A community-deployable SARS-CoV-2 screening test using raw saliva with 45 minutes sample-to-results turnaround

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Abstract

Up to 70% of SARS-CoV-2 infections in working- and school-age people are asymptomatic (Poletti et al., 2020), creating anxiety over reopening workplaces and schools around the world. In the absence of effective treatments or a vaccine, peace of mind will come only with community-based SARS-CoV-2 screening, where many people are tested on a regular basis. However, recent models show that short sample-to-answer turnaround time will be a critical property of effective screening strategies (Larremore et al., 2020). Here, we describe an RT-LAMP test for SARS-CoV-2 in raw saliva that takes about 45 minutes from sample to answer and requires only simple equipment (pipettes and a heating source). The assay has a limit of detection of 100 virions per microliter, and targets two separate regions of the SARS-CoV-2 genome. By combining rapid sample-to-answer turnaround time with the use of saliva, our RT-LAMP assay provides a low-complexity, portable, and robust system for real-time community screening.
Introduction

Infectious disease screening is one of the most basic and powerful tools in the public health arsenal. Screening tests are administered to many individuals in a defined population and are designed to identify an underlying illness in apparently healthy or asymptomatic individuals. Starting in the early 20th century, screening tests have been used to effectively dampen the spread of infectious diseases such as syphilis and HIV, and to trigger lifesaving interventions in those found to be infected (Morabia and Zhang, 2004). In the ongoing HIV/AIDS pandemic, the public health “trio” of screening focused on high-risk persons, contact tracing, and (eventually) the administration of effective drugs significantly curbed new infections by this devastating virus (Branson et al., 2006; DiNenno et al., 2017; Edelman et al., 2020). In the United States, tens of millions of HIV tests are administered every year (CDC, 2008; Hurt and Powers, 2014). Key to the current success and scale of HIV/AIDS testing was the aggressive development of infrastructure for HIV screening throughout communities, including in non-clinical settings such as homeless shelters, parks, and social services storefronts (Bowles et al., 2008; Thornton et al., 2012). In these settings, people who test positive receive critical guidance on how to prevent infecting others while they await confirmatory testing and care in clinical settings. In the case of HIV, client satisfaction with community-based screening is high (Simeone et al., 2017; Thornton et al., 2012).

For the current SARS-CoV-2 pandemic we similarly need to employ community-level surveillance and occupational screening, especially until more effective therapeutics become available. This has taken on new importance as we have learned that potentially as many as 70% of individuals under the age of 60 will not show symptoms of SARS-CoV-2 infection, yet may maintain the ability to spread the virus to others (Poletti et al., 2020). However, compared to HIV the spread of SARS-CoV-2 operates on a far less-forgiving timeline, as respiratory
infections spread faster and more broadly to the general population than sexually transmitted infections.

Modeling approaches show that one of the most important factors in screening for SARS-CoV-2 will be the speed with which infected people receive their test results (referred to as turnaround time) (Larremore et al., 2020). The longer it takes for people to receive their results, the more time goes by where they might unwittingly infect others. Rapid turnaround time has previously been identified as critical in the diagnosis of other respiratory pathogens, such as tuberculosis (Somoskovi and Salfinger, 2015). For SARS-CoV-2 screening, turnaround time appears to be nearly as important as the frequency of screening, and more important than the assay limit of detection (Larremore et al., 2020). Many SARS-CoV-2 tests require that biospecimens be collected, transported to centralized labs, logged, queued, processed, analyzed, and then results communicated back to the agency that sent the sample for testing, and finally delivered to the person who had the test (Goswami et al., 2010; Holland et al., 2005). For this reason, SARS-CoV-2 testing in the United States is currently slow, commonly requiring one or more days before results can be returned (Babiker et al., 2020). At this turnaround time, screening will have a weakened effect on controlling the pandemic (Larremore et al., 2020).

Effective SARS-CoV-2 screening requires tests with rapid turnaround time and broad participation, enabled by a combination of two key factors. First, removing the dependence on sophisticated laboratories will eliminate the need for sample transport from collection to laboratory. Second, using an easily sampled biospecimen such as saliva will decrease behavioral barriers to testing resulting from the discomfort of sampling, particularly for those without symptoms. Many researchers quickly realized that reverse transcription loop-mediated isothermal amplification (RT-LAMP) was an attractive method that could satisfy these criteria for SARS-CoV-2 (Baek et al., 2020; Bhadra et al., 2020; Huang et al., 2020; Jiang et al., 2020;
Kitagawa et al., 2020; Lalli et al., 2020; Lamb et al., 2020; Lau et al., 2020; Lu et al., 2020; Park et al., 2020; Rabe and Cepko, 2020; Yan et al., 2020; Yu et al., 2020). RT-LAMP is fast (typically < 1 hour from sample to result), robust to sample contaminants in crude specimen preparations, and requires no sophisticated equipment. RT-LAMP assays amplify nucleic acids at a constant temperature, requiring only inexpensive equipment that divorces them from clinical laboratories altogether (Notomi et al., 2000). In fact, RT-LAMP assays have been used in remote locations to screen mosquitoes directly for the presence of Zika virus (Brewster et al., 2018; Chotiwan et al., 2017). These assays 1) are specific because they employ four to six primers for each amplification reaction which in principle reduces off-target effects when compared to PCR, 2) are robust in that they can produce on the order of $10^9$ copies of the target in an hour-long reaction (Notomi et al., 2000), and 3) can be performed anywhere because they simply require pipettors and a heating source (e.g. water baths, heat blocks, or thermal cyclers) as equipment. Here, we present a rapid RT-LAMP assay for SARS-CoV-2 RNA detection and present evaluations of this test that relate to the design and performance criteria required by the U.S. Food and Drug Administration (FDA). If such a test were to be used for community screening outside of CLIA-certified diagnostic labs, effective interventions could be taken immediately while awaiting confirmatory tests at partner CLIA labs.
Results

At a high level, our screening test has four fundamental steps:

1. **Saliva self-collection:** The person to be tested provides a 1-mL saliva sample into a tube by spitting a few times. They then use a simple dispenser to dispense a fixed amount of stabilization buffer (described herein) into their own saliva. They close the tube, invert several times, and place it on ice. The remaining steps are done exclusively by testing staff.

2. **Sample preparation by boiling:** The tube is immersed in a 95°C water bath for 10 minutes to inactivate any SARS-CoV-2 virions in the sample. The tube is now presumed safe to open. This step also liberates viral RNA to be measured in the next step.

3. **RT-LAMP assay:** A small amount of the boiled saliva sample is added to three tubes containing a pre- aliquoted RT-LAMP enzyme mixture. Each of the three tubes contains a unique primer set, one that amplifies a host control RNA (positive control), and two that amplify different regions of the SARS-CoV-2 genome. The three tubes are placed in a 65°C heating source for 30 minutes, and reactions are then stopped by incubating at 80°C for 5 minutes.

4. **Read results:** Tubes are removed and visually inspected. If a color change from pink to yellow occurs in all three tubes, the reaction is positive, indicating that the saliva sample does contain the SARS-CoV-2 genome. The tested individual should be directed to a healthcare provider for confirmation testing, and should self-quarantine until their status is resolved.
The following sections provide detailed descriptions of the development and performance of this screening test.

**Optimized RT-LAMP primers targeting multiple regions of the SARS-CoV-2 genome**

A critical parameter in the design of RT-LAMP tests is the primer design. Most RT-LAMP reactions require a combination of 4-6 primers all working together (Notomi et al., 2000). Of published primer sets, we found that the “As1e” set, developed by Rabe et. al. and targeting the ORF1ab region of the SARS-CoV-2 genome, performs well (Rabe and Cepko, 2020). However, in order to target two distinct regions from the SARS-CoV-2 genome, we designed and tested a large number of additional primer sets. Two of our custom sets, “ORF1e” targeting the ORF1ab gene, and “N2” targeting the N gene, exhibited similar sensitivity and amplification efficiency as the As1e set, as determined using real-time fluorescence monitoring of RT-LAMP products (Figure 1A). In these reactions, raw saliva samples were mixed 1:1 with a stabilization buffer (described in detail below), boiled at 95° for 10 minutes, spiked with in vitro transcribed SARS-CoV-2 RNA to reach the indicated concentrations, and 4 µl of this processed saliva sample was used in a final reaction volume of 20 µl with NEB’s WarmStart LAMP 2x Master Mix. Reactions were incubated at 65°C and a fluorescence reading was taken every 30 seconds. All reactions became saturated between 20-25 minutes, and we therefore proceeded with further analyses using these primer sets and a reaction incubation time of 30 minutes at 65°C. From this point forward, we also include a control RT-LAMP primer set, “RNaseP,” which amplifies the human-derived mRNA produced from the POP7 gene, and therefore serves as a positive control that the reaction worked. This primer set was developed in 2018 (Curtis et al., 2018).
Figure 1: Optimized RT-LAMP parameters for detecting SARS-CoV-2 in human saliva. A) Three RT-LAMP primer sets targeting the SARS-CoV-2 genome (As1e, ORF1e, and N2) were tested with real-time RT-LAMP. Saliva samples were mixed 1:1 with stabilization buffer, boiled at 95°C for 10 minutes, spiked with in vitro transcribed SARS-CoV-2 RNA to reach the indicated concentrations, and 4 µl was used as a template in each RT-LAMP reaction with a final volume of 20 µl. EvaGreen was used to monitor amplification products in real-time (X-axis) using a QuantStudio3 qPCR machine. There are 9 lines for each of the three primer sets because three concentrations of spiked in SARS-CoV-2 RNA were each tested in triplicate (0, 200, 400 copies/µl). The normalized change in fluorescence signal (ΔRn) is shown on the Y-axis. B) Saliva samples were diluted 1:1 with stabilization buffer and then spiked with the indicated concentrations of in vitro transcribed SARS-CoV-2 RNA either before or after sample was boiled at 95°C for 10 minutes, and then subjected to RT-LAMP at 65°C for 30 minutes. If reactions remain pink then no amplification occurred, whereas tubes turn yellow if there is an amplification event. An RT-LAMP primer set targeting the human
RNaseP transcript is included as a host and extraction control in addition to the three SARS-CoV-2 primer sets shown in panel A. C) Saliva samples were diluted 1:1 with stabilization buffer before being spiked with the indicated concentrations of heat-inactivated SARS-CoV-2 virions. Samples were then boiled at 95°C for 10 minutes, and subjected to RT-LAMP similarly to the experiment shown in panel B. D) Saliva samples were diluted 1:1 with stabilization buffer before being spiked with the indicated concentrations of heat-inactivated SARS-CoV-2 virions. Samples were then boiled at 95°C for the indicated amount of time, and subjected to RT-LAMP similarly to the experiment shown in panel B. All reactions contain the As1e primer set. Duplicates are presented at each time point.

Stabilizing saliva: Liberating RNA genomes from virions, protecting RNA from degradation, and dealing with pH variability in human saliva

Multiple studies have developed methods for using saliva samples in combination with RT-LAMP specifically with a colorimetric readout (Lalli et al., 2020; L’Helgouach et al., 2020; Rabe and Cepko, 2020). However, the inherent variance in the acidity of saliva samples in a population, which can vary between 6.8 and 7.4 (Cameron et al., 2015), poses a significant problem because the colorimetric readout for RT-LAMP is pH-dependent. In colorimetric versions of the RT-LAMP assay, reactions remain pink in tubes where no amplification occurs, whereas tubes in which an amplification event takes place experience a pH drop to pH 6.0 – 6.5 and turn yellow (Tanner et al., 2015). The RT-LAMP reaction utilizes phenol red as the visual indicator, which turns strongly yellow at pH 6.8 and below, but remains pink at higher pH. For this reason, we find that about 10% of human saliva samples are naturally acidic enough to turn the reaction yellow (positive) immediately when added to the LAMP reaction, before the 30-minute amplification reaction even begins.

There has been at least one attempt to optimize a stabilization buffer that can be added to saliva after collection to normalize pH and alleviate this issue (Rabe and Cepko, 2020), however, we have found that the problem still persists for a significant proportion of individuals.
To address this issue, we further optimized a saliva stabilization buffer by titrating into it various concentrations of NaOH to determine at what concentration saliva samples do not immediately produce false positives upon addition to colorimetric RT-LAMP reaction mix, while still preserving the pink-to-yellow color change upon amplification. We found that a final concentration of 14.5 mM NaOH after mixing 1:1 with raw saliva is optimum to inhibit false positives caused by saliva acidity (Supplementary Figure 1A) without affecting the intended color change during amplification (Supplementary Figure 1B).

Boiling samples at 95°C has been used as a means to both inactivate infectious samples and to liberate SARS-CoV-2 genomic RNA from virions (Batejat et al., 2020; Pastorino et al., 2020; Ranoa et al., 2020). However, the use of in vitro transcribed SARS-CoV-2 RNA as a standard requires that it be spiked into saliva samples after boiling. This is because RNA spiked in before boiling is degraded, although apparently not if it is inside cells (compare host RNaseP control to viral RNA in Figure 1B). The FDA’s most recent guidelines for SARS-CoV-2-related Emergency Use Authorization (EUA) (last updated July 2, 2020) explicitly state that the preferred method when evaluating a potential test is to spike inactivated virions into a sample matrix (in our case saliva) before sample processing. As such, we proceeded to spike-in heat-inactivated SARS-CoV-2 virions into saliva before boiling and were able to show that the viral RNA is preserved through boiling under such conditions (Figure 1C). Therefore, we use heat-inactivated SARS-CoV-2 virions as our standard of choice for the remainder of this study.

When boiling saliva samples for downstream analysis of RNA, one must balance boiling long enough to liberate the target RNA from virions with not boiling for so long that the target RNA will be degraded. RNA degradation during boiling can be minimized with the addition of a chelating agent which we include in our stabilization buffer (1 mM EDTA final concentration). We evaluated the effect of various boiling times on saliva by testing 0, 5, 10, 15, and 30-minute
time points (Figure 1D). Without boiling, no SARS-CoV-2 RNA can be detected with RT-LAMP, presumably because virions remain intact and RNA is not accessible by the amplification enzymes. Amplification is somewhat inconsistent at 5 and 30 minutes of boiling presumably because at 5 minutes hardly any RNA has been liberated, and at 30 minutes it has been largely degraded. However, 10 or 15 minutes of boiling appear to provide just the right balance between liberating and preserving RNA. Therefore, to minimize overall processing time we chose a 10-minute incubation of saliva samples at 95ºC. Recent studies suggest that incubation for 3 minutes at 95ºC is sufficient to inactivate SARS-CoV-2 virions (Batejat et al., 2020), so our test procedure is purposely designed such that testing personnel avoid handling open tubes until after this step.

Stability of saliva samples from the time of collection to the time of processing and analysis is important if testing cannot be performed immediately, or if the tests are being conducted in batches. Using our optimized saliva stabilization buffer (2X stabilization buffer: 5 mM TCEP, 2 mM EDTA, 29 mM NaOH, 100 µg/mL Proteinase K, diluted in DEPC-treated water), we tested saliva collection and storage over a range of SARS-CoV-2 virion spike-in concentrations. Saliva samples containing virions and diluted in stabilization buffer were stored at 4ºC for 24, 48, or 96 hours before being boiled and analyzed using colorimetric RT-LAMP (Supplemental Figure 2). We observed no significant changes in sample stability over this time course, suggesting that saliva samples stored in stabilization buffer at 4ºC are stable for at least 4 days.

From the experiments described above we selected the final optimized conditions for our colorimetric RT-LAMP assay: 1) raw saliva mixed 1:1 with stabilization buffer, 2) sample boiled at 95ºC for 10 minutes, 3) 4 µl processed saliva added to a colorimetric RT-LAMP reaction at a
final volume of 20 µl, 4) reactions incubated at 65°C for 30 minutes, 5) reactions deactivated at 80°C for 5 minutes, and 6) reactions analyzed for pink-to-yellow color change. These conditions provide an end-to-end processing and analysis time of approximately 45 minutes.

Limit of detection (LOD)

We next initiated validation studies to evaluate the limit of detection for not just one, but a combination of primer sets which together would compose a full testing kit. In accordance with EUA guidelines, we began by analyzing six independent replicates for each of four primer sets (RNase P serves as an extraction and reagent performance control). The As1e, ORF1e, and N2 primer sets each amplified three distinct regions of the SARS-CoV-2 genome over a range of SARS-CoV-2 virion spike-in concentrations (Figure 2A). The lowest concentration at which an acceptable number of positive reactions were observed (red box, summary table in Figure 2A) was 100 virions/µl of stabilized saliva. We refer to this concentration as ‘1X LOD’ in subsequent analyses.

We next tested 20 replicates at 1X LOD concentration using all four primer sets (Figure 2B). In this experiment, EUA guidelines require that 19/20 reactions give a positive result. As can be seen, this threshold was achieved with each of the RNaseP, As1e, and N2 primer sets (red box, summary table in Figure 2B). The ORF1e primer set was not consistent in its performance at 1X LOD. Therefore, we decided to eliminate the ORF1e primer set from our testing panel and define a final colorimetric RT-LAMP test that includes primer sets RNaseP, As1e, and N2.
Figure 2: SARS-CoV-2 virion limit of detection using RT-LAMP and saliva samples. A) Saliva samples were diluted 1:1 with stabilization buffer before being spiked with the indicated concentrations of heat-inactivated SARS-CoV-2 virions (top). Samples were then boiled at 95°C for 10 minutes, and subjected to RT-LAMP at 65°C for 30 minutes in 6 replicates. Each panel represents a unique primer set (listed at the bottom of each panel). The table at the bottom shows a summary of positive reactions observed (yellow). Red box indicates our selection of limit of detection (LOD). B) Saliva samples were diluted 1:1 with stabilization buffer before being spiked with heat-inactivated SARS-CoV-2 virions at a concentration of 1X LOD (100 virions/µL). Samples were then boiled at 95°C for 10 minutes and twenty replicates of RT-LAMP with the indicated primer set were incubated at 65°C for 30 minutes. The table at the bottom shows a summary of positive reactions (yellow). Red box indicates our selection of primer sets to advance to subsequent analysis.
Clinical evaluation using contrived clinical specimens

We designed this test as a screening tool for asymptomatic populations. Very recent updates to the EUA guidelines (June 10, 2020) formally recognize the need for such a testing application. However, these guidelines request that 20 SARS-CoV-2 positive samples be collected from the target populations (here, asymptomatic individuals). Additionally, matched nasal swabs are required for these samples. Assuming a SARS-CoV-2 prevalence between 0.5-1.0% (prevalence currently varies dramatically by location and over time), such a trial would require between 2000 and 4000 participants to achieve the 20 positive sample request. At the time of writing this manuscript, IRB approval is being sought to carry out such a study.

To nevertheless estimate the performance of this test, we carried out a simulated clinical evaluation using contrived clinical specimens, a strategy recommended in previous versions of the EUA guidelines. One member of our personnel spiked-in SARS-CoV-2 virions into 30 saliva samples at various concentrations (1X – 10X LOD) and also prepared 30 samples with no SARS-CoV-2 virion spike-in. Sample identification was recorded, sample tubes were shuffled randomly, and then passed blindly to a second member of our personnel to carry out our colorimetric RT-LAMP test. After running the test and scoring samples based on the metrics in Table 1, sample identification was predicted and compared with the original record. Out of these 60 blinded samples, only one sample did not score correctly (Figure 3). This positive sample was scored as inconclusive because one of the SARS-CoV-2 primer sets (N2) failed.

Summary statistics of results are presented in Tables 2 and 3. All negative samples were scored correctly (100% specificity, binomial 95% confidence interval [88%,100%]). Conservatively counting the inconclusive test as a false negative leads to a sensitivity estimate
of 97% (binomial 95% confidence interval [93%,100%]). In summary, the test appears to have high specificity and sensitivity, even though it can be conducted in only 45 minutes.

| Table 1. Assay results and interpretation |
|------------------------------------------|
| As1e | N2 | RNaseP | Result Interpretation | Report        |
| +    | +  | +/-    | SARS-CoV-2 detected   | Positive SARS-CoV-2 |
| If only one of the two targets is positive | +/- | Inconclusive result | Inconclusive |
| -    | -  | +      | SARS-CoV-2 not detected | Not detected   |
| -    | -  | -      | Invalid Result | Invalid |

If only one of the two targets is positive

If both targets are positive

If either of the two targets is negative

If both targets are negative
Figure 3: Blinded sample evaluation. Plain saliva, or saliva spiked with heat-inactivated SARS-CoV-2 virions at different concentrations, was boiled at 95°C for 10 minutes. Samples were then analyzed using RT-LAMP by a researcher that did not know the identification of each sample. Experiments in figure) For each sample, three reactions were performed as indicated by each triplet of tubes. By looking at the patterns of yellow and pink results in each triplet, samples were scored according to Table 1, and then compared to the true status of each sample. The true status of each sample is shown with a white box on the triplet if the sample contained SARS-CoV-2, or with no white box if the sample was plain saliva. The red box indicates the only sample that did not score correctly. This sample did have SARS-CoV-2 spiked into it, but one of the SARS-CoV-2 primer sets failed (N2). This failed reaction is still pink (negative) even though the tube has 2xLOD virus. Throughout this work, 1X LOD = 100 virions / µl. Summary statistics for this experiment are provided in Tables 2 and 3.
**Table 2.** Summary of results from the contrived specimen study with saliva samples, stratified by target level and measurand

| Virions / µL saliva | Number Tested | RNase P | As1e | N2 | Positive (%) |
|---------------------|---------------|---------|------|----|--------------|
| 1X LOD              | 100           | 9       | 9 (100%) | 9 (100%) | 9 (100%) |
| 2X LOD              | 200           | 11      | 11 (100%) | 11 (100%) | 10 (91%) |
| 5X LOD              | 500           | 5       | 5 (100%) | 5 (100%) | 5 (100%) |
| 10X LOD             | 1000          | 5       | 5 (100%) | 5 (100%) | 5 (100%) |
| Negative            | 0             | 30      | 30 (100%) | 0 (0%) | 0 (0%) |
| Total               | 60            |         |       |    |              |

**Table 3.** Summary of positive and negative agreement with contrived saliva samples

| Saliva RT-LAMP Test | Contrived Specimen Type | Positive | Negative | Total |
|---------------------|-------------------------|----------|----------|-------|
| Positive            | 29                      | 0        | 29       |
| Inconclusive        | 1                       | 0        | 1        |
| Negative            | 0                       | 30       | 30       |
| Total               | 30                      | 30       | 60       |

Positive Agreement: 97% (29/30)

Negative Agreement: 100% (30/30)
Discussion

In this work we describe a rapid, simple-to-perform test for the SARS-CoV-2 virus that does not require sophisticated laboratory equipment (Figure 4). With minimal set-up this test could be performed in diverse settings such as factories, meat-packing plants, office buildings, or schools. For instance, imagine a simple lab set up at the exit of a factory. Workers provide a saliva sample before they leave for the day, RT-LAMP is performed, and the employer sends workers with positive results a text directing them to not come to work the next day and instead to seek confirmatory testing and care. This is a win-win because 1) the person who is possibly infected gets early notification, allowing them to seek treatment and to protect their families, 2) their coworkers are safer because the flagged person does not come to work until they are cleared, and 3) the employer has peace of mind that they are operating a safe work environment. This, and other rapid tests being developed for SARS-CoV-2, could be deployed for surveillance in a broad range of settings and thereby impact the current course of the pandemic. A critical step in this process will be a new mindset at governmental regulatory agencies that recognizes the importance of such surveillance tests and therefore rapidly approves their use, not for clinical testing, but for surveillance testing. In this manner, such rapid tests for SARS-CoV-2 could be considered a more sensitive and specific version of temperature screening, which is already used broadly throughout society to identify individuals with high probability of infection.
Figure 4. Assay overview in four steps. **Upper left**) Person provides 1 ml of saliva, and 1 ml of stabilization buffer is added to it. This sample can be processed immediately, or stored in the refrigerator at 4°C for at least 4 days. **Upper right**) A heating step at 95°C for 10 minutes serves to liberate viral RNA from virions and to inactivate virions such that they are not infectious to handlers in downstream steps (although appropriate safe-handling precautions should always be taken). **Lower left**) 4 µl of stabilized saliva is pipetted into each of three test tubes, pre-filled with the RT-LAMP master mix and primers. The only thing that different between the three tubes is the primer set included, with each set targeting something different as written underneath each of the three tubes. **Lower right**) After incubation, the reaction will turn from pink to yellow if the target RNA is present in saliva. An example of a positive and a negative test are shown. Graphic by Annika Rollock.

RT-LAMP offers many solutions to a nation-wide shortage of COVID-19 testing. Combined with the protocol presented here which enables the use of raw saliva samples, multiple barriers to mass and frequent screening of asymptomatic populations are solved: 1) use of saliva eliminates invasive swab-based sampling for which kits are in short supply, 2) an optimized saliva stabilization buffer allows for the neutralization of a broad range of naturally-
variable saliva samples while maintaining compatibility with a colorimetric RT-LAMP assay, 3) preservation of saliva samples for at least 4 days before processing is possible if necessary, 4) inactivation of infectious samples onsite improves biosafety, 5) a group of RT-LAMP primer sets developed here targets two regions of the SARS-CoV-2 genome, and 6) rapid turnaround time enables same-day reporting of results.

In the case of HIV, rapid community testing provides diagnoses to individuals in populations that may not otherwise seek HIV testing. This gives these individuals access to treatment, reducing their risk of short-term morbidity and mortality as well as resulting in the public health benefit of lower rates of virus transmission (Thornton et al., 2012). In the case of SARS-CoV-2, rapid community testing would provide more frequent tests to those who face the risk of transmission every day but who would not have the time or resources to seek tests in a clinical setting. Moreover, more rapid and inexpensive tests would allow facilities the option of testing daily before workers are mingling in enclosed spaces. As businesses, schools, and government organizations are looking for reopening options in the absence of a vaccine or effective drugs, we argue that an inexpensive, rapid test such as that described herein will provide a safer path forward.
Conflict of interest statement

Some of the authors of this study (NRM, QY, CLP, SLS) are founders of Darwin Biosciences, who licenses the RT-LAMP assay described herein.

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Methods

LAMP primer design

Regions of the SARS-CoV-2 genome that are conserved among strains were identified using genome diversity data from NextStrain (nextstrain.org/ncov/global). Next, nucleotide-BLAST (blast.ncbi.nlm.nih.gov) was used to filter out the genome sequence that shares high sequence homology with other seasonal coronavirus genomes. Finally, PrimerExplorer V5 (primerexplorer.jp/e/) was used to design RT-LAMP primers targeting the specific regions of SARS-CoV-2 genomes. The F3, B3, FIP, BIP, Loop F and Loop B primers were selected for optimal melting temperature and complementarity using A plasmid editor (ApE). All primers were ordered from IDT in desalted form. In all cases, a 10X concentration of primer sets was made containing 16 μM FIP and BIP primers, 4 μM LF and LB primers, and 2 μM F3 and B3 primers.
| Primer Set Name | LAMP Primer Component | Primer Sequence (5' - 3') |
|-----------------|-----------------------|---------------------------|
| “RNaseP”        | F3                    | TTGATGAGCTGGAGCCA          |
|                 | B3                    | CACCCCTCAATGCAGAGTC         |
|                 | Loop F                | ATGTGGATGGCCTAGTGTTTTT    |
|                 | Loop B                | CATGCTGAGTACTGGACCTC        |
|                 | FIP                   | GTGTGACCTGAAGACTCGGTATTTGACCTCAGGATC |
|                 | BIP                   | CTCCTCGATATGGCTCTCTTTGACCTCAGGATC |
| “As1e”          | F3                    | CGGTGGACAAATTGTCAC          |
|                 | B3                    | CTTCTCTGGATTTAACACATT      |
|                 | Loop F                | TTACAAGCTAAAGATGTCTGAACACT |
|                 | Loop B                | TTGAATTTAGGTTAAGACATTTTGTACAG |
|                 | FIP                   | TCAGCAGCAAAGCCAAAATTTATTTCTGTCGCCAAAGGAAATTAAGGAG |
|                 | BIP                   | TATTTGTTGAGCTAAACTCAAGCCTTTTCTGTAACACTCTTTCGTCAGGATG |
| “N2”            | F3                    | CGGCAGTCAAGCCTCTTCT       |
|                 | B3                    | TTGCTCAAAGCTGTCTCAA       |
|                 | Loop B                | ATGGGCAGTATGTCTCCTT       |
|                 | FIP                   | TCCCCCTACTGCTGTGCGTCCAGCTCAGTAAGTACGTG |
|                 | BIP                   | TCTCTCTGTAGAATGGCTGCTCATGCTCAAGCAGCAGCAGAAG |
| “ORF1e”         | F3                    | GGCTAACTAAACATCTTTGGC      |
|                 | B3                    | GTCAAGCACAACAGGGCAA        |
|                 | Loop F                | TCTTCAAGCCAAATCAAGGAC      |
|                 | Loop B                | TTGTCCGTGGGCAAATTTGT       |
|                 | FIP                   | TCTCTAAAGAAACTCTACACTTCTCTCTCTCTTTCTGTGTTATGAAAAAATCTCAAAACC |
|                 | BIP                   | TATCTCAACCTGTGCTTTGGAATTTTAGATGTCTGAACACTCTCTCT |

**SARS-CoV-2 RNA and virion standards**

Synthetic SARS-CoV-2 RNA control (Twist Bioscience #102019) was obtained and its copy number of 1x10^6 copies/µl was confirmed using RT-qPCR in conjunction with a DNA plasmid control containing a region of the N gene from the SARS-CoV-2 genome (IDT #10006625).

Heat-inactivated SARS-CoV-2 virion control (ATCC #VR-1986HK) was obtained and its concentration of 3.75x10^5 virions/µl was confirmed using RT-qPCR in conjunction with both the
synthetic SARS-CoV-2 RNA control and a DNA plasmid control containing a region of the N gene from the SARS-CoV-2 genome. SARS-CoV-2 RNA was added to saliva samples after being mixed 1:1 with stabilization buffer and boiling at 95°C for 10 minutes, whereas heat-inactivated SARS-CoV-2 virions were added to saliva samples after being mixed 1:1 with stabilization buffer but before being boiled. Concentrations reported throughout this study represent the final concentration of standards in raw saliva mixed 1:1 with stabilization buffer.

**Saliva collection, stabilization, and deactivation**

Saliva samples (1 mL) were collected in sterile, nuclease-free 5 mL conical screw-cap tubes (Eppendorf #0030122330). 2X Stabilization buffer (5 mM TCEP, 2 mM EDTA, 29 mM NaOH, 100 µg/mL Proteinase K, diluted in DEPC-treated water) was added at a 1:1 ratio. Samples were shaken vigorously for 5-10 seconds and incubated at 95°C for ten minutes. Samples were then placed on ice before being used in downstream analyses. Before any downstream processing occurred, samples were thoroughly mixed to resuspend any cellular debris pellets that may have formed.

**Real-time RT-LAMP**

For each reaction, 10 µL WarmStart LAMP 2X Master Mix (NEB #E1700) was combined with 1 µL 20X EvaGreen Dye (Biotium #31000), 2 µl 10X primer mix and 3 µl DEPC-treated water. The combined reaction mix was added to MicroAmp Optical 96-Well Reaction Plate (ThermoFisher #N8010560) and then 4 µl processed saliva sample was added. The reaction was mixed using a multi-channel pipette and incubated in Applied Biosystems QuantStudio3 Real-time PCR system. The reaction proceeded at 65°C for 30 minutes with fluorescent signal being captured.
every 30 seconds. The results were visualized and analyzed using ThermoFisher’s Design and Analysis software.

**Colorimetric RT-LAMP**

WarmStart Colorimetric LAMP 2X Master Mix (NEB #M1800) was used in all colorimetric RT-LAMP reactions. Each reaction was carried out in a total of 20 µl (10 µl WarmStart Master Mix, 2 µl 10X primer mix, 4 µl processed saliva sample, and 4 µl DEPC-treated water). Reactions were set up in PCR strip tubes on ice. Saliva template was added last and tubes were inverted several times to mix samples and briefly spun down in a microfuge. Reactions were incubated in a thermal cycler at 65ºC for 30 minutes and then deactivated at 80ºC for 5 minutes. The incubation was carried out without the heated lid to simulate a less complex heating device. Images of reactions were taken using a smartphone.
Supplementary Figure 1: Optimized stabilization buffer allows processing of acidic saliva samples while also maintaining color change of the RT-LAMP reaction. **A)** Various final concentrations of NaOH were tested in the stabilization buffer to optimize sample collection over a range of saliva acidity. Red box indicates final optimum concentration after 1:1 mixture of saliva with stabilization buffer. Samples were boiled at 95°C for 10 minutes and then analyzed in an RT-LAMP reaction by incubating at 65°C for 30 minutes. All reactions contained a primer set targeting the human RNaseP transcript. **B)** Optimized buffer was used to confirm no effect on limit of detection of SARS-CoV-2 *in vitro* transcripts in RT-LAMP. Four or two replicates were analyzed at the indicated amount of SARS-CoV-2 RNA spike-in. SARS-CoV-2 RNA was added to samples after being mixed 1:1 with stabilization buffer and boiling at 95°C for 10 minutes. Each replicate was tested with RNaseP primer set and three distinct SARS-CoV-2 primer sets (As1e, ORF1e, and N2).
Supplementary Figure 2: Saliva samples in stabilization buffer are stable at 4°C for at least 4 days before processing. Saliva samples were mixed 1:1 with stabilization buffer and spiked with heat-inactivated SARS-CoV-2 virions at the indicated concentration. Samples were stored at 4°C for either 24, 48, or 96 hours. Samples were then boiled at 95°C for 10 minutes and analyzed using RT-LAMP and the indicated primer sets. Reactions were incubated at 65°C for 30 minutes.
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