	10.87
13 SARS-CoV-2 strains		0	0	0	0	0

Table 3. LODs of colorimetric iNAATs for extracted SARS-CoV-2 genomic RNA

Viral RNA copies (per reaction)	Ratio of positive tests to the total test number					
	LAMP		Lyophilized LAMP		CPA	PSR
	Orf1ab	*N*	*Orf1ab*	*N*	*Orf1ab*	*N*
1725.89	3/3	3/3	3/3	3/3	3/3	3/3
862.95	-	-	-	-	3/3	3/3
431.47	-	-	-	-	3/3	1/3
215.74	-	-	-	-	0/3	0/3
172.59	3/3	3/3	3/3	3/3	0/3	0/3
86.30	3/3	3/3	3/3	3/3	0/3	0/3
43.15	3/3	3/3	3/3	3/3	0/3	0/3
21.57	3/3	0/3	0/3	0/3	0/3	0/3
17.26	0/3	0/3	0/3	0/3	0/3	0/3
1.73	0/3	0/3	0/3	0/3	0/3	0/3
LOD	21.57	43.14	43.14	43.14	431.47	862.95

Figure Legends

Figure 1. Colorimetric iNAATs to detect the presence of SARS-CoV-2 targeted gene. (A) The color of iNAAT mixtures changed from red to yellow after incubation in the presence of the synthesized DNA template (1 ng) of SARS-CoV-2. The lowest panel: the reactions were added with SyBr Green I (Invitrogen, California, USA) and illuminated by a 470 nm light source. The reactions were incubated at 60 °C (for LAMP) and 63 °C (for CPA and PSR) for 30 min. (B) The amplified products in (A) were analyzed with gel electrophoresis and compared with the routine PCR result. Abbreviation, L: DNA ladder.

Figure 2. Optimization of the incubation time and temperature for iNAATs. The LAMP (in A), CPA (in B) and PSR (in C) reactions were incubated from 10 to 60 min at 60 °C (LAMP) and 63 °C (CPA and PSR) (left panels) and at different temperatures from 50 to 70 °C for 30 min (right panels). One ng of the synthesized DNA template was used.

Figure 3. The specificity of the colorimetric iNAATs. The specificity of LAMP (in A), CPA (in B) and PSR (in C) assay was evaluated with the synthesized DNA (1 ng) of SARS-CoV-2, SARS-CoV, MERS-CoV, and bat SARS-like-CoV.

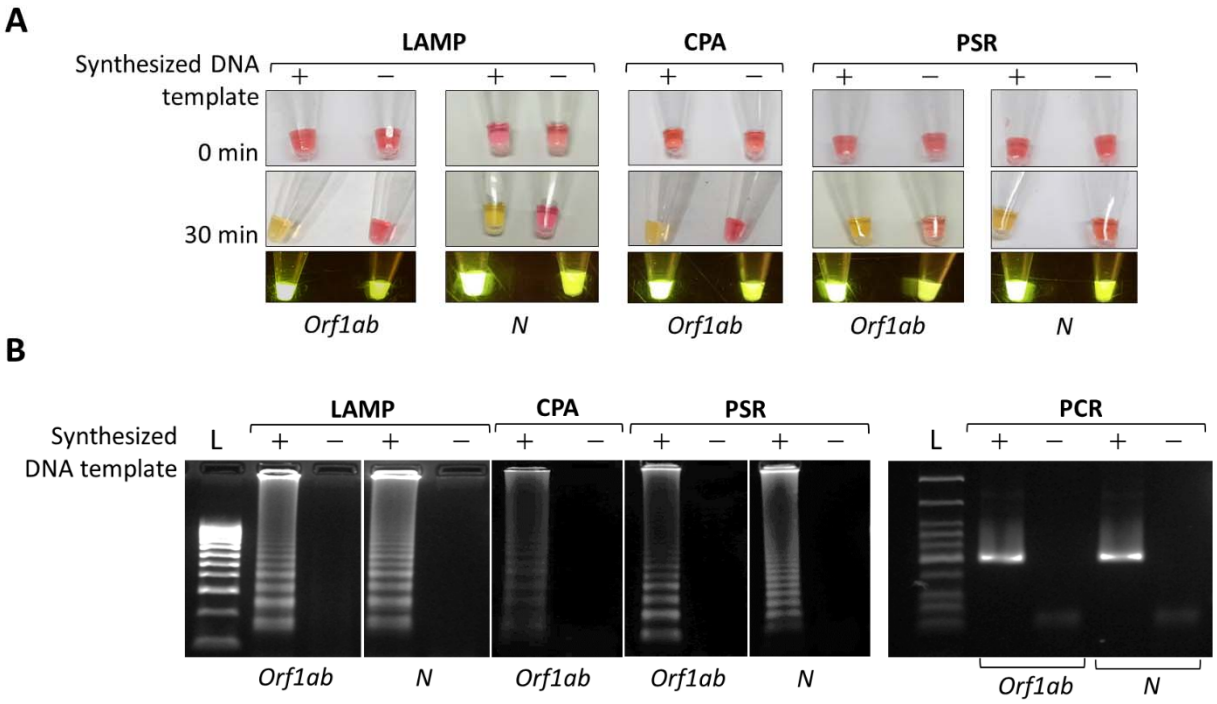
Figure 4. The LOD values of colorimetric iNAATs. LOD of LAMP (in A), CPA (in B) and PSR (in C) assay were evaluated. The synthesized DNA template was serially diluted in nuclease-free water to the indicated concentrations and 1 µl of the diluted DNA templates was added to the reactions.

Figure 5. The accuracy of iNAATs for detecting SARS-CoV-2. Different amounts of the synthetic-DNA template were spiked into the nasopharyngeal and oropharyngeal swab samples to simulate the clinical samples containing SARS-CoV-2, and 1 µl of the 10-fold diluted specimens was added to the LAMP (in A), CPA (in B) and PSR (in C) reactions. The number indicates the copy of template per reaction. Abbreviation, N: negative control; P: positive control.

Figure 6. Colorimetric LAMP assays for detection of SARS-CoV-2 genomic RNA in simulated samples. Various concentrations of genomic RNA of SARS-CoV-2 were spiked into the nasopharyngeal and oropharyngeal swab samples to simulate the clinical specimens containing SARS-CoV-2. The mimicked samples were 50-fold diluted and 5 µl of the diluted specimens were added to the LAMP reactions. The number indicates the copy of viral RNA per reaction. Abbreviation, N: negative control; P: positive control.

Figure 7. The performance of SARS-CoV-2 colorimetric lyophilized LAMP kit. **(**A) LODs of lyophilized LAMP kit evaluated in the simulated samples. The defined amount of synthetic DNA templates were spiked into the nasopharyngeal and oropharyngeal swab samples, followed by the 10-fold dilution of simulated specimens into water and 1 µl of the diluted samples was added to the reaction. The sensitivity of lyophilized LAMP kit was examined with mimicked clinical specimens containing different amounts of spiked synthetic DNA (in B) and SARS-CoV-2 genomic RNA (in C). The number indicates the copy number of synthetic DNA or viral RNA in reaction. Abbreviation, N: negative control; P: positive control.

Figure 8. The testing process of SARS-CoV-2 using the colorimetric LAMP kit.



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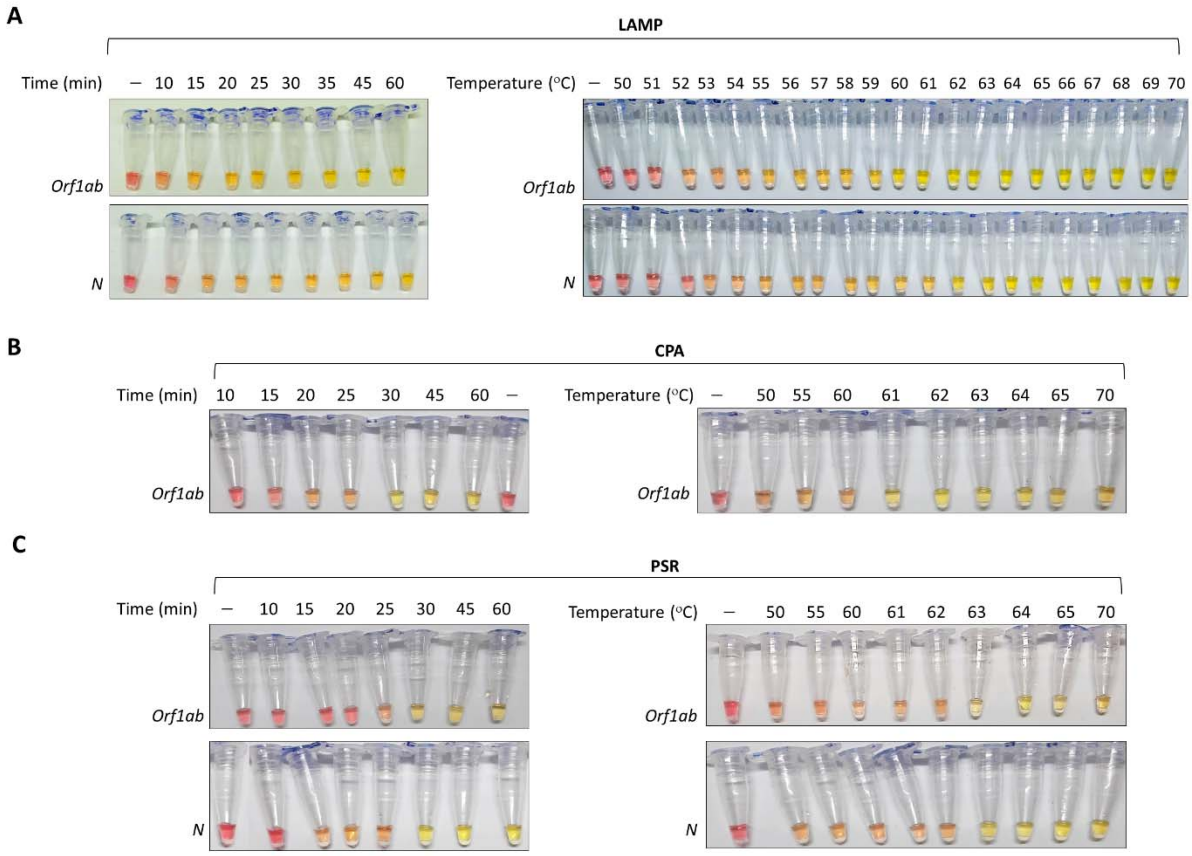

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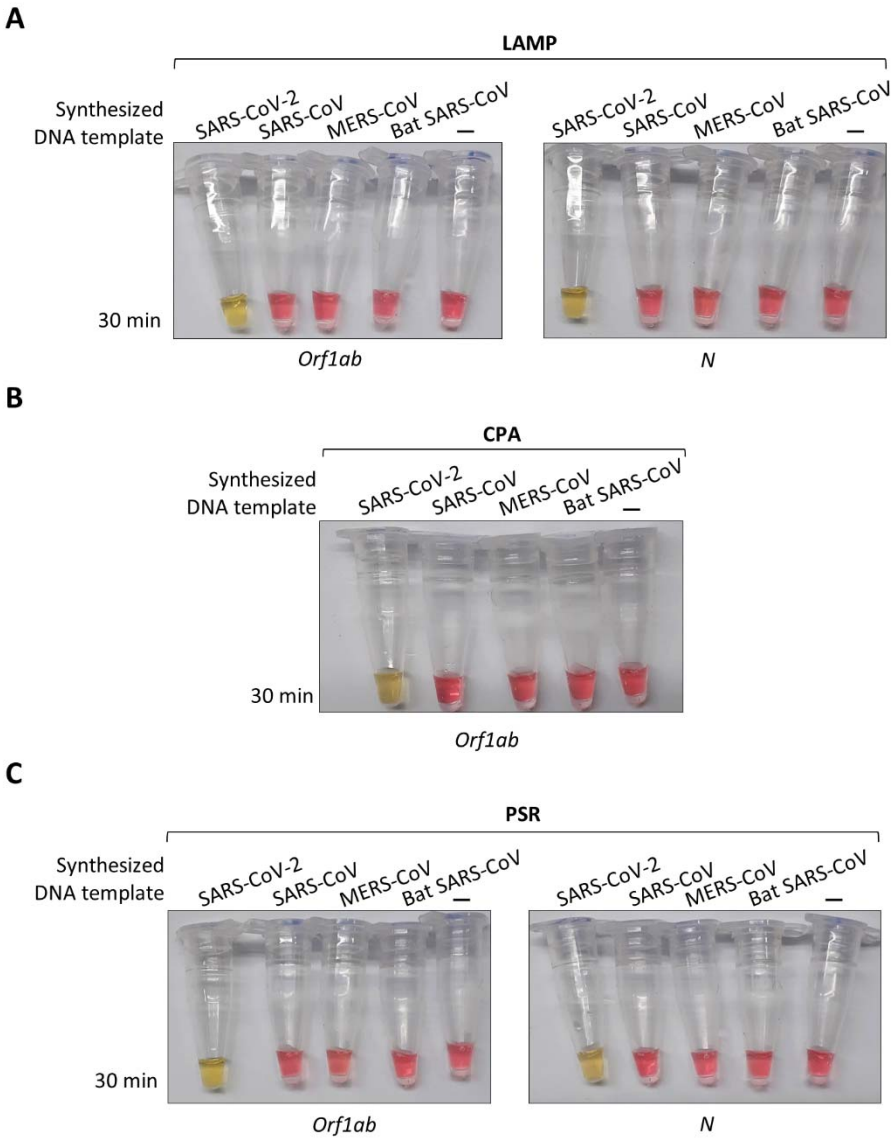

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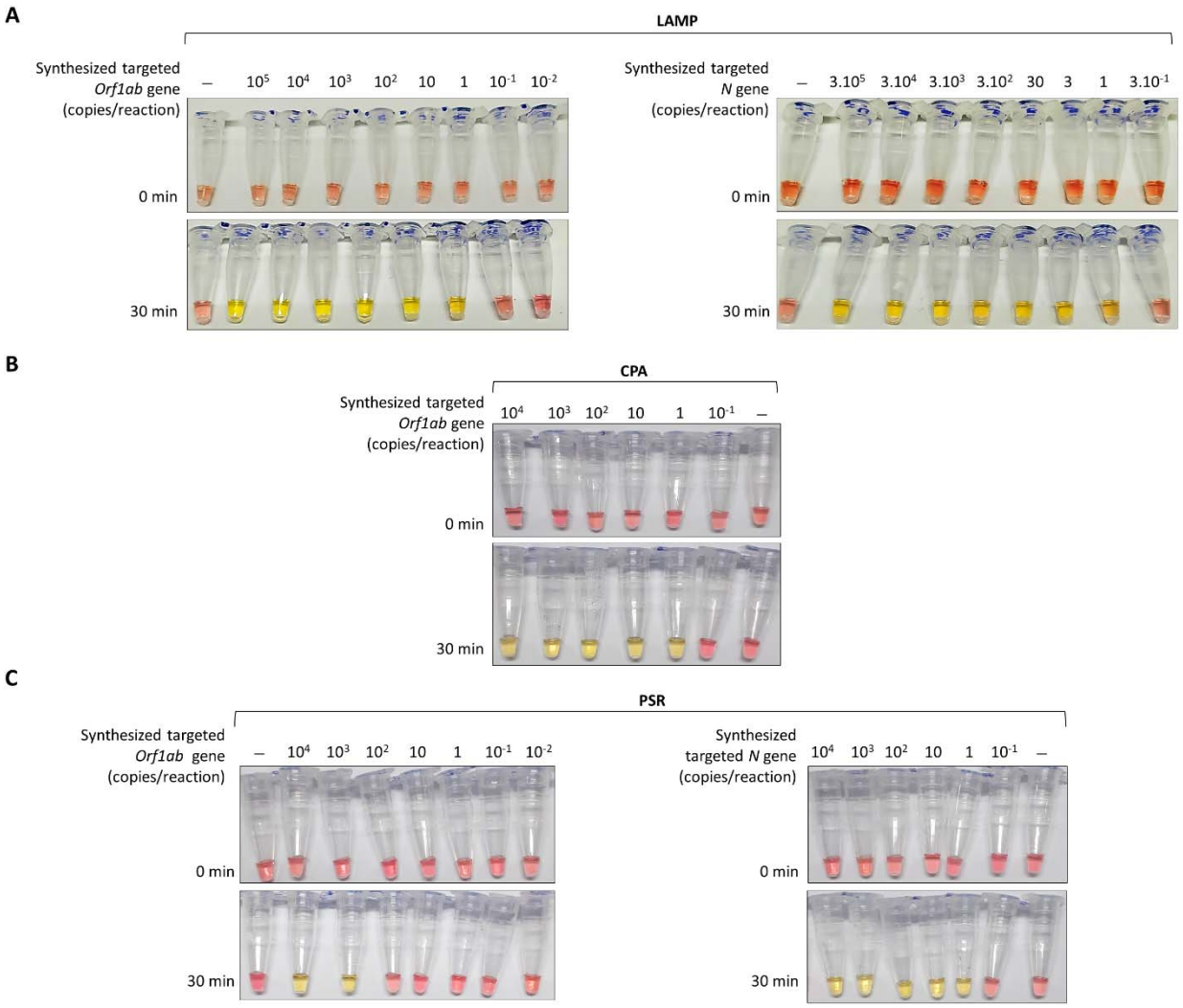

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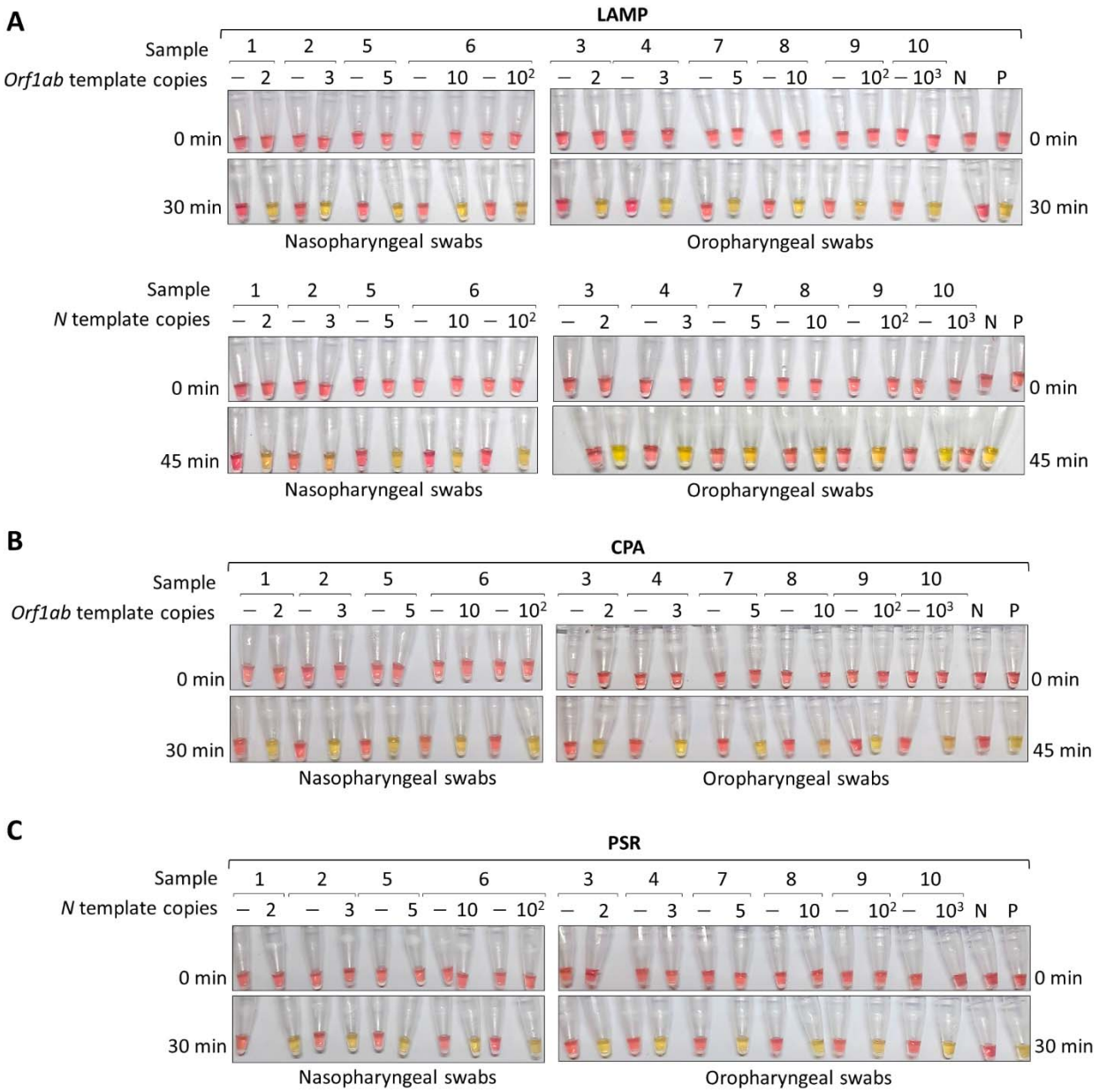

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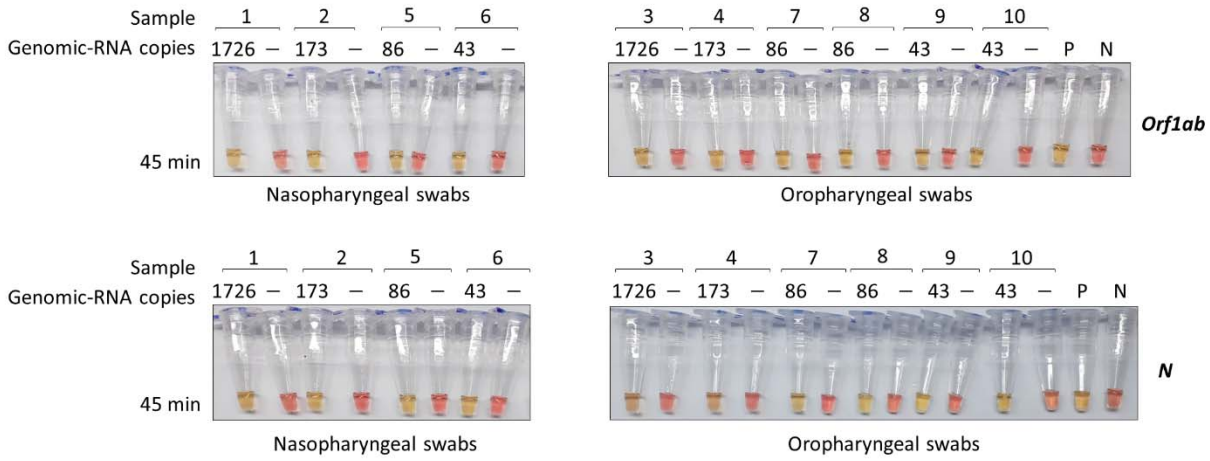

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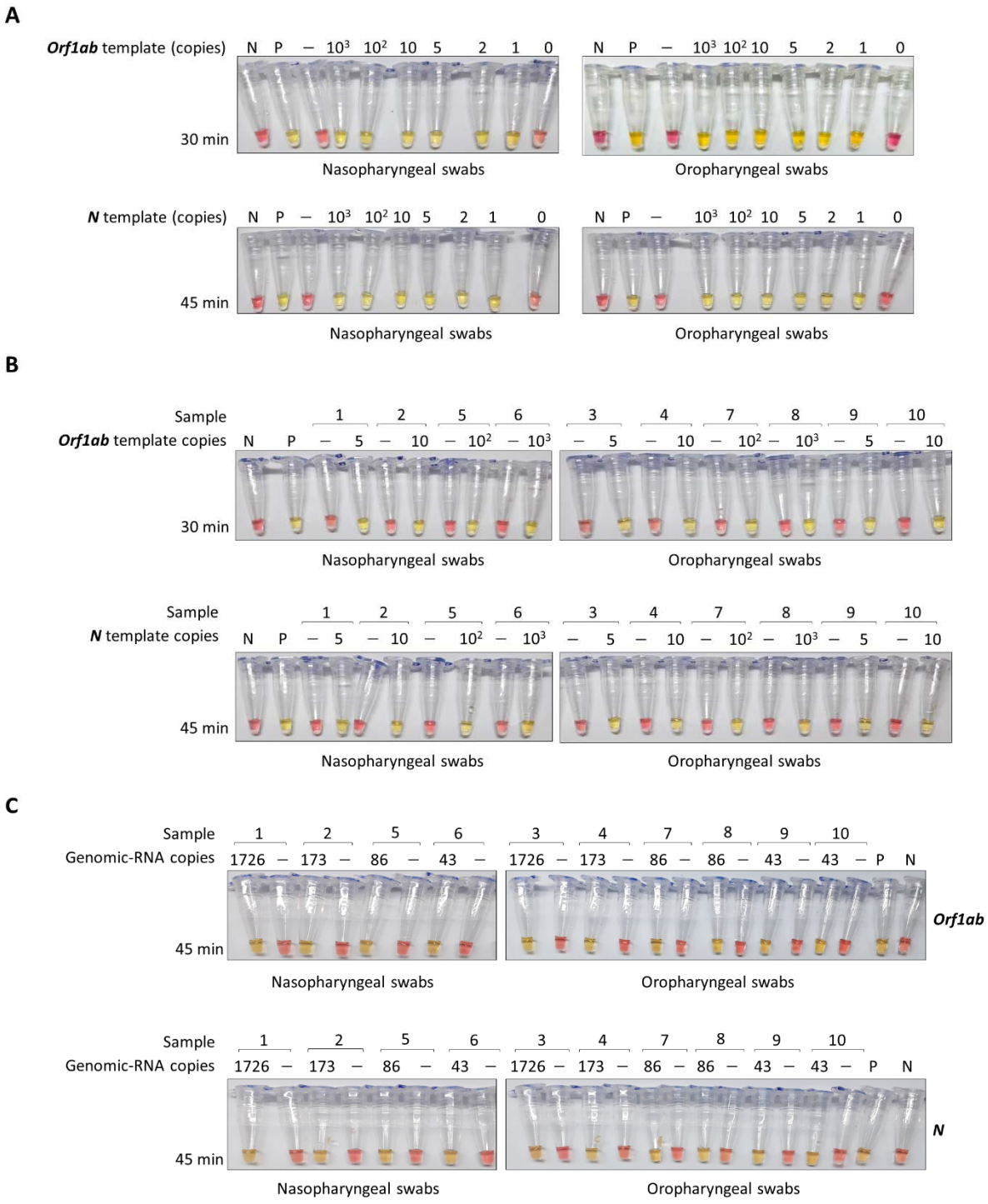

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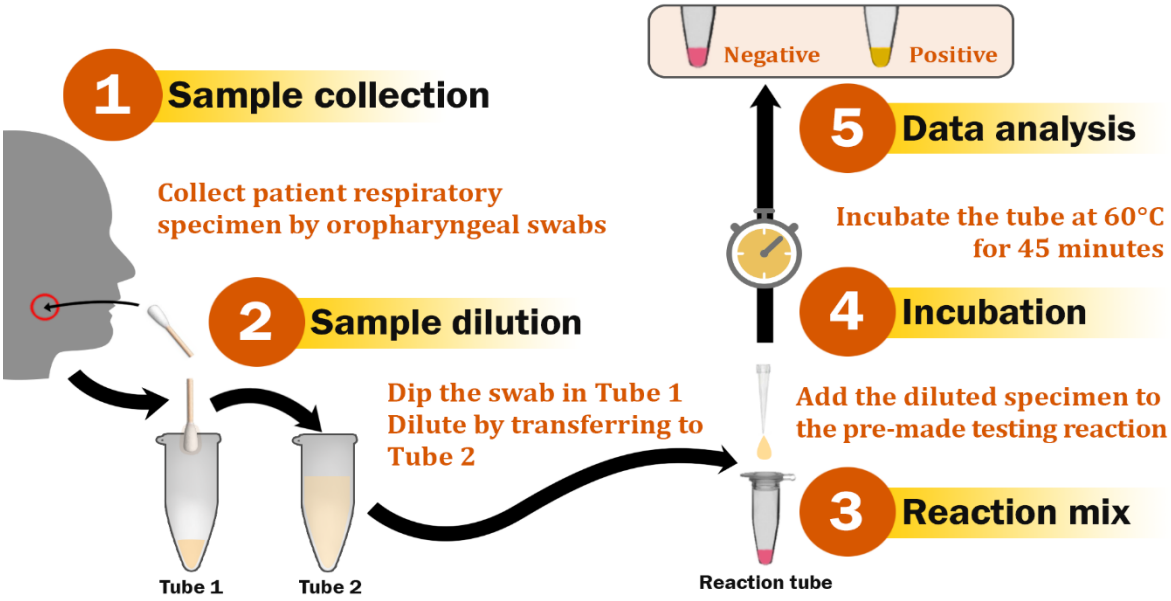

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