Rapid and extraction-free detection of SARS-CoV-2 from saliva by colorimetric reverse-transcription loop-mediated isothermal amplification

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Keywords

SARS-CoV-2, COVID-19, acute respiratory syndrome, LAMP, RT-LAMP, saliva, rapid diagnosis, point-of-care testing, high-throughput testing, cost-effective, preventive medicine

List of abbreviations:

Coronavirus disease 2019 (COVID-19), reverse-transcription (RT), loop-mediated isothermal amplification (LAMP), quantitative polymerase chain reaction (qPCR), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), nasopharyngeal (NP), Tris EDTA (TE), phosphate buffered saline (PBS), quantitative real-time fluorescent-based LAMP assay (qLAMP), cycle threshold (Ct), limit of detection (LOD), nucleocapsid gene (N), envelope small membrane protein gene (E)
Abstract

**Background:** Rapid, reliable, and widespread testing is required to curtail the ongoing COVID-19 pandemic. Current gold standard nucleic acid tests are hampered by supply shortages in critical reagents including nasal swabs, RNA extraction kits, personal protective equipment, instrumentation, and labor.

**Methods:** To overcome these challenges, we developed a rapid colorimetric assay using reverse-transcription loop-mediated isothermal amplification (RT-LAMP) optimized on human saliva samples without an RNA purification step. We describe the optimization of saliva pretreatment protocols to enable analytically sensitive viral detection by RT-LAMP. We optimized the RT-LAMP reaction conditions and implemented high-throughput unbiased methods for assay interpretation. We tested whether saliva pretreatment could also enable viral detection by conventional reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Finally, we validated these assays on clinical samples.

**Results:** The optimized saliva pretreatment protocol enabled analytically sensitive extraction-free detection of SARS-CoV-2 from saliva by colorimetric RT-LAMP or RT-qPCR. In simulated samples, the optimized RT-LAMP assay had a limit of detection of 59 (95% confidence interval: 44-104) particle copies per reaction. We highlighted the flexibility of LAMP assay implementation using three readouts: naked-eye colorimetry, spectrophotometry, and real-time fluorescence. In a set of 30 clinical saliva samples, colorimetric RT-LAMP and RT-qPCR assays performed directly on pretreated saliva samples without RNA extraction had accuracies greater than 90%.
Conclusions: Rapid and extraction-free detection of SARS-CoV-2 from saliva by colorimetric RT-LAMP is a simple, sensitive, and cost-effective approach with broad potential to expand diagnostic testing for the virus causing COVID-19.
Introduction

Establishing rapid and widespread testing for coronavirus disease 2019 (COVID-19) is essential to containing the pandemic and safely reopen society. The current gold standard test measures viral nucleic acids extracted from clinical swabs by reverse transcription quantitative polymerase chain reaction (RT-qPCR). This assay requires trained medical personnel, specialized instrumentation, supply-limited reagents, and substantial technical labor. Isothermal nucleic acid amplification tests are an alternative to conventional PCR methods that do not require expensive instruments to perform the reaction or interpret the results. Specifically, loop-mediated isothermal amplification with simultaneous reverse-transcription (RT-LAMP) allows for rapid and analytically sensitive detection of nucleic acids within one hour in an easily interpretable colorimetric assay that requires only a heat source (1,2).

Several groups are currently developing LAMP-based protocols for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus causing COVID-19 (3–13). The analytical sensitivity of LAMP on purified RNA compares well to RT-qPCR, and LAMP may achieve higher sensitivity on crude clinical samples (5). Robustness of LAMP to PCR inhibitors makes it especially well-suited and widely used for pathogen detection in unpurified samples (14). This confers a major potential advantage over current testing protocols as it enables skipping the cost-, labor-, time-, and reagent-consuming RNA extraction step.

Saliva is a promising sample for expanding and facilitating testing due to the ease, safety, and non-invasive nature of its collection and its relatively high viral load (15,16). Direct comparison of saliva to nasopharyngeal (NP) swabs from the same
individuals revealed that saliva samples provided more consistent and clinically sensitive results for SARS-CoV-2 detection (17). Here, we sought to establish and optimize a simple RT-LAMP assay for the qualitative detection of SARS-CoV-2 directly from saliva without an RNA extraction step.

**Materials and Methods**

**LAMP Reactions**

20 µL LAMP reactions containing 3 µL of sample were performed following New England Biolab’s recommended protocol using WarmStart Colorimetric LAMP 2X Master Mix (NEB, M1800L). Primer sequences are provided in online Supplemental Table 1.

**SARS-CoV-2 Standards and Controls**

*In vitro* transcribed RNA standards were prepared as described (18). Individual aliquots (10 µL aliquots were frozen at -80°C in 8-tube strips to prevent multiple freeze-thaws. Heat inactivated SARS-CoV-2 particles were acquired from the US Centers for Disease Control and Prevention (CDC) through BEI Resources. DNA plasmid coronavirus controls corresponding to SARS-CoV-2 and MERS were obtained from IDT as plasmid DNA solutions.

**Saliva Pretreatment Protocols**

We initially implemented a heat treatment of 55°C for 15 min followed by 95°C for 5 min. We later optimized heat treatment to 65°C for 15 min followed by 95°C for 5 min.
Samples were cooled to 4°C for 5 min before being assayed. In initial experiments, Proteinase K from NEB (#P8107S) was added to undiluted saliva at 1/10 volume (5 µL in 50 µL saliva). Later, Proteinase K was used at 100X in samples diluted 1:1 in TE buffer (10 mM Tris, 0.1 mM EDTA) or phosphate buffered saline (PBS). Optimized experiments included 1X RNAsecure (25X, ThermoFisher, AM7006). We tested the HUDSON method (19), and other pretreatment protocols (online Supplemental Materials file).

**RT-qPCR**

RT-qPCR reactions were performed according to CDC Emergency Use Authorization guidelines using TaqPath 1-Step RT-qPCR Master Mix GC (ThermoFisher, A15300) and the nCoV-N1 probe from the 2019-nCoV RUO Kit (IDT). Reactions were performed on Quantstudio 3 and 6 Real-Time PCR systems (ThermoFisher). 3 µL sample was used as input to match the LAMP protocol.

**High-Throughput Colorimetric Assay**

Assay scale-up was performed in a 96-well plate format (BioRad) with minor modifications to the LAMP reaction. 4 µL of saliva samples were used in 25 µL total volume reactions and pretreated with the original heat treatment, proteinase K, and RNasin (Promega). Samples were run in technical triplicate at each dilution. Samples were analyzed using a BioTek Epoch microplate spectrophotometer measuring absorbances at 430 nM and 560 nM wavelengths (online Supplemental Materials file).
**Quantitative real-time LAMP (qLAMP)**

qLAMP was performed by adding the DNA-binding dye SYTO 9 (ThermoFisher) to the colorimetric LAMP reaction (1 µM) and performing the reaction on a QuantStudio 3 or 6 Real-Time PCR system. Machines were programmed to run 90 or 120 isothermal cycles of 30 s at 65°C, then slowly ramped up to 95°C for inactivation and melt-curve analysis.

**Data Analysis**

Data were analyzed and plotted in R (v3.5.1) using ggplot2. qLAMP/RT-qPCR experiments were analyzed using Quantstudio Design & Analysis Software (v2.3.3, ThermoFisher), or exported for analysis in R. For sensitivity analysis, we fit a probit regression model to estimate the limit of detection (LOD).

**Clinical Samples**

Saliva collection was approved by the institutional review board at Washington University School of Medicine (WU350, IRB#202003085). Informed consent was obtained for all participant samples. Saliva samples from COVID-19 positive and negative individuals were diluted 1:1 in PBS to facilitate pipetting and then frozen. Some samples were heat-treated at 56 °C for 30 minutes for viral inactivation. Most samples underwent several freeze-thaw cycles prior to assaying.

**Results**

*LAMP Primer Screening*
We compared the performance of five sets of recently developed LAMP primer sets targeting different regions of the SARS-CoV-2 genome (3–6). Of these, the NEB Gene N-A (3) and Lamb et al. (4) primers targeting the nucleocapsid (Gene N) and Orf1ab regions respectively had the highest analytical sensitivity, lowest rates of false positives in water-only controls, and no cross-reactivity with MERS coronavirus controls (online Supplemental Figure 1).

RT-LAMP Reaction Optimization in Simulated Samples

Next, we validated these primers on both RNA standards and heat-inactivated viral particles spiked into water or human saliva to simulate clinical samples (online Supplemental Figure 2A). Saliva strongly inhibited LAMP detection of SARS-CoV-2 compared to water (online Supplemental Figure 2B). Particles were weakly detected in saliva whereas their detection in water was on par with detection of RNA (online Supplemental Figure 2C) indicating the presence of an inhibitor in saliva that impaired the assay. Colorimetric interpretation was time-sensitive with many samples, including negative controls, turned yellow in LAMP reactions longer than 40 min due to non-specific amplification (20,21). A 30-min incubation provided a reliable readout.

To neutralize or otherwise reduce inhibitors in human saliva, we tested several approaches that have been demonstrated to improve viral RNA detection in crude samples (19,22–25). First, we found that simple dilution of saliva into water enabled sensitive detection of SARS-CoV-2 particles using LAMP (Figure 1A, top). Heat treatment with or without proteinase K further improved LAMP assay sensitivity (Figure 1A) and enabled SARS-CoV-2 particle detection in undiluted human saliva samples.
(Figure 1B). This simple pretreatment conferred a consistent LOD on the order of $10^2$ particles per reaction, representing a 10,000-fold improvement in sensitivity over assays on untreated saliva.

We experimented with additional heat and chemical pretreatments including the HUDSON protocol (19) and various detergents, but each of these conditions decreased assay sensitivity or interfered with colorimetry (online Supplemental Figure 3A-C). Varying the amount of crude sample input to the LAMP reaction, we found that adding up to 8 µL of direct saliva was compatible with the assay (online Supplemental Figure 3D).

**Multiplexing LAMP Primer Sets**

To further improve assay accuracy, we sought to multiplex LAMP primer sets in a single reaction. Combining primers can potentially increase sensitivity through additive signals of simultaneous amplification reactions (8). Non-specific primer interactions, however, could result in increased rates of false positives. We compared pairwise combinations of NEB Gene N-A primers with the other four primer sets targeting various regions across the SARS-CoV-2 genome. All pairs of primer sets outperformed the NEB Gene N-A primer set alone, with no apparent increase in spurious background amplification (online Supplemental Figure 4).

We next tested whether multiplexing primer sets could improve signal detection in untreated and heat and chemical treated particle-containing saliva (Figure 1C). Heat treatment alone gave a marked improvement in SARS-CoV-2 particle detection from saliva (Figure 1D, $p < 1e-5$, two-sided t-test). Heat treatment plus proteinase K further
improved assay sensitivity compared to heat alone ($p < 0.003$, two-sided $t$-test). Multiplexed primer sets slightly improved the sensitivity of the assay, increasing the frequency of detection in samples with $\sim 10^1$ particles per reaction. At this sensitivity, the multiplexed LAMP assay would detect the vast majority of COVID-19 positive samples based on reported saliva viral loads (median $\sim 10^2$-$10^3$ viral copies per µL) (16,17), and virtually all infectious individuals (26). As viral loads and contagiousness peak around the time of symptom onset, LAMP would have the highest accuracy at this critical timepoint for isolating infectious carriers (27).

To determine whether our extraction-free protocol also improved the sensitivity of SARS-CoV-2 detection by conventional RT-qPCR, we performed RT-qPCR using the CDC Gene N1 hydrolysis probe set directly on untreated and treated simulated saliva samples. We found that RT-qPCR had similar analytical sensitivity to LAMP on crude samples, reliably detecting SARS-CoV-2 in all samples down to $\sim 10^1$ particles per reaction (Figure 1E). We observed strong improvements in cycle thresholds (Ct) using either heat alone or heat plus proteinase K ($p < 1e^{-3}$, two-tailed paired $t$-tests), increasing the sensitivity of viral RNA detection by RT-qPCR by 3 to 4-fold. Taken together, our results show that a simple, extraction-free pretreatment protocol can significantly improve the LOD of downstream nucleic acid-based assays.

*Initial Assay Validation on Clinical Samples*

We validated the initial colorimetric RT-LAMP assay on five COVID-19 positive samples. Four of five samples pretreated with heat plus proteinase K tested positive after a 30-minute RT-LAMP reaction (online Supplemental Figure 5A). We performed
RT-qPCR directly on the untreated and treated samples. Results were qualitatively concordant with the RT-LAMP results, identifying the same 4 positives (online Supplemental Figure 5B). Pretreatment significantly improved viral detection by RT-qPCR (online Supplemental Figure 5C). Together these results demonstrated the feasibility of our assay on actual clinical samples.

**Establishing a High-throughput Quantitative Assay**

To enable substantial scale-up of testing capacity using RT-LAMP, we adapted our protocol to a 96-well plate format measuring absorbance. Spectrophotometric plate scanning before and after the assay provided an unbiased, quantitative interpretation. Heat treatment with and without proteinase K enabled unbiased and sensitive detection of viral particles in saliva samples down to $10^2$ particles per reaction (Figure 2A).

We next sought to establish a quantitative real-time fluorescent-based LAMP assay (qLAMP) using the DNA intercalating dye SYTO 9 (28). qLAMP offers several potential advantages over colorimetric LAMP including real-time reaction monitoring and melt curve analysis to discriminate false positives. We benchmarked qLAMP using contrived samples of known amounts of viral particles in diluted saliva, and we determined that a Ct of 50 (25 min) reliably discriminated positive reactions from nonspecific amplifications (Figure 2B). SYTO 9 did not interfere with colorimetric RT-LAMP allowing assay interpretation by colorimetry or fluorimetry (Figure 2C) with qualitatively concordant results (Figure 2D). Spectrophotometry and real-time LAMP therefore represent two alternative modalities for high-throughput, unbiased LAMP implementation.
Improving Compatibility with Point-of-Care Testing

We sought to develop a saliva pretreatment compatible with a single isothermal heat source to reduce equipment requirements and facilitate point-of-care testing (online Supplemental Materials file). Pretreatment at 65°C for 15 min of diluted saliva improved detection (online Supplemental Figure 6). RNAsecure, guanidine hydrochloride (40 mM), and primer multiplexing further enhanced assay sensitivity. Pulse spinning samples in a microfuge prior to the LAMP reaction improved assay reliability. While not quite as sensitive as pretreatments including a 95°C heat step, these optimizations enable assaying saliva by colorimetric RT-LAMP using a single heat source, simplifying point-of-care testing.

Re-optimizing the RT-LAMP assay

Given the improvements we observed in assay sensitivity using RNAsecure, guanidine, and sample dilution into TE, we incorporated these into an optimized protocol. We increased the 55°C stage of the original heat treatment to 65°C for better inactivation of virus, RNases, and reaction inhibitors. In these conditions, we observed a low rate of nonspecific amplification arising in experiments with multiplexed primers (20) (online Supplemental Figure 7). Switching to primer sequences redesigned by NEB targeting the nucleocapsid and envelope small membrane protein (E) genes (NEB-N2 and NEB-E1 primers) (13) solved this issue.

We validated the performance of the new primers with both colorimetric RT-LAMP and qLAMP. These experiments indicated the new NEB-N2 primer set
outperformed the previous primers in both sensitivity and time to threshold (online Supplemental Figure 8A,B). RNAsecure improved analytical sensitivity across all primer sets (online Supplemental Figure 8C). Multiplexing NEB-N2 with NEB-E1 gave consistent results with no false positives in saliva-only controls (online Supplemental Figure 8D). Guanidine improved both the speed and sensitivity of the LAMP reactions ($p < 0.001$, online Supplemental Figure 8D-E) (13).

**LOD Analysis of Optimized Assay**

We assayed serial dilutions of viral particles to estimate the concentration of target viral particles that could be detected with a probability of 0.95 (LOD95). To account for variation across donors, we spiked viral particles into saliva from at least three donors. In parallel, we tested whether proteinase K inclusion was beneficial. Our optimized assay was highly sensitive across all donors, with a LOD95 of 59 [44-104] particle copies per reaction, and 100% [86-100] specificity (online Supplemental Figure 9A).

Proteinase K significantly improved SARS-CoV-2 detection, especially in samples with the lowest viral amounts ($p = 0.006$, online Supplemental Figure 9B). For samples that included proteinase K, LOD95 was estimated to be 27 [22-47] particles per reaction (online Supplemental Figure 10A), a major improvement compared to assays without proteinase K treatment (LOD95: 79 [55-175], online Supplemental Figure 10B).

We re-tested the optimal amount of saliva that should be added to the reaction and found that increasing amounts of pretreated saliva impeded reaction times (online Supplemental Figure 9E). Based on this observed reaction inhibition at higher levels of
input saliva, we recommend adding 1-4 µL of pretreated saliva per 20 µL LAMP reaction. Reaction volumes and saliva amounts can be scaled up proportionally to increase assay sensitivity but at higher costs per reaction.

Finally, our optimized saliva pretreatment protocol again markedly improved the detection of SARS-CoV-2 by RT-qPCR (online Supplemental Figure 9F). Compared to untreated saliva, heat and proteinase K pretreatment improved viral detection by an average 3.4-fold ($p < 3e-9$, $t$-test). RT-qPCR on direct saliva without RNA extraction is thus another viable option to overcome bottlenecks limiting widespread testing.

**Clinical Validation of Optimized Assay**

We tested our optimized colorimetric RT-LAMP and RT-qPCR protocols on 30 additional clinical samples (20 positive and 10 negative samples). By naked-eye interpretation of colorimetric RT-LAMP, 17/20 positive samples were correctly called positive and 9/10 negative samples were called negative (Figure 3A). The false positive result was called negative when re-tested with an alternate set of LAMP primers, indicating possible carry-over contamination of LAMP amplicons (29). This can be prevented by including uracil (dUTP) and uracil glycosylase in the reactions (30), as implemented in clinical RT-qPCR. qLAMP Cts had strong overall agreement with colorimetric results. qLAMP Cts for five samples were too high to distinguish between weak positive signal or non-specific amplification (Figure 3B). RT-qPCR correctly called 19/20 positive samples (Figure 3B, purple squares), and 10/10 negative samples (Table 1). RT-qPCR Cts were well-correlated with LAMP Cts (Figure 3C). One positive sample
was undetected by either method, suggesting sample degradation or very low viral levels.

We included a quantitative dilution series of particles in the RT-qPCR assay to estimate viral loads in clinical samples. The median estimated viral copy number in positive samples was ~500 copies per µL (range 16-126,000). All false negatives in the LAMP assay had fewer than 100 estimated viral copies per µL which is likely below the threshold for viral transmission (26). Nevertheless, among 8 samples below this level, LAMP still detected virus in half the samples. Above this level, the assay achieved 100% [71.5 to 100] sensitivity. Therefore, colorimetric RT-LAMP on pretreated saliva samples without RNA extraction is a cheap, fast, and accurate method for SARS-CoV-2 testing.

Discussion

Our proposed approach combines three promising avenues to enable rapid and widespread SARS-CoV-2 testing: 1) colorimetric RT-LAMP, 2) self-collected saliva specimens, and 3) direct testing on crude saliva samples without RNA extraction. This approach solves two major bottlenecks in massively scaling up COVID-19 nucleic acid testing: sample collection and RNA extraction, and it enables test result turnaround times less than an hour. Using both colorimetric RT-LAMP and RT-qPCR directly on treated samples without RNA extraction, we demonstrated high accuracy in simulated and actual clinical saliva samples.

Due to its ease of use, rapid amplification of nucleic acids, and high specificity RT-LAMP has been widely used for pathogen detection. Sensitive diagnostic assays
have been developed for viruses including Zika (31) and such assays are being
developed for SARS-CoV-2 by several groups including ours (3–13). Its low cost, fast
turnaround time, and simple colorimetric readout make RT-LAMP an effective solution
for ramping up global testing capacity. Further, because it does not require specialized
equipment or training for performing or interpreting the assay, colorimetric RT-LAMP is
especially well-suited for point-of-care detection.

NP swabs are uncomfortable and must be carefully performed by a trained
health-care worker using personal protective equipment. Mid-nasal swabs are a
promising alternative to NP swabs because they can be self-administered and contain
high viral loads (32–34). We instead focused on saliva due to its ease of collection, high
viral load, and potential swab shortages (17). Saliva is a challenging clinical matrix due
to variability across individuals in pH and viscosity and the presence of reaction
inhibitors (35). Here, we have overcome these challenges and developed a saliva
pretreatment protocol that enables sensitive detection of SARS-CoV-2 by both RT-
LAMP and RT-qPCR without an RNA extraction step.

While RNA extraction methods improve sample purity and increase viral
collection, they are cost-, labor-, and time-consuming and some reagents are still in
short supply. Several groups are optimizing workarounds to avoid the RNA extraction
step for nucleic acid-based SARS-CoV-2 testing (22,23,25). We achieved a careful
balance between the inactivation of reaction inhibitors and the preservation of viral RNA
with heat and chemical pretreatment. A 1:1 dilution of saliva followed by treatment with
RNAsecure and 65 °C incubation potently reduces reaction inhibitors. This can be
implemented with a single heat source isothermal with the LAMP reaction in a point-of-
care setting. Proteinase K addition and a brief incubation at 95 °C further reduce inhibitors, ease saliva handling, and improve assay sensitivity. For high-throughput testing in a centralized laboratory, this pretreatment protocol can be coupled with colorimetric RT-LAMP using a spectrophotometric or fluorescent readout, or with RT-qPCR.

Our protocol has several potential limitations for clinical implementation. While many studies have shown that saliva samples have higher and more stable viral loads than found in NP swabs (17), NP and other clinical specimens may provide higher sensitivity for viral detection (34). The saliva collection protocol is limited to individuals who can produce enough saliva. Contaminants in saliva, such as food or mucus, might influence downstream assays, although our protocol and additives should mitigate these factors. Carry-over contamination of LAMP amplicons is a major concern for clinical implementation (29), but this can be prevented by including dUTP and a uracil-DNA glycosylase in the reaction (30). While qLAMP facilitated the comparison of multiple conditions, future implementation of digital LAMP could enable more exhaustive optimization of reaction conditions (21). For clinical assay implementation, samples should be tested in duplicate across multiple primer sets targeting the SARS-CoV-2 genome and an internal human RNA control. Finally, the complexity of the optimized two-step protocol of saliva pretreatment and downstream assay still requires some training and equipment that may preclude at-home implementation.

Direct colorimetric RT-LAMP on saliva has broad potential to increase COVID-19 screening speed and capacity and has high flexibility in implementation depending on equipment availability. Extension of testing to asymptomatic individuals and increased
test frequency would promote the application of predictive, preventive, and personalized medicine (36). Expanded testing would improve the predictive reliability of modeling disease spread, inform better containment policies, and identify and protect vulnerable populations (37). Integrating test results into contact tracing tools would help provide personalized risk assessments, enabling the self-isolation of exposed individuals and the avoidance of high-risk areas by healthy individuals (38). Expanded testing and contact tracing are essential for successful management of the pandemic.

In summary, we have developed a saliva pretreatment protocol which enables sensitive SARS-CoV-2 detection from unpurified samples in an optimized colorimetric RT-LAMP assay and RT-qPCR. This optimization overcomes the burdensome step of RNA extraction, and alleviates some of the time, labor, and reagent bottlenecks of the current gold standard nucleic acid-based tests. Our extensive optimizations have enabled reliable detection below $\sim 10^2$ viral genomes per reaction from saliva samples. COVID-19 positive individuals with viral loads below this level are likely not infectious (26). Colorimetric RT-LAMP and RT-qPCR assays achieved high accuracy on clinical saliva specimens without RNA extraction. Because of the flexibility of implementation and read-out, our assay can be deployed as a point-of-care test or in a centralized laboratory facility and has broad potential to expand diagnostic testing for the virus causing COVID-19.
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M.A. Lalli, X. Chen, S.J. Langmade, C.S. Sawyer, L.C. Burcea, M.N. Wilkinson, and W.J. Buchser developed and optimized the LAMP assay with significant intellectual contribution from M. Heinz, R.D. Mitra, R.S. Fulton, R.D. Head, J. Milbrandt. Clinical samples were obtained, prepared, and tested by M.A. Lalli, C.C. Fronick, S.J. Langmade, and R.S. Fulton. Spectrophotometric assay scale-up and quantitative read-out were implemented and analyzed by C.S. Sawyer, L.C. Burcea, M. Heinz, and R.D. Head. Fluorescent assay scale-up was implemented and analyzed by M.A. Lalli, X. Chen, S.J. Langmade, M.N. Wilkinson, and W.J. Buchser. M.A. Lalli analyzed the data and prepared the manuscript, with editing and revision by all authors.

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### Tables

Table 1. Accuracy of LAMP and RT-qPCR assays on pretreated clinical saliva samples.

|                | True Positive | False Negative | True Negative | False Positive | Clinical Sensitivity (%), 95% CI | Clinical Specificity (%), 95% CI | Accuracy (%), 95% CI |
|----------------|---------------|----------------|---------------|----------------|----------------------------------|----------------------------------|---------------------|
| **Colorimetric LAMP** | 17            | 3              | 10*           | 0*             | 85 (62.1-96.8)                  | 100 (69.1-100)                 | 90 (73.5-97.9)      |
| **RT-qPCR**     | 19            | 1              | 10            | 0              | 95 (75.1-99.9)                  | 100 (69.1-100)                 | 96.7 (82.8-99.9)    |

* after re-testing.
Figure Legends

Figure 1. Dilution, heat, and Proteinase K treatments improve SARS-CoV-2 detection from saliva. A) Dilution of particle-containing saliva into water improved LAMP detection by at least two orders of magnitude from undetectable to $\sim 10^3$ particles per reaction. Heat treatment and heat treatment plus proteinase K further increased LAMP sensitivity to $\sim 10^2$ viral genome equivalents per reaction. *Replicate 3 used Lamb et al. primers but gave nearly identical results to NEB Gene N-A primers. B) Heat treatment with or without proteinase K increased LAMP sensitivity from $10^6$ to $\sim 10^2$ viral genome equivalents in undiluted saliva. C) Multiplexed primers improved LAMP sensitivity. LAMP reactions using NEB Gene N-A primers alone or in combination with Yu et al. or Lamb et al. primers are shown. S = negative control saliva. Viral particles per reaction are indicated. D) Saliva pretreatments significantly improved LAMP sensitivity. Heat treatment improved limit of detection ($p = 6e-6$, t-test, two-tailed vs ‘No Treatment’). Proteinase K treatment further improved heat treatment ($p = 0.002$, t-test, two-tailed vs ‘Heat’). Multiplexed primers increased the frequency of detection at $\sim 10^1$ particles / reaction. N = NEB Gene N-A. E) RT-qPCR on crude saliva using the CDC N1 probe showed increased sensitivity with either heat or proteinase K treatment ($p < 1e-3$ for either treatment, two-tailed paired t-test).
Figure 2. Establishing high-throughput LAMP assays with quantitative readouts. A) RT-LAMP assay was adapted to a high-throughput 96-well plate format with a quantitative absorbance readout, achieving a limit of detection $< 10^2$ particles per reaction from saliva samples. Absorbance for 430 nM (yellow) and 560 nM (red) wavelengths was measured before and after the LAMP reaction and normalized to negative controls. Heat indicates 55 °C for 15 minutes, 95 °C for 3 minutes, with or without proteinase K (ProK). Two biological replicates were each run in triplicate. B) Real-time quantitative fluorescent LAMP results are shown for a dilution series of particles in saliva. Change in fluorescence (delta Rn) is monitored over 120 ‘cycles’ of 30-second incubations at 65 °C. Cycle thresholds (Cts), indicated by red triangles, represent the time at which total fluorescence reaches a given level. Samples with higher viral loads reach this threshold earlier. Nonspecific amplification may arise after 50 cycles, corresponding to 25 minutes. C) Colorimetric and D) fluorescent results for the same reaction show that the fluorescent dye does not interfere with colorimetric interpretation. Results are concordant with colorimetric LAMP, and fluorescent results are more quantitative.
Figure 3. Clinical Validation of Optimized Colorimetric LAMP Assay and RT-qPCR. A) Colorimetric LAMP results on 30 clinical samples (20 positives, 10 negatives). Correct calls are numbered in black. Incorrect calls are numbered in red. B) qLAMP and RT-qPCR results on pretreated samples are shown for each sample. Red numbers indicate false negative calls by qLAMP. C) qLAMP and RT-qPCR Ct values were highly correlated. Thresholds of 40 cycles were used for determining positivity for LAMP and RT-qPCR as indicated. Samples negative by either of these methods are colored red. D) Estimates of viral genome copy number were made for each sample using a quantitative standard curve by RT-qPCR. Samples called negative by LAMP are colored red. Red-dotted line indicates 100 viral genomes. ND = Not Detected.