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Article

Single- and Two-Stage, Closed-Tube, Point-of-Care, Molecular Detection of SARS-CoV-2

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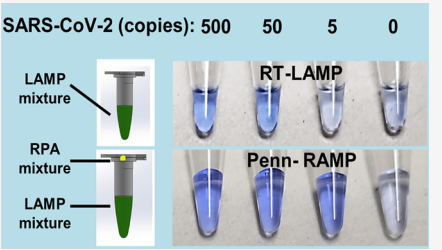
ABSTRACT: Short of a vaccine, frequent and rapid testing, preferably at home, is the most effective strategy to contain the COVID-19 pandemic. Herein, we report on single-stage and two-stage molecular diagnostic tests that can be carried out with simple or no instrumentation. Our single-stage amplification is reverse transcription-loop mediated isothermal amplification (RT-LAMP) with custom-designed primers targeting the ORF1ab and the N gene regions of the virus genome. Our new two-stage amplification, dubbed Penn-RAMP, comprises recombinase isothermal amplification (RT-RPA) as its first stage and LAMP as its second stage. We compared various sample preparation strategies aimed at deactivating the virus while preserving its RNA and tested contrived and patient samples, consisting of nasopharyngeal swabs, oropharyngeal swabs, and saliva. Amplicons were detected either in real time with fluorescent intercalating dye or after amplification with the intercalating colorimetric dye LCV, which is insensitive to sample's PH. Our single RT-LAMP tests can be carried out instrumentation-free. To enable concurrent testing of multiple samples, we developed an inexpensive heat block that supports both the single-stage and two-stage amplification. Our RT-LAMP and Penn-RAMP assays have, respectively, analytical sensitivities of 50 and 5 virions/reaction. Both our single- and two-stage assays have successfully detected SARS-CoV-2 in patients with viral loads corresponding to the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) threshold cycle smaller than 32 while operating with minimally processed samples, without nucleic acid isolation. Penn-RAMP provides a 10-fold better sensitivity than RT-LAMP and does not need thermal cycling like PCR assays. All reagents are amenable to dry, refrigeration-free storage. The SARS-CoV-2 test described herein is suitable for screening at home, at the point of need, and in resource-poor settings.



INTRODUCTION

Coronaviruses are a large family of RNA viruses including human coronaviruses (HCoV)-229E, OC43, NL63, and HKU1 that typically cause mild to moderate respiratory illnesses[1,2] with the exceptions of the fatal severe acute respiratory syndrome coronavirus (SARS-CoV)[3,4] and the Middle East respiratory syndrome coronavirus (MERS).[5] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019, causing a global pandemic, infecting tens of millions of individuals, and causing over a million deaths and severe economic disruption. Short of a vaccine, effective control of the pandemic requires frequent, rapid turn-around screening of asymptomatic individuals.[6,7]

Most medical centers use reverse transcription-polymerase chain reaction (RT-PCR) to detect SARS-COV-2 in nasopharyngeal swab samples, oropharyngeal swab samples, saliva, bronchoalveolar lavage fluid, sputum, and feces.[8] RT-PCR tests are, however, carried out in well-equipped laboratories that lack the capacity to frequently screen the entire population. Furthermore, a significant time gap between sample collection and the results delays the isolation of potentially contagious individuals challenging pandemic control efforts. The need to deliver samples to collection sites exposes individuals to infection risk and is inconvenient. Rapid, point-of-care molecular diagnostic tests for COVID-19 are needed.

Early in the COVID-19 pandemic, we reported on two simple closed-tube molecular tests for COVID-19 that can be carried out in the clinic and at home by minimally trained personnel without any sophisticated equipment.[9] We selected the loop mediated isothermal amplification (LAMP)[10] that does not require thermal cycling and can be incubated with a heat block, a water bath, or even equipment-free, with an exothermic chemical reaction.[11−13] For amplicon detection, we use either (A) colorimetric intercalating dye Leuco Violet Crystal[14] that changes from colorless in the absence of amplicons to violet in the presence of dsDNA and can be detected by the unaided eye or (B) fluorescent intercalating dye that can be excited and monitored with a smartphone or

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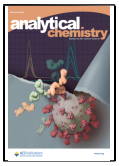

with a simple USB camera.[15,16] We prefer the LCV colorimetric dye over the more commonly used phenol red pH indicator[17] because samples such as saliva vary in their pH and may alter the color of the pH indicator even in the absence of amplification, yielding false positives.

Our second test, dubbed Penn-RAMP, relies on two-stage amplification. The first stage of Penn-RAMP is recombinase polymerase amplification (RT-RPA)[18] and its second stage is LAMP. We developed the Penn-RAMP for high-level, nested multiplexing to co-detect multiple co-endemic pathogens (as many as 16 different targets were demonstrated).[18] Serendipitously, Penn-RAMP provides better sensitivity, by as much as a factor of 10, and is less inhibited by contaminants than LAMP alone. Hence, Penn-RAMP is beneficial even in a single plex setting. Here, we carry out both tests: Penn-LAMP and Penn-RAMP in a closed tube, avoiding the need to open an amplicon-rich tube and risking contamination of the work area.

At the time of our earlier report,[9] we tested our assays with contrived samples consisting of synthesized DNA that mimics the actual viral sequence since COVID-19 cases in the USA were rare and patient samples were not available. Unfortunately, this is no longer the case. Here, we describe refinements to our tests, compare and optimize sample preparation strategies, and report on tests of patient samples in comparison with the RT-PCR gold standard. Furthermore, to enable easy adaptation of our test for use at the point of need, we have developed a simple heat block to incubate polymerase amplification.

EXPERIMENTAL SECTION

Sample Collection and RNA Extraction. Clinical specimens were collected during the first week after hospitalization (likely a few days after symptoms' onset) from confirmed SARS-CoV2-positive patients in the Hospital of the University of Pennsylvania following informed consent under protocols approved by the institutional review board (protocol no. 823392). Because SARS-CoV-2 titers peak around the time of symptom onset and fall thereafter,[19] most samples contained low viral load. Nasopharyngeal samples were collected using flocked swabs (Copan Diagnostics) and eluted in 2 mL of CDC-compliant viral transport medium (VTM) containing Hanks' buffered salt, 2% fetal bovine serum (FBS), penicillin, streptomycin, gentamycin, and amphotericin[20] or in water. Saliva samples were self-collected by the patient. All samples were first deactivated in biosafety level 2 plus laboratory by being added directly to the lysis buffer (Qiagen kit, Cat. No. 52904/52906), vortexed, and incubated at room temperature for 10 min or by heating at 56 °C for 1 h in the absence or in the presence of the RNase inhibitor.

SARS-CoV-2 virus (USA-WA1/2020 strain) was obtained from BEI and propagated in the Vero E6 cells.

All viral RNA were extracted from 140 μL of samples using the Qiagen QIAamp Viral RNA Mini kit (Qiagen, Cat. No. 52904/52906), suspended in 50 μL of ddH_2O, and quantified with standard qRT-PCR (see the Supporting Information) by utilizing Twist Synthetic SARS-CoV-2 RNA Control (MN908947.3, Twist Bioscience) as the reference.

For direct RT-LAMP, RT-RPA, and Penn-RAMP, a 2−4 μL unprocessed clinical specimen or contrived sample was directly added to the RT-LAMP and the Penn-RT-RAMP reaction buffers. The standard qRT-PCR was carried out in parallel with extracted RNA from these clinical specimens to serve as the "gold standard" results.

LAMP Primer Design. Complete genome sequences of various SARS-CoV-2 (Table S1) were aligned and analyzed with Clustal X (http://www.clustal.org/clustal2/) to identify conserved sequences, which were then compared with sequences of other coronaviruses (Table S1) to assure differentiation. We elected to target conserved sequences within the ORF1ab and the N gene (Figures S1 and S2) because of their high homology among SARS-CoV-2 sequences and high divergence from all other known coronaviruses. Moreover, infected cells express subgenomic mRNA,[21] increasing abundance of the N gene sequences in the samples and enhancing assay sensitivity.

We designed our LAMP primer sets with the PrimerExplorer V5 software (Eiken Chemical Co., Ltd.) and verified primers' specificity with a BLAST search of the GenBank nucleotide database. A few LAMP primer sets were synthesized (Integrated DNA Technologies, Coralville, IA) and tested, and the ones documented in Table S2 were selected for further use.

RT-LAMP Assay. The LAMP reaction mix contained 1× Isothermal MasterMix (ISO-001, OptiGene, U.K.) and primers (Table 1). During assay development, LAMP amplification was carried out with a 10 μL reaction mix that included 1 μL of synthesized templates (Table S3) or purified RNA of various concentrations and incubated in a Thermal Cycler (BioRad, Model CFD3240) at 63 °C for 40 min. For RNA detection, 0.2 U/μL AMV reverse transcriptase (Promega) was added to each LAMP reaction mix. Nontemplate controls (NTC) were included with each run to ensure the absence of false positives.

Table 1. Sensitivity of Direct RT-LAMP, RT-RPA, and Closed-Tube Penn-RAMP for the Detection of SARS-CoV-2 Eluted from Nasal Swabs (Contrived Samples)[a]

swab elution media	spiked samples	RT-LAMP[b]	RT-RPA[b]	Penn-RAMP[b]
VTM	10 000 particles/reaction	4/4	4/4	4/4
	1000 particles/reaction	4/4	4/4	4/4
	100 particles/reaction	2/4	1/4	4/4
	10 particles/reaction	0/4	0/4	0/4
	0 particles/reaction	0/4	0/4	0/4
water	10 000 particles/reaction	4/4	4/4	4/4
	1000 particles/reaction	4/4	4/4	4/4
	100 particles/reaction	4/4	4/4	4/4
	10 particles/reaction	0/4	0/4	3/4
	0 particles/reaction	0/4	0/4	0/4

[a]We submerged a nasal swab in 3 mL of VTM and water and spike SARS-CoV-2 into each of them to prepare contrived nasal swab samples. [b]The table documents the fraction of positive results.

Colorimetric RT-LAMP Detection of SARS-CoV-2. We prepared LCV[14] solution containing 0.5 mM crystal violet (CV), 30 mM sodium sulfite, and 5 mM β-cyclodextrin and stored at −20 °C until use. The prepared LCV dye (5.5 μL), 0.5 μL of AMV Reverse Transcriptase (10 U/μL), and 2−4 μL of the sample were added to a 25 μL LAMP reaction volume. For field use, we dried LCV.[14] A 10 μL volume of the mixture was dispensed into a 200 μL microtube and dried at 60 °C for 60 min in a vacuum oven to remove the solvent. When running RT-LAMP with dried LCV, a 10 μL of the LAMP reaction buffer was added to rehydrate the dried LCV dye. The RT-LAMP reaction was performed with either the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Japan) or

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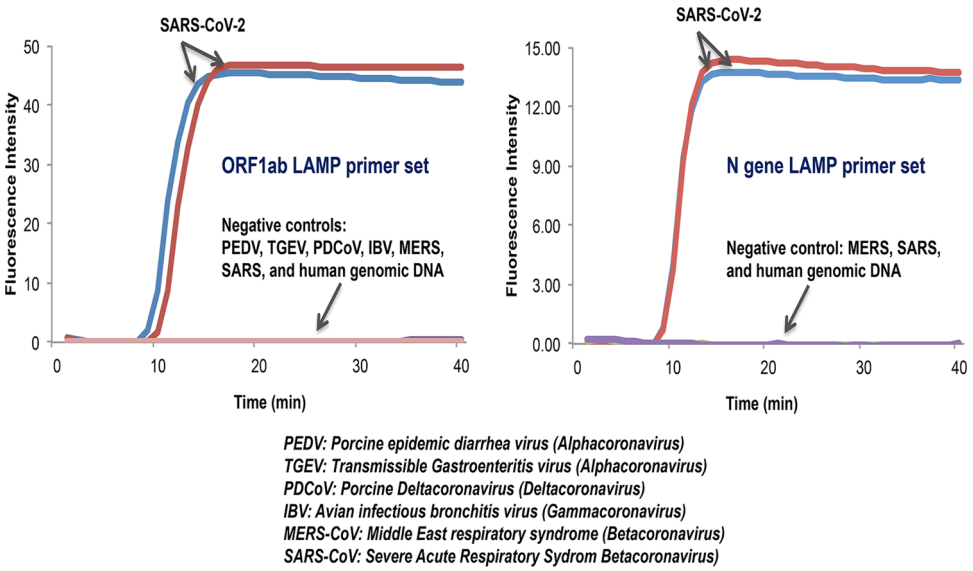

Figure 1. SARS-CoV-2 ORF1ab and N gene LAMP primer sets are specific. Only samples with SARS-CoV-2 produced a positive signal, while negative controls (PEDV, TGE, PDCoV, IBV, MERS-CoV, and SARS-CoV) did not show any amplification signal. Copies (10^4) of coronaviruses genome RNAs (PEDV, TGE, PDCoV, IBV), cDNA (MERS-CoV) or synthetic DNA (SARS-CoV) were added to each reaction. Reverse transcriptase (Promega) was included in the LAMP reaction mix.

OptiGene kit (ISO-001, OptiGene). For concurrent fluorescence monitoring and LCV colorimetric detection, the OptiGene kit is preferred. The reaction mixes were incubated with our bench top thermal cycler (BioRad, Model CFD3240), miniPCR 8 (miniPCR Bio), and our custom-made block heater at 63 °C for up to 40 min. The LCV color change was observed at the end of the incubation process by the naked eye and, if desired, can be recorded with a smartphone.

Closed-Tube Penn-RAMP. Penn-RAMP consists of two isothermal amplification processes: RT-RPA (38 °C) and LAMP (63 °C). We carried out the RT-RPA amplification in the lid of the tube and the LAMP in the tube itself. The RT-RPA reaction mix included 480 nM of each LAMP F3 and B3 primers, 1× rehydrated twistAmp Basic buffer (twistAmp Basic kit, TwistDx Limited, Cambridge, U.K.), 14 mM Mg-$(CH_3COO)_2$, 0.2 U/μL AMV reverse transcriptase (Promega), and 1−2 μL of purified SARS-CoV-2 RNA or the patient's sample. The sample was inserted in the tube lid together with the RPA mix. The LAMP reaction mix is as described above but without F3/B3 primers and without the target. The ratio of the volume of RPA/LAMP reaction mixtures' volumes was kept at 1:9 to prevent the inhibition of the LAMP reaction.[22] Typically, we used an RPA volume of 5 μL and a LAMP volume of 45 μL. After loading the tube lid with the RPA mix and the tube itself with the LAMP mix, the tube was sealed and remained so throughout the entire process, protecting the work area from exposure to amplicons. The closed tube was first incubated in our bench top thermal cycler or in the miniPCR (for colorimetric detection) with the lid and block temperatures at 38 °C. After 15−20 min, the tube was either centrifuged or flipped back and forth a few times to blend the RPA and LAMP reaction volumes. The tube was then incubated with both the lid and block temperatures at 63 °C for 40 min with real-time signal monitoring and/or endpoint colorimetric detection.

RT-RPA. The RT-RPA experiment was carried out with 10 μL of the rehydrated (1×) RPA reaction mix (TwistAmp Exo kit) containing 420 nM each of LAMP F3/B3 primers (Table 1), 14 mM magnesium acetate (MgOAc), 120 μM Exo-RPA Probe (synthesized by Sangon Biotech Co., Ltd., Shanghai, China, Table S4), 0.2 μL of AMV Reverse Transcriptase (10 U/μL), and 1 μL of SARS-CoV-2 RNA (National Sharing Platform for Reference Materials, China). The reaction mix was kept on ice prior to incubation. After vortexing, the reaction mix was incubated in a Thermal Cycler (BioRad, Model CFX96) at 38 °C with a plate-read every 30 s.

Inhibition Effect of the Swab Collection Medium and of Saliva on Direct RT-LAMP. Contrived swab and saliva samples were prepared by spiking inactivated SARS-CoV-2 virions into VTM, saline solution (0.9%), water, and saliva. These samples were treated with and without heating and in the presence and absence of our home-made RNase inhibitor TCEP/EDTA containing 1 mL of 0.5 M EDTA (pH = 8); 2.5 mL of 0.5 M Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl; Millipore Sigma, 580567); 0.575 mL of 10 N NaOH (final concentration 1.15 N); and 0.925 mL of UltraPure water to a final volume of 5 mL.[23] After RNA extraction, CDC SARS-CoV-2 RT-PCR was used to quantify the virus titer with a previously prepared calibration curve (Figure S3). The SARS-CoV-2 titer of the contrived samples was adjusted to ∼40 virions/μL (Figure S3C). The threshold time provided a metric to evaluate inhibition effects of the swab collection medium and saliva on direct RT-LAMP.

RESULTS AND DISCUSSION

Specificity of Our RT-LAMP Assay. In addition to examining COVID-19 RT-LAMP specificity in silico (Figures S1 and S2), we tested samples of other available coronaviruses, such as alphacoronaviruses (PEDV and RGEV), gammacoronavirus (IBV), deltacoronavirus (PDCoV), and betacoronavirus (MERS-CoV and SARS-CoV). Only SARS-CoV-2 samples produced a positive signal (Figure 1A,B). We did not observe any false positives.

Analytical Performance of COVID-19 RT-LAMP, RT-RPA, and Penn-RT-RAMP Assays. We prepared dilution series and carried out RT-LAMP (Figure 2), RT-RPA with F3/

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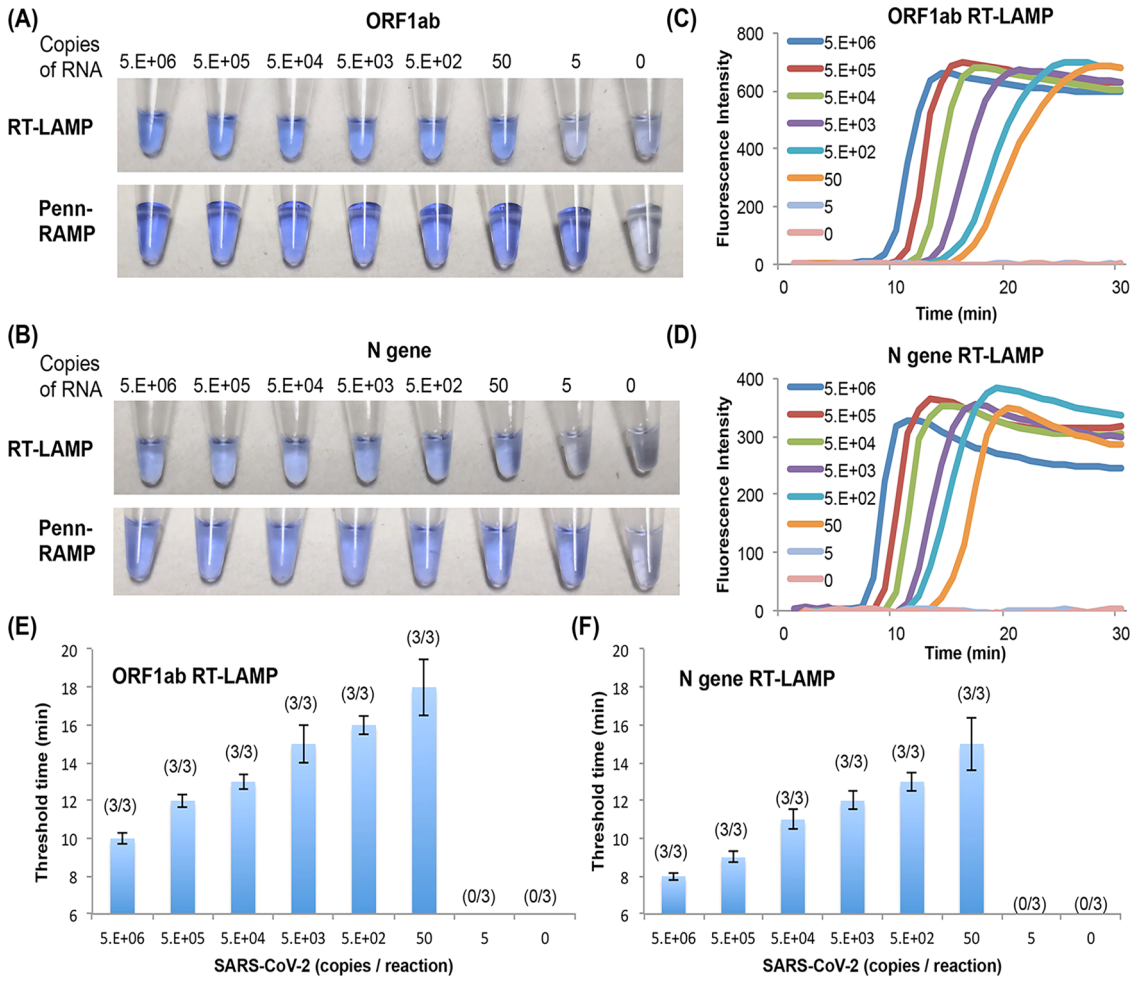

Figure 2. Real-time and colorimetric detection of SARS-CoV-2. (A, B) Visual endpoint detection (LCV dye) of SARS-CoV-2 amplicons with our direct COVID-19 RT-LAMP and our closed-tube Penn-RAMP with ORF1ab and N gene LAMP primer sets, respectively. (C, D) LAMP amplification curves corresponding to RT-LAMP in (A, B). (E, F) LAMP threshold time in (C, D) as a function of SARS-CoV-2 concentration (n = 3).

B3 primers (Figure S4) and Penn-RT-RAMP (Figure 2) with the ORF1ab and N gene primer sets and purified SARS-CoV-2 RNA as templates. We used, respectively, 25 and 50 μL of OptiGene buffer augmented with LCV dye for RT-LAMP and Penn-RAMP. The presence of LCV dye in the LAMP buffer had no apparent inhibitory effects and allowed us to monitor the LAMP in real time with the fluorescent dye included in the OptiGene buffer. The RT-RPA was monitored in real time with fluorescent EXO-RPA probes (Table S4). All experiments were carried out in triplicate.

RT-RPA successfully amplified the targets while operating with somewhat shorter F3/B3 LAMP primers (18−22 nt) than common (28−35 nt). The RT-LAMP with both colorimetric LCV dye and fluorescent dye (Figure 2E,F) and the RT-RPA detect as few as 50 RNA copies/reaction with either ORF1ab or N gene primers. The LAMP primer set targeting the N gene amplified 2−5 min faster compared to the other reported SARS-CoV-2 LAMP primer sets due to the shorter amplicon (Figure 2F).

Since visual detection does not require any instrumentation, the colorimetric LAMP assay is attractive for home use. The LCV dye is nearly colorless in the absence of dsDNA and turns deep violet in the presence of dsDNA, enabling detection of amplicons by the eye. The results of our colorimetric RT-LAMP detection are consistent with our real-time amplification curves, detecting as few as 50 targets/reaction (Figure 2A (top), B (top)). Penn-RAMP provides better sensitivity than standalone RT-LAMP (and standalone RT-RPA), changing color with as few as 5 copies/reaction with both the ORF1ab and the N primer sets (Figures 2A (bottom), B (bottom) and S5). In addition, the LCV dye-based colorimetric detection has advantages over phenol red (Supplementary Results and Discussion).

Infectivity of Coronavirus after Heat Treatment. We evaluated various rapid sample preparation methods, aiming to inactivate the virus while maintaining viral RNA's integrity. Out of safety concerns, we carried out our initial experiments with avian γ-coronavirus infectious bronchitis virus (IBV) isolates ($10^{3.2}$ EID_{50}/mL) as a surrogate for SARS-CoV-2 (Supplementary Methods). The IBV intact virus was inactivated entirely (no infected cells were observed) after incubation at 95 °C for ≥5 min, 70 °C for ≥10 min, and 56 °C for ≥30 min (Table S5). At shorter incubation times such as 5 min at 70 °C, viral activity was observed at high virus concentrations. We also incubated SARS-CoV-2 for 5 min at 70 °C and at 95 °C in a BSL3, obtaining similar results to the ones obtained with IBV.

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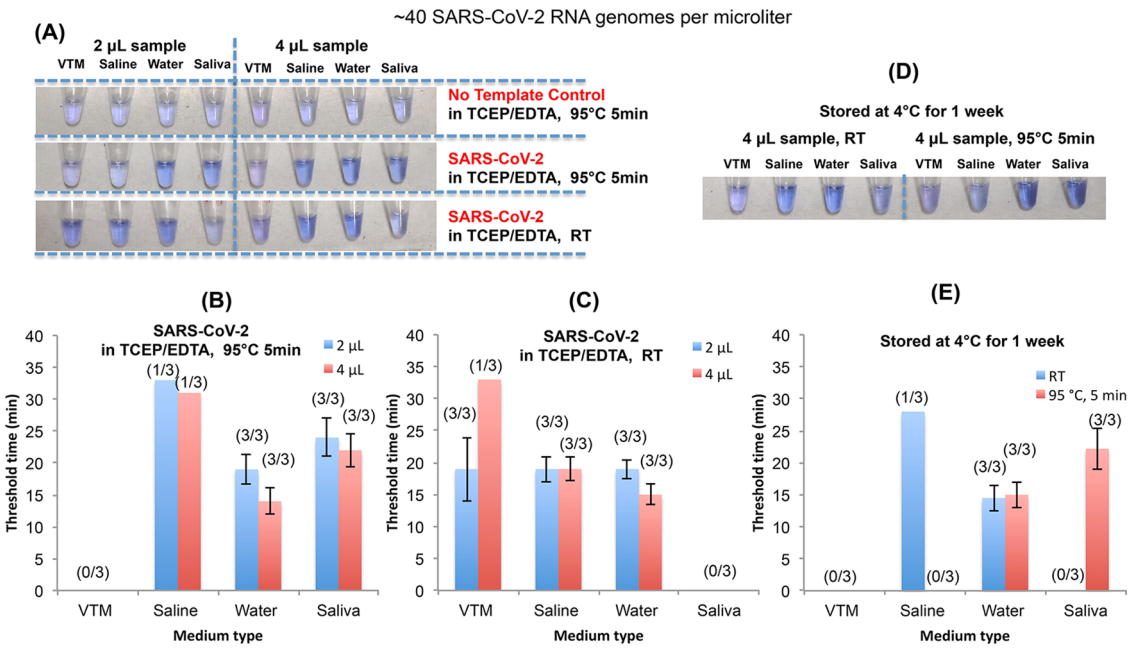

Figure 3. Inhibition effect of swab sample collection medium and saliva on direct RT-LAMP. (A) Impact of VTM, saline, water, and saliva on colorimetric detection of SARS-CoV-2 with LCV dye in the presence and absence of TCEP/EDTA and/or heat treatment. (B, C) LAMP threshold time as a function of medium type in the presence (B) and the absence of heat treatment (C) (n = 3). (D) Colorimetric detection of SARS-CoV-2 after storing the samples at 4 °C for a week. (E) LAMP threshold time in (D) as a function of medium type and heat treatment (n = 3). RT = room temperature. All LAMP experiments were carried out with OptiGene master mix (ISO-001) and the N gene LAMP primer set.

Effect of RNase Inhibitors on RNA Integrity after Heat Treatment. Next, we examined the effect of heat treatment and RNase inhibitors on RNA Integrity, using RT-qPCR's threshold cycle (C_t) as the figure of merit (Supplementary Methods). We carried out our experiments with IBV suspended in water in the absence of the RNase inhibitor, in the presence of the RNase inhibitor iNtRON (optimal working temperature 42 °C, Cat. No. 25011, iNtRON Biotechnology, Seongnam, Korea), and in the presence of our custom-made TCEP/EDTA RNase inhibitor (Figure S6). As a result of university safety regulations, SARS-CoV-2 had to be deactivated prior to our experiments, which forced us to use iNtRON at temperatures greater than that recommended by the manufacturer. In all cases, the threshold cycle (C_t) increased as the incubation time and temperature increased, indicating degradation of RNA. Specifically, the addition of our custom RNase inhibitor (TCEP/EDTA) resulted in a 2-fold (5 min incubation at 70 °C) and ∼4-fold (95 °C, 5 min) reduction in the number of templates. Five minutes heating at 70 and 95 °C resulted, respectively, in ∼8-fold and ∼16-fold reduction in the number of templates both in the presence and absence of iNtRON, indicating that iNtRON had little effect, if any. Among samples incubated under similar conditions, the C_t values decreased from pure water to the commercial RNase inhibitor (optimal temperature 42 °C) to our custom-made RNase inhibitor buffer (TCEP/EDTA). We see little benefit from the commercial RNase inhibitor, probably because its working temperature is too low to protect RNA well at 70 and 95 °C.

Although our preferred sample preparation is 5 min incubation at 95 °C in the presence of TCEP/EDTA, at the time of our experiments, our office of environmental safety has only approved incubation at 56 °C for 1 h for patient sample deactivation. Thus, all our patient samples were incubated at 56 °C for 1 h in the presence of the RNase inhibitor RNasin (Cat. N2615, Promega) with optimal temperatures ranging from 50 to 70 °C. Samples treated with RNasin exhibited better results (Table S6, shaded area) than samples untreated with RNasin. TCEP/EDTA yielded similar results to that of RNasin (Table S6).

Effect of Swab Elution Media on RNA Integrity after Heat Treatment. The swab elution media plays a key role in RNA degradation and polymerase inhibition (in the absence of purification). To investigate the optimal swab elution media, we spiked the heat-inactivated SARS-CoV-2 high-titer patient sample into VTM, saline solution, and water in the presence of TCEP/EDTA. The concentration of templates in each medium was quantified with the CDC-approved SARS-CoV-2 RT-PCR after RNA extraction. The final concentration of viral RNA genomes from intact and damaged viral particles in these spiked samples was ∼40 copies/μL. Then, we carried out direct OptiGene RT-LAMP (without RNA isolation) on these samples.

A viral transport medium (VTM) is frequently used to elute and preserve viral particles collected with swabs and to maintain viral viability for virus culturing. Although preservation of viral activity is neither needed nor desired for molecular tests, many laboratories still use VTM for molecular tests. Thus, we examined the effect of VTM after heat treatment on direct RT-LAMP. VTM samples spiked with SARS-CoV-2 (40 virions/μL) yielded true positives in the absence of heat treatment but puzzlingly false negatives in the presence of heat treatment (95 °C, 5 min). We suspect that heat treatment of VTM in the presence of TCEP/EDTA resulted in RNA degradation, reducing the number of templates available to the amplification process. To test this hypothesis, we carried out RT-qPCR with purified heat-treated samples and observed a significant delay in the threshold cycle of virions suspended in VTM (Figure S7) possibly due to the presence of RNase activity in the VTM (perhaps introduced with the fetal bovine

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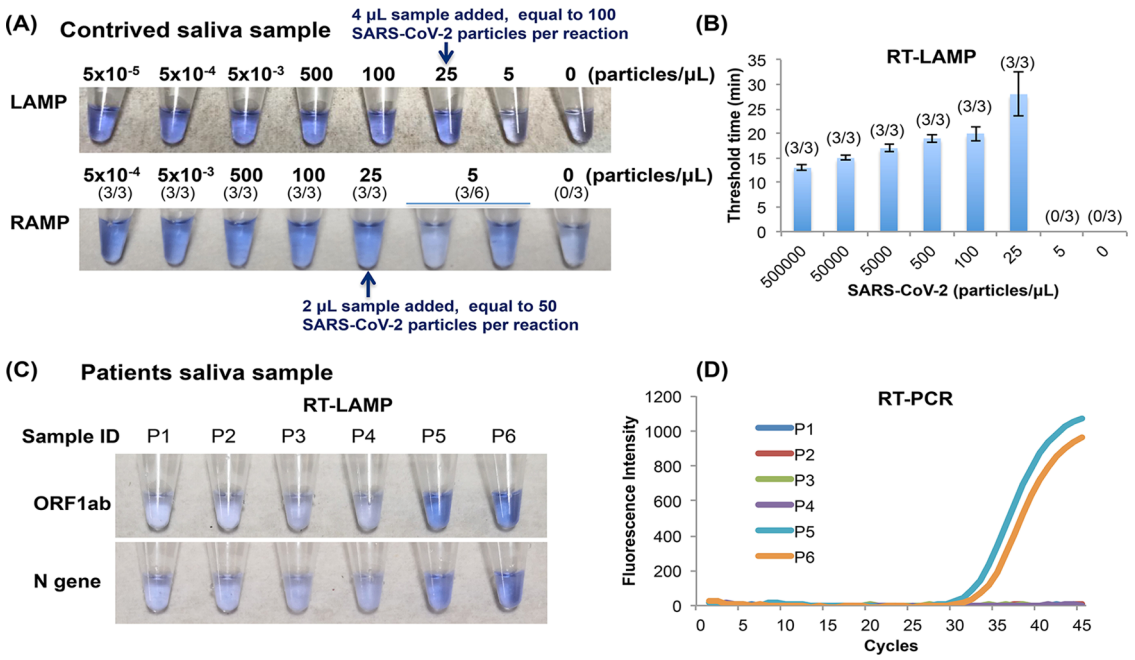

Figure 4. Saliva sample detection. (A) Colorimetric detection of the SARS-CoV-2 virus in contrived samples contained virions spiked in saliva. (B) RT-LAMP threshold time as a function of the SARS-CoV-2 virus concentration (n = 3). (C) Colorimetric detection of SARS-CoV-2 in saliva samples from suspected COVID-19 patients. A 4 μL of saliva was directly added to the RT-LAMP reaction mix. (D) RT-PCR amplification curves of the saliva samples in (C).

serum) that was not completely suppressed by incubation and presence of TCEP/EDTA. Others[24] have reported positive tests with heat-treated VTM at 95 °C for 1 min but at much greater virus concentrations than in our experiments.

In our hands, like in the case of VTM, heat treatment of saline solution spiked with the virus had an adverse effect (Figure 3B), perhaps due to RNA degradation. Surprisingly, the virus spiked in molecular water provided nearly the same threshold times in the presence and absence of heat treatment. Incubation at 95 °C (5 min) had little adverse effects, if any, on virions suspended in water (Figure S7).

VTM even when refrigerated did not serve as an effective storage medium, while molecular water provided the best storage medium with nearly the same threshold time after a week of refrigeration (4 °C) as that of freshly prepared samples (Figure 3D,E). In summary, water/TCEP/EDTA appears to be a superior swab collection medium and by far less expensive than VTM.

Inhibitory Effects of Swab Elution Media on Direct RT-LAMP. Next, we examine the inhibitory effects of swab elution media on direct RT-LAMP. Without heat treatment of the contrived swab samples, water and saline solution, both with TCP/EDTA, were the best elution media, providing the smallest threshold times and enabling positive identification of all spiked samples without any false positives (100% specificity) (Figure 3A,C). When the sample is virions suspended in water, our direct RT-LAMP detects down to 80 virions/reaction and produces a much lower threshold time than VTM (Figure 3C), suggesting that VTM inhibits polymerase. Such inhibition would not affect tests operating with purified RNA but has an adverse effect when unprocessed samples are added directly into the reaction mix. This is evident from samples with 4 μL of VTM having significantly greater threshold times than samples with 2 μL of VTM (half the number of templates) (Figure 3C).

Inhibitory Effects of Saliva on Direct RT-LAMP before and after Heat Treatment. Saliva is becoming the sample of choice for SARS-CoV-2 screening because of its ease of collection, amenability to self-collection, minimal risk to health care workers, and the absence of need for swabs and storage media that may be in short supply. In contrast to VTM, saline solution, and water, 95 °C incubation for 5 min in the presence of TCP/EDTA enhanced our ability to detect virions spiked in saliva (Figure 3B). In the absence of heat treatment, all our saliva samples yielded false negatives (Figure 3C). It appears that heat treatment diminishes inhibitors in saliva and affects favorably saliva's rheology. Doubling the saliva volume in the reaction mix reduced threshold time, suggesting that the incubation process neutralized LAMP inhibitors. Saliva samples incubated at 95 °C for 5 min in the presence of TCP/EDTA were successfully stored for a week without any significant degradation of viral RNA.

Direct Closed-Tube Penn-RAMP Outperforms Standalone Direct RT-LAMP and Standalone Direct RT-RPA. When testing purified RNA, Penn-RAMP provided about 10-fold better analytical sensitivity than standalone RT-LAMP and standalone RT-RPA (Figures 2 and S4). Does this advantage carry over when operating with minimally processed samples? We compared the detection of SARS-CoV-2 eluted from nasal swabs (contrived samples) (Table 1). Penn-RAMP detected successfully 4/4 of the samples with 100 virions in VTM, while standalone RT-LAMP and standalone RT-RPA detected, respectively, only 2/4 and 1/4 of similar samples as positives. All three assays detect 4/4 of samples with 100 virions in water as positive. Penn-RAMP successfully identifies 3/4 of the samples with 10 virions in water as positive, while standalone RT-LAMP and standalone RT-RPA yield false negatives for all of these samples.

Clinical Performance of COVID-19 Direct RT-LAMP and Closed-Tube Penn-RAMP Assays for Colorimetric

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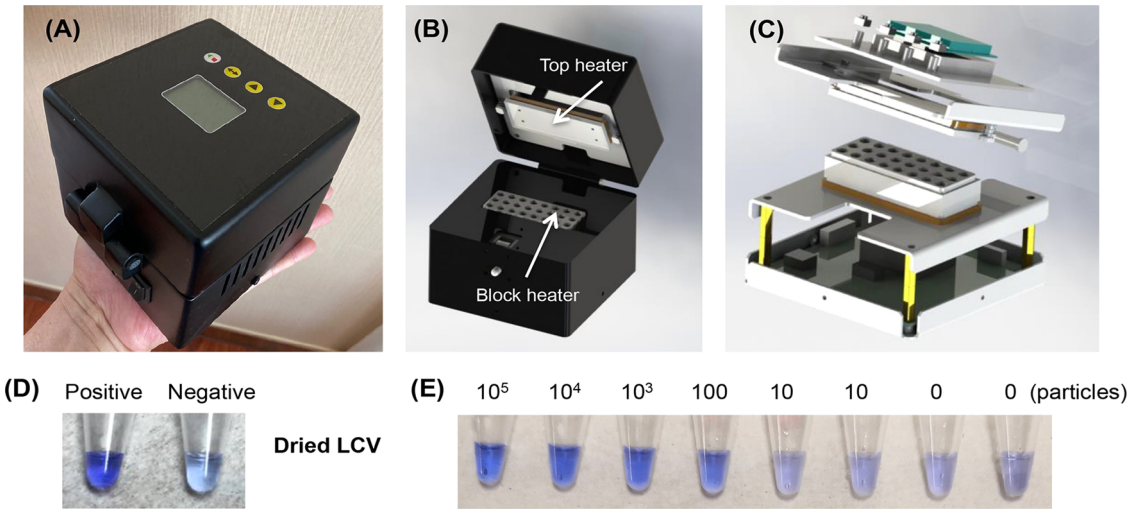

Figure 5. Block heater and dried reagents. (A), (B), and (C) outside, inside, and exploded views of our block heater. (D) Colorimetric detection of SARS-CoV-2 with the dried reaction mix and LCV dye. SARS-CoV-2 virions (10^3) used in the positive control. (E) Colorimetric detection of SARS-CoV-2 with the dry LAMP reaction mixture in the presence of 10^5, 10^4, 10^3, 100, 10, and 0 virions/reaction. These reactions were incubated with our custom-made block heater.

(LCV) Detection of Swab VTM and Water Samples. To examine the performance of our closed-tube molecular tests with minimal sample preparation such as might be used at home and in poor resource settings, we evaluated the COVID-19 direct RT-LAMP and Closed-Tube Penn-RAMP assays with clinical samples obtained by eluting swabs in VTM (Table S7, $N = 40$). After incubating the sample at 56 °C for 1 h, 2 μL of the VTM was added to the reaction mix. Our closed single-stage RT-LAMP and our Penn-RAMP had, respectively, sensitivities of 9/19 (47%) and 16/19 (84%) compared to the CDC EUA RT-PCR gold standard. Our RT-LAMP and Penn-RAMP detected, respectively, samples with RT-qPCR $C_t < 28$ and $C_t < 36$ without any false positives (100% specificity). Importantly, both our assays operated without nucleic acid isolation. Penn-RAMP outperformed RT-LAMP likely because of its higher amplification efficiency and greater tolerance to inhibitors in the VTM.

Next, we eluted different swabs collected from the same patient into water/TCEP/EDTA and VTM/RNasin and compared the RT-LAMP performance for detecting SARS-CoV-2 in these two media (Table S8, $N = 5$). Our direct RT-LAMP identified all (5/5) RT-qPCR positive ($C_t \leq 32$) eluates in water but only 4/5 RT-qPCR-positive eluates in VTM. The VTM sample with RT-qPCR $C_t > 28$ yielded a false negative. Consistent with our previous data, water eluates produced shorter threshold times than VTM eluates.

Performance of COVID-19 Direct RT-LAMP and Closed-Tube Penn-RAMP Assays for Colorimetric (LCV) Detection of Virions in Saliva. Saliva collection is both noninvasive and convenient and has been advocated as a reliable medium for SARS-CoV-2 screening.[25−27] Here, we investigate colorimetric detection of saliva with our direct RT-LAMP (Figure 4A (top), B) and closed-tube Penn-RAMP (Figure 4A (bottom)). We carried out our experiments with both dilution series of contrived samples and with actual patient samples.

When operating with contrived samples, RT-LAMP targeting the N gene has successfully detected SARS-CoV-2 in 4 μL of saliva at a titer of 25 virions/μL (100 virions/reaction) (Figure 4A (top), B), while Penn-RAMP successfully detected 2 μL of saliva at a titer of 25 virions/μL (50 virions/reaction) and less reliably (3/6) at a titer of 5 virions/μL (Figure 4A (bottom)).

At the time of our experiments, the Hospital of the University of Pennsylvania did not collect saliva samples from patients. Hence, we had only limited access to actual patient saliva samples. We tested six patient saliva samples. Each of these samples was subjected to the standard RT-qPCR test. Only two of the samples were positive with an estimated viral load of 25 virions/μL (Figures 4D and S3B). We added TCEP/EDTA to all patient samples, heated the samples to 95 °C for 5 min, and then tested each of the samples with our direct RT-LAMP; with one assay targeting the ORF1ab and the other targeting the N gene (Figure 4C). All of the positive samples were detected as positive and all negative samples were detected as negative by our direct RT-LAMP, attesting to the efficacy of our proposed assay.

Block Heater and Dried Reagents. Although our direct RT-LAMP and closed-tube Penn-RAMP can be incubated either in a water bath, in a domestic oven with temperature control, or with an exothermic chemical reaction without any electrical power,[11,12] we developed an inexpensive (~$75), portable block heater (Figure 5A−C), capable of incubating either RT-LAMP or Penn-RAMP, to enable testing in centralized locations such as offices and clinics.

Furthermore, for home and field use, it is desirable to avoid the need for a cold chain. To this end, we augmented the lyophilized OptiGene master mix (ISO-DR004) that has both polymerase and reverse transcriptase activities, with vacuum-dried LCV. The performance of the dry reagents was equivalent to that of our wet reagents (Figure 5D). Our RT-LAMP incubated with our block heater successfully detected 100 SARS-CoV-2 virions/reaction (Figure 5E).

CONCLUSIONS

We have designed and tested two sets of primers for SARS-CoV-2 RT-LAMP. One set of primers targets a sequence in the ORF1ab region and the other the N gene. Both primer sets provide efficient amplification and are specific, distinguishing SARS-CoV-2 from other coronaviruses. Our RT-LAMP primer

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sets were adapted by others with equally good results.[28,29] Additionally, we have demonstrated colorimetric detection with the intercalating LCV dye that changes from nearly colorless to violet in the presence of amplicons (dsDNA); is visible to the naked eye; and unaffected by sample composition such as PH.

We have used each primer set in our single-stage RT-LAMP and in our newly developed two-stage Penn-RAMP to detect the presence of SARS-CoV-2 in contrived and in clinical samples, without RNA isolation. The contrived samples comprised of synthesized oligos replicating segments of the SARS-CoV-2 genome and cell-cultured virions spiked in various media. The patient samples included swabs and saliva collected in the intensive care unit and the emergency room of the Hospital of the University of Pennsylvania. We examined various elution media for the swab samples, ranging from VTM, saline solution, to molecular water.

When testing contrived samples, our RT-LAMP and Penn-RAMP assays successfully detected, respectively, down to 50 and 5 virions/reaction. Our assays operated reasonably well with samples mixed with the RNase inhibitor with and without thermal incubation. Our direct RT-LAMP tolerated up to 8% of minimally processed samples that included saliva and swab-collected samples eluted in VTM, water, or saline solution. Among the various swab elution media, we find molecular water to provide the best results while VTM inhibits polymerase.

Penn-RAMP has better sensitivity and tolerance to contaminants than RT-LAMP alone. Also, it has better specificity and does not require molecular probes or lateral flow devices for amplified product analysis compared with standalone RPA.

In contrast to NEB colorimetric assay that uses phenol purple to detect proton production during polymerase and is susceptible to pH variations among samples, occasionally leading to false positives (Figure S8D), our LCV dye is unaffected by sample pH variability. Our reaction mix and dye are amenable to dry storage, eliminating the need for a cold chain.

Since our direct RT-LAMP and the second-stage LAMP in Penn-RAMP tolerate, respectively, relatively small volumes of the unprocessed sample and first-stage RPA products, there are limitations to our assays' sensitivities. These can potentially be addressed with the development of more robust LAMP enzymes and by modifying the second-stage LAMP buffer composition to tolerate a greater volume of first-stage products.

We hope that our test and similar ones can be adapted for home use, enabling individuals to test themselves every morning prior to leaving their house and quarantine themselves when warranted. This modus operandi that provides prompt test results, eliminates the need for potentially contagious individuals queuing at sample collection sites and posing a risk to themselves and others and provides timely information to policymakers to make rational decision critical to the management of the pandemic.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c03016.

Experimental details of qRT-PCR, calibration curve establishment, verification of the specificity of the LAMP Assay, investigation of infectivity of coronavirus and RNA integrity after heat treatment, blocker heater fabrication; results and discussion on the comparison of Direct RT-LAMP with OptiGene Reaction Mix and NEB Colorimetric Master Mix; and additional experimental data (PDF)

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Notes

The authors declare the following competing financial interest(s): University of Pennsylvania has applied for a patent on Penn-RAMP with J.S. and H.H.B. listed as co-inventors.

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