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Reverse Transcription Loop-Mediated Isothermal Amplification Assay for Ultrasensitive Detection of SARS-CoV-2 in Saliva and Viral Transport Medium Clinical Samples

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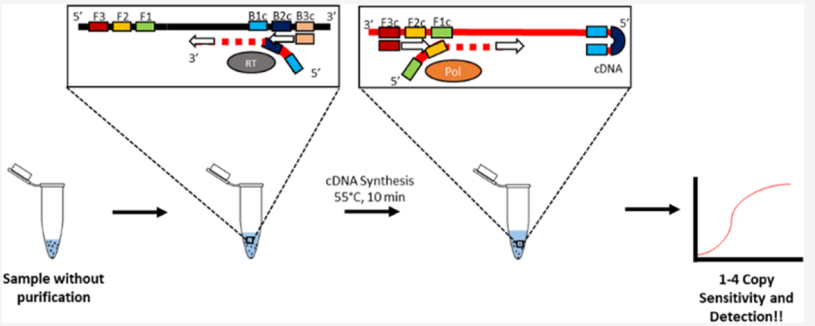

ABSTRACT: The COVID-19 pandemic has underscored the shortcomings in the deployment of state-of-the-art diagnostics platforms. Although several polymerase chain reaction (PCR)-based techniques have been rapidly developed to meet the growing testing needs, such techniques often need samples collected through a swab, the use of RNA extraction kits, and expensive thermocyclers in order to successfully perform the test. Isothermal amplification-based approaches have also been recently demonstrated for rapid severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection by minimizing sample preparation while also reducing the instrumentation and reaction complexity. In addition, there are limited reports of saliva as the sample source, and some of these indicate inferior sensitivity when comparing reverse transcription loop-mediated isothermal amplification (RT-LAMP) with PCR-based techniques. In this paper, we demonstrate an improved sensitivity assay from saliva using a two-step RT-LAMP assay, where a short 10 min RT step is performed with only B3 and backward inner primers before the final reaction. We show that while the one-step RT-LAMP demonstrates satisfactory results, the optimized two-step approach allows detection of only few molecules per reaction and performs significantly better than the one-step RT-LAMP and conventional two-step RT-LAMP approaches with all primers included in the RT step. We show control measurements with RT-PCR, and importantly, we demonstrate RNA extraction-free RT-LAMP-based assays for detection of SARS-CoV-2 from viral transport media and saliva clinical samples.

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The outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has become a major challenge for national healthcare systems, infecting millions of people, burdening daily life, and causing heavy economic losses.[1,2] The fast spread of the COVID-19 pandemic has underlined the shortcomings of the existing technologies and testing paradigm for viral diagnostics and has propelled the need for alternate rapid and accurate diagnostic approaches for SARS-CoV-2 detection. As the SARS-CoV-2 vaccine is broadly disseminated, testing has been highlighted as a critical step to be continued and to suppress the spread.[3] However, due to the current cost of the test, the time required to obtain results, and other logistical difficulties for massive deployment, there is further need for additional scientific improvements for the deployment of testing solutions.

The current gold standard approaches for viral detection primarily rely on the polymerase chain reaction (PCR), but such assays often need extensive and time-consuming RNA

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purification steps and expensive thermocyclers to successfully perform the test. These assays are also challenging to perform at the point of care. Isothermal amplification techniques such loop-mediated isothermal amplification (LAMP)[4] have generated much interest, especially in eliminating the need for precise thermal cycles to achieve the nucleic acid amplification.[5] Likewise, LAMP has also shown to be robust against crude samples and inhibitors that often slow PCR.[6,7] In addition, its use of 4−6 primers to identify 6−8 regions of a target genome for amplification increases its specificity in comparison to PCR.[4,8] The robustness of the Bst polymerase and reverse transcriptase enzymes allow direct detection of RNA targets from crude samples without any RNA extraction or purification.[6,9] However, the sensitivity of these assays in detecting RNA viruses has remained low and to the best of our knowledge, never reached detection limits to a few molecules.[10−12] In the particular case of SARS-CoV-2, recent studies indicate that the sensitivity of reverse transcription (RT)-LAMP is inferior to that of some PCR-based techniques for COVID-19 in saliva specimens.[13]

Besides the detection technique, the sample specimen is also critical when developing assays that could be massively scaled or used for asymptomatic testing. Toward this end, although saliva is becoming a preferred and scalable option for reliable and massive testing, very few of the Emergency Use Authorization (EUA) approved PCR-based tests detect the SARS-CoV-2 RNA from saliva specimens,[14] and to the best of our knowledge, to date, there are no saliva-to-RT-LAMP EUA-approved tests.[15] The use of saliva as a sample has demonstrated not only to serve as an alternative upper respiratory tract specimen type for SARS-CoV-2 detection[16−18] but also saliva offers a number of advantages over nasopharyngeal (NP) and nasal swabs when considering the aforementioned criteria for mass testing efforts. The use of NP swabs can cause discomfort and irritation that could promote sneezing and coughing and increase the risk of exposure for the medical providers.[19,20] Collection of NP swabs has been associated with variable, inconsistent, and false-negative test results.[18] On the other hand, saliva does not require a certified swab, specific collection receptacle, or transport media, and does not have to be obtained by a skilled healthcare provider, all of which increase diagnostic associated costs. Despite the interest generated and the mentioned advantages, only three papers in the preprint form[21,22] or peer-reviewed[13] have been reported regarding RT-LAMP detection of SARS-CoV-2 RNA from saliva specimens. Although these papers highlight the possibility of bypassing the RNA extraction step,[21,22] they note the limitation on sensitivity of traditional RT-LAMP when detecting SARS-CoV-2 RNA from saliva. For instance, it is mentioned that RT-LAMP reaction was still not sufficiently sensitive to detect fewer than 200 viral copies/μL in saliva and that significant modifications to the RT-LAMP technique were needed to achieve the single-copy detection levels.[21] Also, an RT-LAMP colorimetric assay enabled detection of ∼100 viral genomes per reaction,[22] and in a study across 103 saliva specimens, RT-LAMP showed only 70.9% of sensitivity, lower than that of some PCR-based techniques.[13]

In this paper, we address the above limitations and present an optimized RT-LAMP approach for detecting viral RNA down to a few copies from NP swabs (viral transport media) and saliva specimens. By adding a short 10 min RT-incubation step, with B3 and backward inner primer (BIP) (B2 + B1c) specifically separated to improve primer annealing with the target, we increase the sensitivity of the RT-LAMP reaction by 2 orders of magnitude over the current one-step or two-step RT-LAMP reactions. We first characterize the approach and quantitatively demonstrate the improvement in the limit of detection (LOD). We use our optimized assay for detecting SARS-CoV-2 viral RNA and inactivated viruses in buffer down to a few copies per reaction. Next, we show the robustness of our assay by doing direct detection of inactivated SARS-CoV-2 viruses in viral transport medium (VTM) and saliva samples, without extensive sample processing such as RNA extraction or purification, with a detection limit down to 1 copy/μL within 40 min and with a sample-to-result time of less than 1 h. Finally, we tested our RT-LAMP reactions detecting SARS-CoV-2 virus in 50 VTM and 34 saliva clinical samples. It is important to note that our protocol gives superior sensitivity to the conventional two-step RT-LAMP protocol, where RT is separately performed with all primers, followed by an amplification step.[23−25] We believe, with our improved LOD and simple RNA extraction-free protocol, our approach will allow rapid scaling of testing and detection of cases, which might have been otherwise missed due to low viral loads.

EXPERIMENTAL SECTION

Clinical Samples. VTM clinical samples used are discarded VTM prior to the RNA purification step from 25 samples from patients who were tested positive for COVID-19 and from 25 samples from patients who were tested negative for COVID-19 at OSF Healthcare (Peoria, IL) by a RT-PCR test performed at OSF Healthcare. The samples were received deidentified, frozen, and were obtained through an approved institutional review board (OSF Peoria IRB # 1602513 through the University of Illinois College of Medicine with waiver for consent).

Saliva clinical samples were collected from 34 in-patients at Carle Foundation Hospital (Urbana, IL) who were tested COVID-19 positive (RT-PCR) when they were admitted in the hospital (or at most 14 days before admission) through an approved institutional review board (Carle IRB # 20CRU3150). For saliva samples, after consent, the subjects were asked to let their saliva pool in their mouth and then to open their mouth and let the saliva fall/dribble into the collection tube. On the same day as saliva collection, nasal swab samples were also collected from the same subject at Carle Foundation Hospital and analyzed by the RT-PCR technique at the Carle clinical lab. Immediately after collection, the saliva samples were mixed with TE buffer (1:1). Samples were received deidentified and either fresh (during the collection day) or frozen (the next day after the collection day).

RT-LAMP Reactions. The following components comprised the RT-LAMP assay: 4 mM $MgSO_4$ (New England Biolabs), 1× final concentration of the isothermal amplification buffer (New England Biolabs), 1.025 mM each of deoxyribonucleoside triphosphates, and 0.29 M Betaine (Sigma-Aldrich). Individual stock components were stored according to the manufacturer's instructions, and a final mix including all of the components was freshly created prior to each reaction. Along with the buffer components, a primer mix consisting of 0.15 μM F3 and B3, 1.17 μM forward inner primer (FIP) and BIP, and 0.59 μM of LoopF and LoopB was added to the reaction. Finally, 0.47 U/μL BST 2.0 WarmStart DNA polymerase (New England Biolabs), 0.3 U/μL WarmStart reverse transcriptase (New England Biolabs), 1 mg/mL bovine serum albumin (New England Biolabs), and 0.735× EvaGreen (Biotium) were included in the reaction. EvaGreen dye is a double-stranded DNA intercalating dye. After

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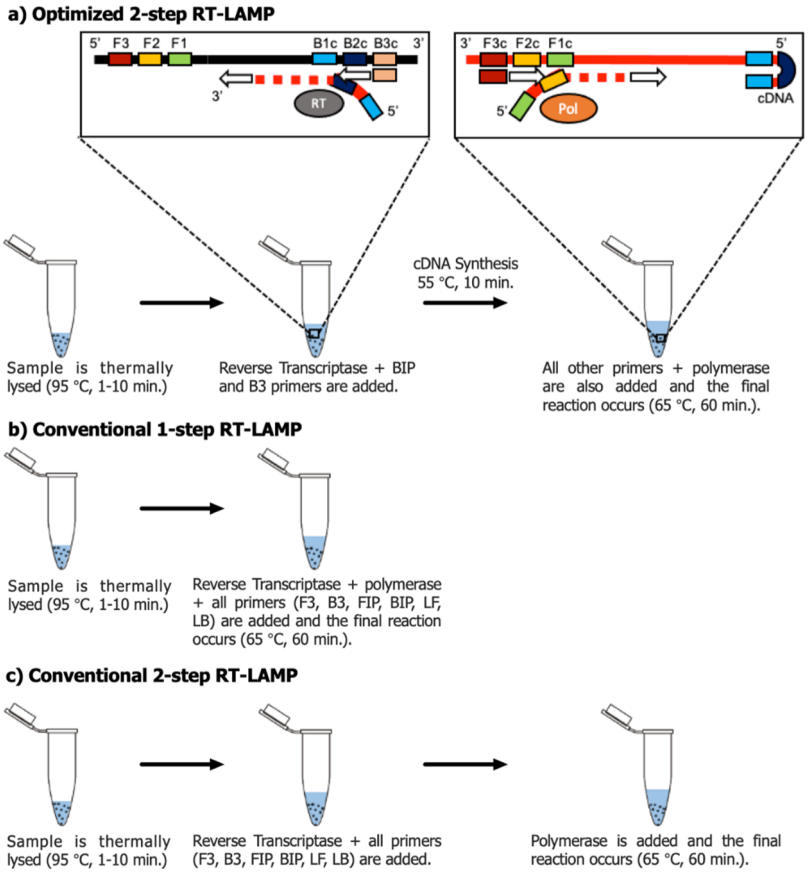

Figure 1. Process flow schematic of RT-LAMP approaches. (a) Optimized two-step RT-LAMP: viral sample is first thermally lysed (95 °C, 1 min). Then, RT-LAMP buffer reagents, B3 and BIP (B1c + B2) primers only, and reverse transcriptase enzyme are added. A short incubation (55 °C, 10 min) is performed for cDNA synthesis to occur. Finally, the rest of the primers and WarmStart Bst 2.0 polymerase are added for the final reaction (65 °C, 60 min); (b) conventional one-step RT-LAMP: after thermal lysis, the RT-LAMP buffer reagents, polymerase and reverse transcriptase enzyme and all primers are added for the reaction to occur (65 °C, 60 min); (c) conventional two-step RT-LAMP: after thermal lysis, the RT-LAMP buffer reagents including the reverse transcriptase enzyme, all primers, and the polymerase are added, and the reaction occurs at 65 °C for 60 min.

addition of the template, the final volume of the reaction was 16 μL (small-volume format) or 96 μL (large-volume format). In the case of the small-volume format, the volume of the template/sample was 2 μL, while in the case of the large-volume format, the volume of the template/sample was 12 μL.

All RT-LAMP assays were carried out in 0.2 mL PCR tubes in an Eppendorf Mastercycler realplex real-time PCR system at 65 °C (60 min). Fluorescence data were recorded every 1 min during the reaction. While all characterization experiments were performed with the number of replicates $n = 3$, $n = 4$ was used for the analysis of clinical samples.

For the one-step RT-LAMP reactions without incubation, the first step was to serially dilute genomic RNA or viruses in H_2O and aliquot 2 or 12 μL sample of the correct concentration into PCR tubes. Samples with viruses were thermally lysed (95 °C, 1 min). Finally, 14 or 84 μL of RT-LAMP reagents were added with final concentrations as mentioned above, and the final reactions were incubated in the thermocycler for amplification.

For the two-step RT-LAMP reactions with RT-incubation, the first step was to perform serial dilutions of the RNA or viral sample as mentioned in the one-step protocol and to aliquot 2 or 12 μL sample of the correct concentration into PCR tubes. Only samples spiked with viruses were lysed (95 °C, 1 min) in a heater. Post thermal lysis, we added 3.28 μL (small-volume format) of a buffer mix with B3 and BIP (B1c + B2) primers as well as reverse transcriptase enzyme and incubated the reaction tubes in a heater for 10 min at 55 °C (RT-incubation step). In this step, BIP and B3 primers anneal to the target and begin cDNA synthesis, without competition from other primers, reducing primer dimers. Post RT incubation, 10.71 μL (small-volume format) of the rest of the reagents for RT-LAMP were mixed in the reaction tubes, and final reactions were incubated in the thermocycler for amplification. Final concentrations of all reagents in the two-step RT-LAMP assay were the same as mentioned above.

All RT-LAMP reactions consisted were performed with at least one set of nontemplate negative controls.

SARS-CoV-2 Genomic RNA and Virus Detection in RT-LAMP Reactions. Reactions done with heat-inactivated viruses and clinical samples included a thermal lysis step. Heat-inactivated viruses were serially diluted in TE buffer and then thermally lysed in a heater at 95 °C (1 min) prior to their addition into the final reaction mix. VTM clinical samples were thermally lysed in a heater at 95 °C (1 min) prior to their addition into the final reaction mix, while in the case of saliva clinical samples the thermal lysis step lasted 10 min. After heat lysis, the tubes were centrifuged (2000g, 1 min) and then maintained at 4 °C (5 min).

In the first analysis of VTM clinical samples, we noticed that samples # 15, 18, 22, and 25 did not show amplification. Likewise, internal controls from samples # 21, 24, and 25 did not show amplification either. During processing, we noticed that

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these samples were highly viscous and contained debris, which may have prevented amplification. We repeated the analysis of these samples but added a 5-min centrifugation step (4000*g*). This additional centrifugation allowed separation of the debris and viscous components from the viral particles. The amplification reaction these samples was repeated using the supernatant postcentrifugation. After the second analysis, internal controls from samples # 21, 24, and 25 did show amplification while from the VTM clinical samples only samples # 15 and 25 did show amplification. Results in Figures 4, 5a,b, and S4 report the second analysis of these samples.

VTM used for assay characterization was obtained from Redoxica (VTM-500ML), aliquoted, and stored in 4 °C away from direct light. Saliva used for assay characterization was pooled human saliva obtained from Innovative Research (IRHUSL5ML) and aliquoted and stored in −20 °C until use for the reactions. The pooled material was collected from donors prior to December 2019.

Serial dilutions of heat-inactivated SARS-CoV-2 viruses were done in VTM to concentrations ranging from 4×10^3 copies/μL to 1 copy/μL in the starting sample. The samples were aliquoted into PCR tubes and lysed (95 °C, 1 min) prior to adding the RT-LAMP reaction mix. For our optimized two-step protocol, the B3 and BIP (B2 + B1c) primers and RT enzyme were added for the RT-incubation step (55 °C, 10 min). The remaining RT-LAMP reagents including F3, FIP, and loop primers were added for a final reaction volume of 16 μL. The amplification reaction was performed at 65 °C for 60 min.

Reactions done with heat-inactivated SARS-CoV-2 viruses in saliva were in the same formats as mentioned above for the one-step and our optimized two-step RT-LAMP assays. Heat-inactivated SARS-CoV-2 viruses were serially diluted and directly spiked in saliva to starting sample concentrations ranging between 4×10^3 copies/μL and 1 copy/μL. The samples were aliquoted into PCR tubes and thermally lysed at 95 °C for 1 min prior to adding the RT-LAMP reaction reagents. In our optimized two-step RT-LAMP assay, 7.85 μL of RT reaction volume including the B3, BIP (B2 + B1c) primers, RT enzyme, and buffer were added to 2 μL of sample for the RT-incubation step (55 °C, 10 min). The remaining RT-LAMP reagents (6.15 μL) were added post RT step for a final reaction volume of 16 μL. The amplification reaction was done in a thermocycler (65 °C, 60 min).

Prior to the thermal lysis step, the clinical samples (VTM and saliva) were aliquoted in two portions. One aliquot was used for internal control and therefore spiked with MS2 bacteriophage (ZeptoMetrix Corporation) in a 1:9 ratio (MS2/sample).

RT-PCR Control. Reactions done for the RT-qPCR control assay with heat-inactivated viruses and clinical samples included a thermal lysis step. Serially diluted heat-inactivated viruses were spiked in saliva. Spiked saliva was thereafter mixed with TE buffer in a one-to-one ratio. These sample were then thermally lysed in a heater at 95 °C (30 min) prior to their addition into the final reaction mix. Saliva heat-treatment, rather than the use of an RNA extraction kit, was selected to follow the EUA-approved procedure developed by the University of Illinois.[32,33] Spiked saliva was mixed with TE buffer to mimic the procedure followed with the saliva clinical samples supplied by Carle Foundation Hospital.

The RT-PCR control assays were performed using the TaqPath RT-PCR COVID-19 kit according to manufacturer's instructions. For more information, please see the Supporting Information.

RESULTS

Improved RT-LAMP Assay. For sensitive detection of viruses, we optimized the standard one-step RT-LAMP process flow to include a short incubation step for cDNA synthesis before the LAMP reaction. Figure 1 compares our optimized two-steps RT-LAMP protocol with the conventional one-step RT-LAMP and two-step RT-LAMP approaches. Our improved RT-LAMP assay is a two-step process that begins with thermally lysed viral samples (95 °C, 10 min). We and others have recently shown that a short thermal lysis prior to the RT-LAMP reaction efficiently exposes the RNA for amplification and is also known to inactivate nucleases in crude samples.[5,26] In our new protocol, post thermal lysis, we add the RT-LAMP reaction buffer, B3 and BIP (consisting of B1 complementary sequence and B2 regions) LAMP primers, and the thermophilic reverse transcriptase enzyme (RT×, NEB) and perform a 10 min RT incubation at 55 °C. The reduced temperature for the RT step (55 °C RT vs 65 °C LAMP) allows for improved annealing between the RNA and the primers and allows for efficient complementary strand synthesis. Since LAMP requires the formation of loop structures for amplification through a defined sequence of steps (binding and extension of inner primer, followed by binding and extension of outer primer), having only B3 and BIP in the RT step reduces the possibility of mispriming events[27,28] and allows for improved LOD, which we characterize in the following sections. After the short RT incubation, the rest of the primers (F3, FIP, LF, and LB) and Bst polymerase are added, and the LAMP reaction is performed at a constant temperature (65 °C, 60 min). It is important to note that our modified protocol involving a separate RT step with only B3 and BIP primers offers superior sensitivity to the conventional two-step RT-LAMP process, where RT incubation is performed with either random hexamers or all of the LAMP primers.[23−25]

Characterization with SARS-CoV-2 RNA and Virus. We evaluated our optimized two-step assay composition and protocol with SARS-CoV-2 genomic RNA and inactivated viruses. Thus, we first confirmed the improved detection limit of our optimized RT-LAMP assay by performing the conventional one-step and our modified two-step reactions to detect SARS-CoV-2 genomic RNA in 2 μL of the starting sample. Figure S2a,b shows the amplification curves for the detection of SARS-CoV-2 genomic RNA in a one-step RT-LAMP reaction and in our optimized two-step RT-LAMP reaction. While in the one-step RT-LAMP reaction, the LOD was 2×10^1 copies/μL, we observed that our optimized two-step assay allowed an improved LOD of 2 copies/μL, with all three replicates amplifying within 25 min. Figure S2c shows the threshold time plot for these reactions. These results highlight that our optimized RT-LAMP assay can amplify 2 copies/μL of SARS-CoV-2 RNA in 2 μL of the starting sample. To note, no amplification for 0 copies/μL indicates that we did not detect false-positive amplification for the time of the reaction; hence, these reactions are considered negative, and the threshold time was not calculated and has been kept at zero.

Next, we compared the results between a one-step RT-LAMP assay and our optimized two-step assay for the detection of inactivated SARS-CoV-2 virus in 2 μL of the starting sample. The LOD was 1 copy/μL using our optimized protocol with a short RT-incubation and separated primers (Figure S2d,e). This is more than 1 order of magnitude sensitive than the LOD of the one-step RT-LAMP assay for SARS-CoV-2 viruses. Figure S2f shows the threshold times plot for these reactions, and we

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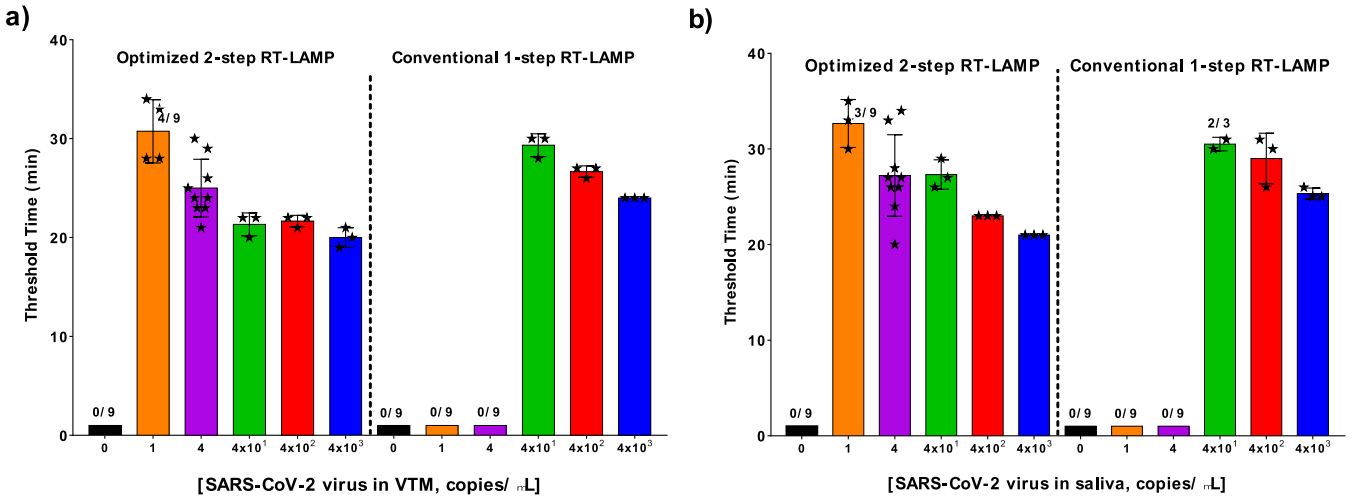

Figure 2. Detection of inactivated SARS-CoV-2 virus in VTM and human saliva. (a) Threshold timings for inactivated SARS-CoV-2 virus in VTM (2 μL starting sample). Optimized two-step RT-LAMP LOD = 1 copy/μL; conventional one-step RT-LAMP LOD = 4×10^1 copies/μL. (b) Threshold timings for inactivated SARS-CoV-2 virus detection in saliva (2 μL starting sample). Optimized two-step RT-LAMP LOD = 1 copy/μL; conventional one-step RT-LAMP LOD = 4×10^2 copies/μL. The bar graphs show mean and standard deviation for three replicates for high concentration samples and nine replicates for reactions with less than or equal to four copies/μL in the starting sample. No amplification events are recorded as "zero", indicating no threshold time for the duration of the reaction.

further observed 3−5 min faster amplification timings for the three highest concentrations of viral sample (4×10^3 to 4×10^1 copies/μL) in the two-step RT-LAMP process. These results highlight that our optimized RT-LAMP assay can show sensitivity for only a few viral copies.

Detection of the SARS-CoV-2 Virus in VTM Transport Media. Current diagnostic testing for SARS-CoV-2 includes collecting NP or anterior nasal specimen swabs with viruses and transferring them into VTM, from which the sample can be collected, purified, and tested using a diagnostic assay.[29] To evaluate if the detection limit of viral samples in VTM is improved by our optimized two-step RT-LAMP reaction, we spiked serial dilutions of inactivated SARS-CoV-2 virus in VTM and performed both the usual one-step and our optimized two-step RT-LAMP reactions on 2 μL of the starting sample. The amplification curves (Figure S3a,b) and threshold times (Figure 2a,b) show a detection limit of 1 copy/μL for our optimized RT-LAMP assay in comparison to the LOD of 40 copies/μL in the one-step RT-LAMP process. These results demonstrate no reduction in sensitivity of our assay in VTM compared to viruses spiked in buffer. It also highlights the fact that for our assay, sensitive detection is possible without any RNA extraction. This pathway can allow potential integration in the current clinical workflow from NP or nasal samples.

Detection of SARS-CoV-2 Virus in Spiked Healthy Human Saliva. The use of saliva has been demonstrated as an alternative upper respiratory tract specimen type for SARS-CoV-2 detection and to offer advantages over the use of NP and nasal swabs. Thus, to evaluate our optimized assay with saliva samples (without extraction or purification of RNA), serial dilutions of inactivated SARS-CoV-2 viruses were spiked in purchased pooled human saliva (Innovative Research). The detection was performed using the conventional one-step and our optimized two-step RT-LAMP reaction. In the amplification curves (Figure S3c,d) and threshold times (Figure 2b), we observed that for the one-step RT-LAMP reaction, a LOD of 4×10^2 copies/μL virus in 2 μL of saliva sample was obtained (with 2/3 replicates amplifying for 4×10^1 copies/μL). In contrast, in our optimized two-step RT-LAMP assay in 2 μL of the saliva starting sample, all three replicates amplified for 4 copies/μL and 1/3 replicates amplified for 1 copy/μL. This highlights the low copy sensitivity, with which we can detect SARS-CoV-2 viruses from human saliva samples without any purification or extraction of RNA.

We compared the LOD for inactivated SARS-CoV-2 virus detection in saliva using RT-LAMP and RT-PCR (as a control experiment). To properly calibrate our RT-LAMP assay with the RT-PCR control, aliquots from the same spiked samples were analyzed using both techniques (Figure S4). We observed an LOD of 1 copy/μL using our optimized two-step RT-LAMP protocol, but the LOD from RT-PCR experiments was 4×10^1 copies/μL. Moreover, threshold timings were in general lower for our optimized two-step RT-LAMP results in comparison to RT-PCR *Ct* values.

Detection of SARS-CoV-2 Virus from Clinical Samples. To demonstrate the applicability of our assay, we also characterized our optimized two-step RT-LAMP reaction using 50 VTM clinical samples (25 known positives and 25 known negatives) and 34 saliva clinical samples (from in-patients at Carle Foundation Hospital, Urbana, IL). While for the analysis of the VTM samples, we used our optimized two-step RT-LAMP reaction (2 μL sample + 14 μL reaction mix), for the characterization of our assay with saliva samples, we compared our two-step RT-LAMP reaction with the conventional one-step RT-LAMP reaction and also using two different reaction volumes to examine differences in sampling. For saliva, we used the small-volume format (2 μL sample + 14 μL reaction mix) and also the large-volume format (12 μL sample + 84 μL reaction mix) for both optimized two-step and traditional one-step reactions. Thus, in the case of saliva, four different reactions were used to analyze each of clinical samples. In addition to the presence of the SARS-CoV-2 virus, the quality of samples (both VTM and saliva) was also tested by spiking an aliquot of the sample with MS2 bacteriophage (internal control). Figure S5 summarizes the samples, techniques, and volume formats used. Each clinical sample (both VTM and saliva) was also tested using the RT-PCR assays from clinical lab results (gold

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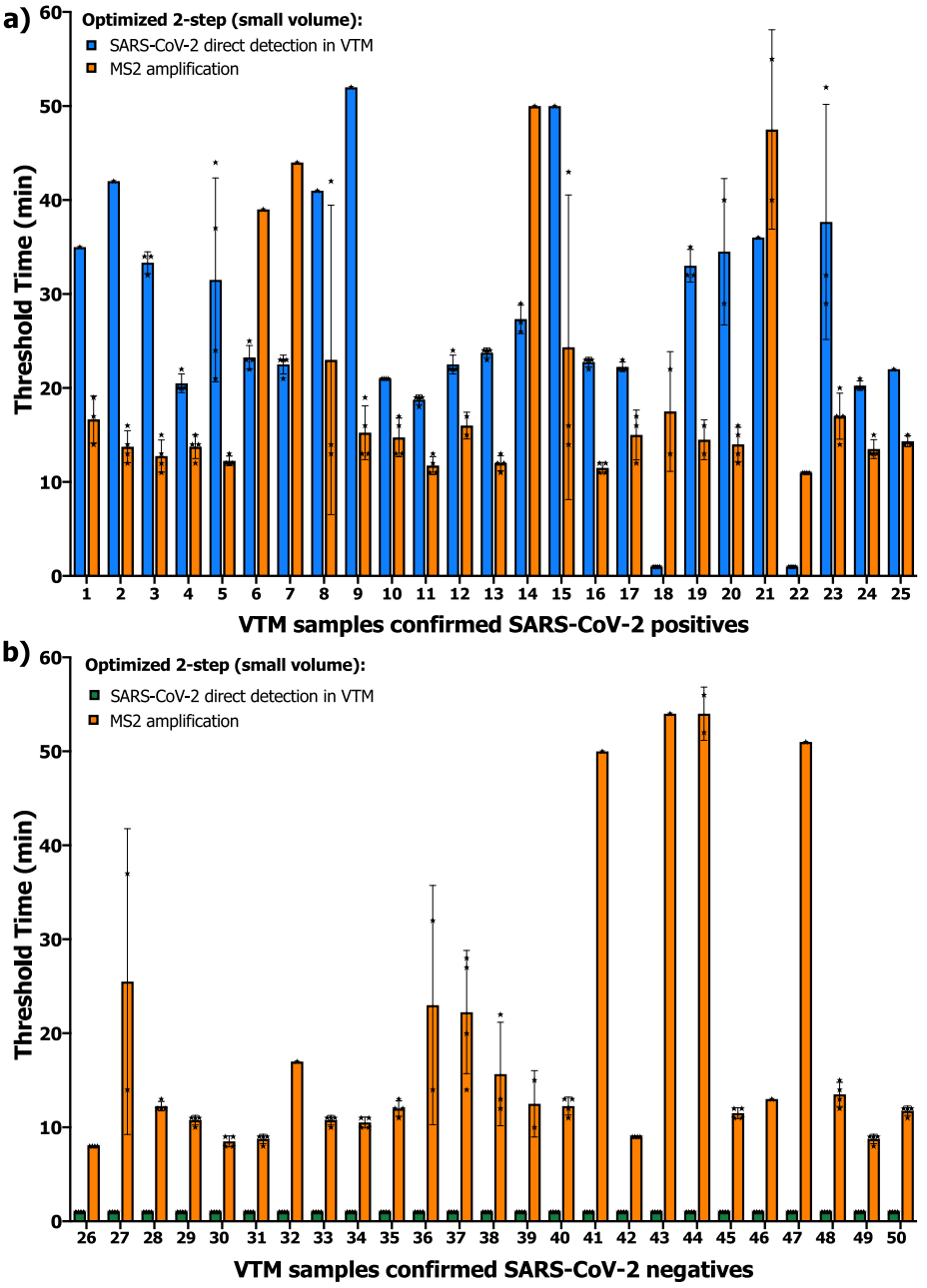

Figure 3. Summary of the results obtained when analyzing VTM clinical samples with our two-step RT-LAMP reaction (small volume). Samples used were clinical VTM discards prior to the standard RNA purification step used in the clinical labs. (a) Confirmed positive samples ($n = 4$). Our reaction detected as positives, 23 of 25 confirmed VTM positive samples. (b) Confirmed negative samples ($n = 4$). Our reaction detected as negatives, 25 of 25 confirmed VTM negative samples. For all positive and negatives samples, internal control was also analyzed ($n = 4$). The bar graphs show mean and standard deviation. Symbols in the bars indicate the results of the individual replicates. No amplification events are recorded as "zero", indicating no threshold time for the duration of the reaction.

standard), which included the RNA purification step, and was used to compare our RT-LAMP results.

The VTM samples used in our work were obtained from Order of St. Francis (OSF) Healthcare (Peoria, IL) through an approved institutional review board (OSF Peoria IRB # 1602513 through the University of Illinois College of Medicine at Peoria with waiver for consent). The samples received were VTM discards prior to the RNA purification step. Along with the samples, we also received the results of the RT-PCR tests performed by OSF Healthcare. The results obtained using these VTM clinical samples are summarized in Figure 3 (raw amplification data is in Figure S6). Our RNA extraction-free two-step RT-LAMP reaction (small volume) was able to detect as positives, 23 of 25 confirmed VTM positive samples, after a thermal lysis step (95 °C, 1 min). Likewise, this reaction was able to detect as negatives, 25 of 25 confirmed VTM negative clinical samples. With these results, the positive predictive value (PPV), negative predictive value (NPV), sensitivity, and the specificity were calculated as 100, 92.6, 92, and 100%, respectively (see the Supplementary Information section for details on calculation of these parameters).

The saliva samples used in this work were obtained from COVID-19 in-patients at Carle Foundation Hospital (Urbana, IL) through an approved institutional review board (Carle IRB #

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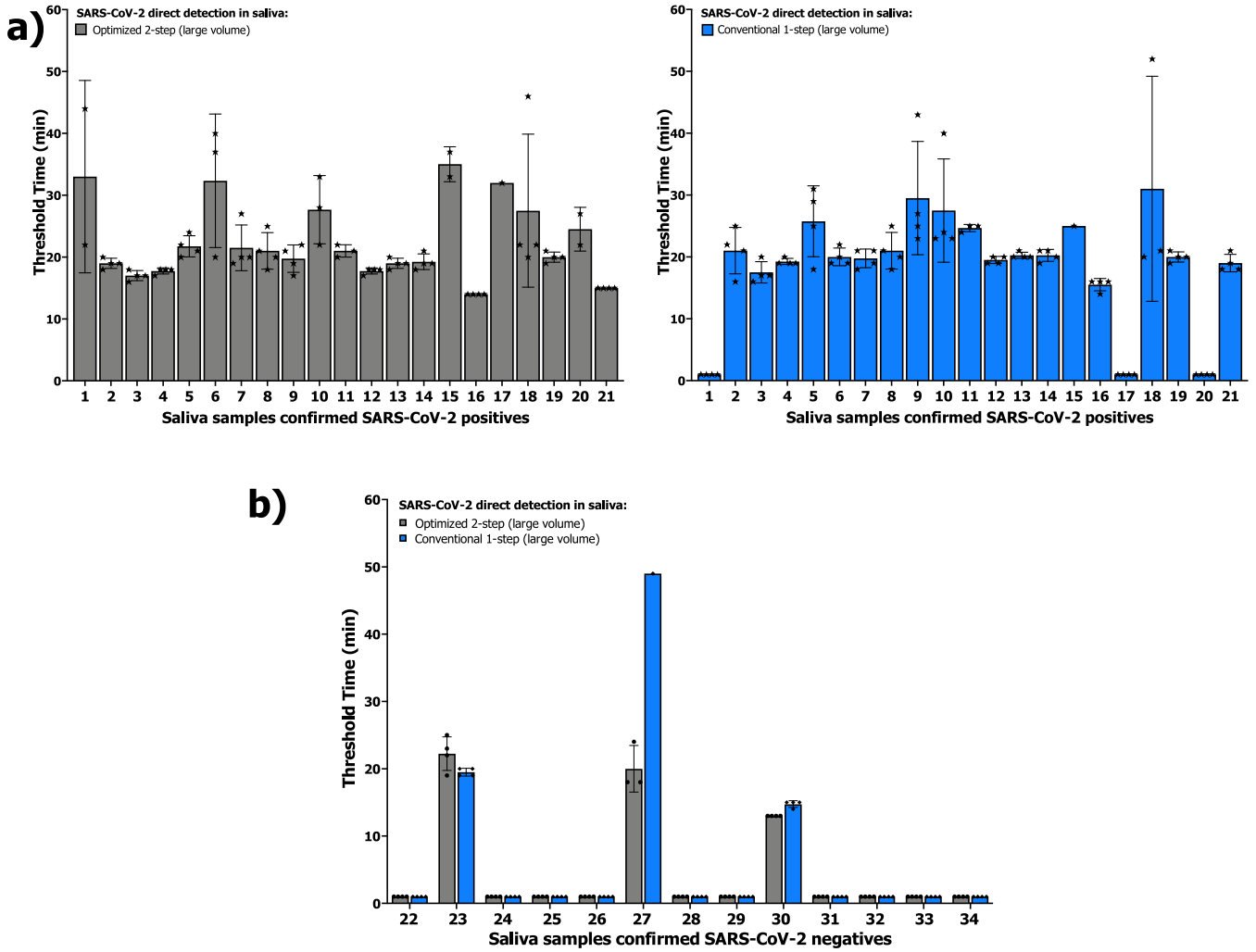

Figure 4. Summary of the results obtained when analyzing saliva clinical samples. Samples used were saliva from in-patients for COVID-19. (a) Confirmed positive samples analyzed with our optimized two-step RT-LAMP reaction (large volume) and with conventional one-step RT-LAMP reaction (large volume) ($n = 4$). (b) Confirmed negative samples analyzed with our optimized two-step RT-LAMP reaction (large volume) and with conventional one-step RT-LAMP reaction (large volume) ($n = 4$). The bar graphs show mean and standard deviation. Symbols in the bars indicate the results of the individual replicates. No amplification events are recorded as "zero", indicating no threshold time for the duration of the reaction.

20CRU3150). Along with the collection of the saliva clinical samples, nasal swab samples were also collected from the same subject at Carle Foundation Hospital and analyzed by the RT-PCR technique at the Carle clinical lab. Immediately after collection, the saliva samples were mixed with TE buffer (1:1). The use of TE buffer reduced the viscosity of the sample, allowing for easier pipetting. The results obtained using these saliva clinical samples are summarized in Figures 4 and S7 (amplification raw data and internal control results are in Figure S8). In addition, to validate our RT-LAMP results in saliva clinical samples, six of the received clinical saliva samples were also analyzed by RT-PCR. Comparative RT-PCR results on clinical samples are in Figure S9.

The results demonstrate that our two-step RT-LAMP reaction (large volume) offered the best results with 100% of sensitivity when analyzing the 21 saliva samples that were confirmed positive with the RT-PCR results from clinical laboratory from the nasal swab control samples. Interestingly, from the 13 saliva samples confirmed negative, 3 were detected as positive with our method. Note that samples (both confirmed positive and negative) are from patients admitted in the hospital who were diagnosed COVID-19-positive by RT-PCR of nasal samples obtained within 14 days of the saliva sample collection date.

DISCUSSION

Although NP, oropharyngeal, and nasal swabs have been recommended by the CDC as the upper respiratory tract specimen types for SARS-CoV-2 viral testing,[29] saliva specimens have been an appealing alternative to the swabs as saliva samples can be collected noninvasively and minimize the contact between healthcare workers and patients and the chance of exposure to the virus.[17,18] In previous analysis of the concordance between NP and saliva samples for detection of SARS-CoV-2 and other respiratory pathogens, it has been demonstrated that sensitivity between the two sample types is highly comparable.[17,18,30,31] Therefore, in this paper, we evaluated the detection of SARS-CoV-2 virus without RNA extraction not only from VTM clinical samples but also from saliva clinical samples using the optimized two-step RT-LAMP protocol in the current clinical workflow. In addition, in the case of saliva, we studied the role of the sampling volume in the assay sensitivity. For saliva clinical samples, we used the small-volume

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a)

Sample	Reaction format	# true positives	#false positives	# true negatives	# false negatives	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	AUC	95% confidence interval	P value
VTM	Optimized 2-step RT-LAMP (small volume)	23	0	25	2	100	92.6	92	100	0.9600	0.8967 – 1.0000	<0.0001
Saliva (before clinical adjudication)	Optimized 2-step RT-LAMP (large volume)	21	3	10	0	87.5	100	100	76.9	0.9121	0.7839 – 1.0000	<0.0001
	Optimized 2-step RT-LAMP (small volume)	17	3	10	4	85	71.4	81	76.9	0.8095	0.6455 – 0.9736	0.0027
	Conventional 1-step RT-LAMP (large volume)	18	3	10	3	85.7	76.9	85.7	76.9	0.8810	0.7644 – 0.9975	0.0002
	Conventional 1-step RT-LAMP (small volume)	16	2	11	5	88.9	68.8	76.2	84.6	0.8370	0.7026 – 0.9714	0.0011
Saliva (after clinical adjudication)	Optimized 2-step RT-LAMP (large volume)	24	0	10	0	100	100	100	100	1.0000	1.0000 – 1.0000	<0.0001
	Optimized 2-step RT-LAMP (small volume)	20	0	10	4	100	71.4	83.3	100	0.9167	0.8214 – 1.0000	0.0002
	Conventional 1-step RT-LAMP (large volume)	21	0	10	3	100	76.9	87.5	100	0.9375	0.8542 – 1.0000	0.0001
	Conventional 1-step RT-LAMP (small volume)	18	0	10	6	100	62.5	75	100	0.8750	0.7591 – 0.9909	0.0007

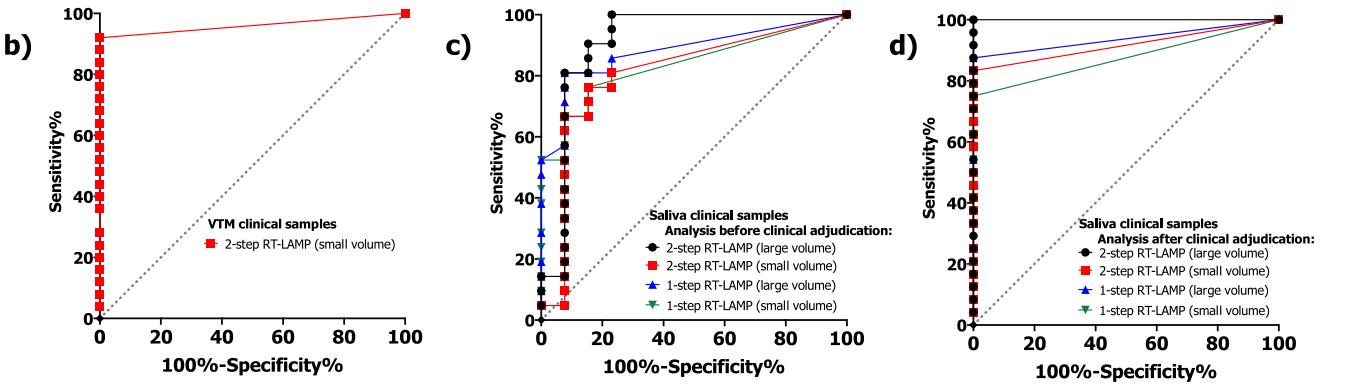

Figure 5. Summary of the performance of the different reaction conditions when analyzing VTM and saliva clinical samples. (a) Summary table including results for VTM and saliva (before and after the clinical adjudication about samples 23, 27, and 30). (b–d) ROC curve analysis for (b) VTM clinical samples, (c) saliva clinical samples (before clinical adjudication), and (d) saliva clinical samples (after clinical adjudication). For each reaction condition, the positive samples were analyzed against the negative samples.

format (2 μL sample + 14 μL reaction mix) and also the large-volume format (12 μL sample + 84 μL reaction mix) for both optimized two-step and conventional one-step RT-LAMP reactions.

The VTM patient samples used in this work were collected and stored frozen prior to the RNA purification step at OSF Hospital (25 confirmed positive and 25 confirmed negative). The saliva samples used in this work were obtained from in-patients for COVID-19 (21 confirmed positive and 13 confirmed negative). In both cases, the presence of SARS-CoV-2 virus was confirmed using the RT-PCR technique from NP (for VTM) and nasal swabs (for saliva) as the control. First, we tested the VTM samples using our optimized two-step RT-LAMP approach with the small-volume format (2 μL sample + 14 μL reaction mix) due to the limited sample amount available. The results (Figure 3) indicated a perfect agreement with the RT-PCR control when testing the negative samples. However, from the 25 positive samples, 2 were detected as negatives with our reaction in the small-volume format. We believe that this could be attributed to the fact that the concentration in these samples was low, and the RNA molecules were not sampled in the volume that we analyzed. Results for PPV, NPV, sensitivity, and specificity were calculated based on these results (VTM in Figure 5a,b). As shown in Figure 5a,b, our optimized two-step RT-LAMP approach (small volume) shows 100% PPV and specificity. However, due to the two false negatives, NPV and sensitivity were 92.6 and 92%, respectively. Based on these results, we decided to study the role of the sampling in the assay sensitivity by including a large-volume format when analyzing saliva samples.

We found that when using a larger saliva sample volume in the optimized two-step RT-LAMP approach, we obtained 100% sensitivity as we were able to detect as positives the 21 confirmed positive samples (Figure 4a, saliva—before clinical adjudication—in Figure 5a,c). However, three of the confirmed negative samples (# 23, 27, and 30) from the in-patients were detected as positives with our reaction, impacting the specificity results (76.9%) (Figure 4b). Similar behavior was observed when using the optimized two-step RT-LAMP approach (small volume) and conventional one-step RT-LAMP approach (large volume) where samples 23, 27, and 30 were also detected as positives. However, in these cases, the sensitivity was worse as these reactions reported four and three false negatives, respectively. Finally, the conventional one-step RT-LAMP approach (small volume) reported the lowest sensitivity (76.2%), although the specificity was a little bit better (84.6%) as only sample numbers 23 and 30 (not sample # 27 in this case) were detected as positives. Interestingly, since the patients were known to be COVID-19 positive from an earlier test when admitted to the hospital due to symptoms and were still in the hospital being treated when the saliva sample and the control nasal swab sample was taken, it could be hypothesized that the saliva samples depict the real presence of the viral RNA and the nasal swab does not. If samples 23, 27, and 30 are considered positives

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(after clinical adjudication), then sensitivity and specificity change, as shown in Figure 5a,d. First, the total number of positives is now 24, while the total number of negatives is now 10. The optimized two-step RT-LAMP approach (large volume) indicates a perfect agreement with the control (PPV, NPV, sensitivity, and specificity = 100%). In addition, for all reaction conditions studied, the specificity would be 100%. We can conclude that increasing the sample volume improves the reaction sensitivity, although this also increases the cost of the assay due to increased use of reagents. Also, the use of the optimized two-step approach improves the assay sensitivity in comparison to the conventional one-step reaction when the same volume of sample is used. An important point to note is that the saliva samples can potentially detect the presence of RNA in in-patient samples diagnosed of COVID-19 when the nasal swabs cannot.

The better performance of the optimized two-step RT-LAMP reaction (large volume) is also highlighted in the receiver operating characteristic (ROC) curves (Figure 5c,d). With this reaction condition (after clinical adjudication), the AUC was 1.00, showing that this reaction can correctly detect positive and negative samples without false positives or false negatives. The saliva results and the study of the volume sampling indicate that the optimized two-step RT-LAMP reaction (small volume) can produce some false negatives. This could explain the two false negatives we obtained when analyzing the VTM clinical samples.

It is important to highlight that, although the sensitivity can vary with the different approaches used in this study, the RT-LAMP reaction achieves 100% specificity when analyzing VTM and saliva samples, independent of the assay conditions. This improved performance along with the high sensitivity of our optimized two-step RT-LAMP approach are very important features when considering the use of RT-LAMP reactions for massive surveillance testing, especially for portable and point-of-care applications.[5]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c05170.

Detection of ZIKA genomic RNA and virus in buffer, detection of SARS-CoV-2 genomic RNA and virus in buffer, amplification curves for detection of inactivated SARS-CoV-2 virus in VTM and human saliva, comparison of RT-LAMP and RT-PCR detection of inactivated SARS-CoV-2 virus in human saliva, VTM and saliva samples collection and pathways of analysis, amplification raw data of the results obtained when analyzing VTM clinical samples with our two-step RT-LAMP reaction (small volume), results obtained when analyzing saliva clinical samples (small volume), saliva clinical samples results, RT-PCR analysis of clinical saliva samples, Zika and SARS-CoV-2 genomic RNA and viruses, primer sequences and primer validation reactions, amplification data analysis, RT-PCR control, characterization with Zika RNA and virus (PDF)

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Author Contributions

A.G., E.V., and R.B. designed the assay and diagnostics study. E.V., S.A.S.d.R., M.J., K.W., J.K., and R.B. designed the clinical sample collection protocols. A.G., A.M., J.B., J.L., E.A., and J.B. performed the experiments. A.B., K.R., M.A., S.A., M.M., P.M., and S.S. supported the clinical study, collected samples, and analyzed data. A.M., A.G., J.B., E.V., and R.B. analyzed data. A.M., A.G., E.V., and R.B. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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