Improving performance of a SARS-CoV-2 RT-LAMP assay for use with a portable isothermal fluorimeter: Towards a point-of-care molecular testing strategy

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Abstract

Frequent and accessible testing is a critical tool to contain the spread of SARS-CoV-2. To develop low-cost rapid tests, many researchers have employed reverse transcription loop-mediated isothermal amplification (RT-LAMP) with fluorescent readout. Fluorescent LAMP-based assays can be performed using cost-effective, portable, isothermal instruments that are simpler to use and more rugged than PCR instruments. However, false positive results due to nonspecific priming and amplification have been reported for a number of LAMP-based assays. In this report, we implement a RT-LAMP assay for SARS-CoV-2 on a portable isothermal fluorimeter and a traditional thermocycler; nonspecific amplification is not observed using the thermocycler, but occurs frequently with the isothermal fluorimeter. We explored four strategies to optimize the SARS-CoV-2 RT-LAMP assay for use with an isothermal fluorimeter and find that overlaying the reaction with mineral oil and including the enzyme *Tte* UvrD Helicase in the reaction eliminates

the problem. We anticipate these results and strategies to be relevant for use with a wide range of portable, isothermal instruments.

Introduction

Loop-mediated isothermal amplification (LAMP) is a promising technology to detect SARS-CoV-2 in clinical samples, with nine LAMP-based tests having received Emergency Use Authorization as of April 2021, and at least two scientific advisory groups recommending its use.[1,2] However, LAMP reactions are known to produce nonspecific amplification products due to the use of six oligonucleotide primers at high concentrations and the continuous amplification that takes place during isothermal incubation.[3] LAMP reaction mixes are available in "WarmStart" formulations (NEB E1700) that include an intercalating fluorescent dye and which may be assembled easily at room temperature. However, intercalating dyes produce signal that is proportional to the total amount of dsDNA, and nonspecific products cannot be easily distinguished from true amplification of the desired target. It is advantageous to have strategies to eliminate false positive results and encourage only on-target amplification when using RT-LAMP assays based on intercalating fluorescent dyes.

In this paper, we describe performance of an RT-LAMP assay for SARS-CoV-2 that was optimized for a commercial thermocycler and then implemented on a portable isothermal fluorimeter without assay modifications. We then describe four strategies to reduce false positive results observed on the portable device: overlaying a reaction with mineral oil, incorporating helicase, mixing during amplification, and performing a brief vortex and centrifuge prior to the reaction.

The use of mineral oil or other substrates to overlay nucleic acid amplifications has been well established before lid heating was a common feature of thermocyclers, as it prevents evaporation that would lead to changes in both the reaction temperature and the effective concentration of primers and enzymes in the reaction. Mineral oil is frequently used to overlay reactions in recent reports of LAMP-based assays in cases where thermocyclers or even reaction tubes are not being used.[4,5] Therefore, we chose to use PCR-grade mineral oil to overlay RT-LAMP reactions when

they were performed in the Axxin T8-ISO. The enzyme *Tte* UvrD helicase was developed by New England Biolabs specifically to reduce non-template amplification in LAMP assays.[6–9] Helicase functions to unwind double-stranded DNA, reducing both spurious and specific products. Specific products are typically much more abundant than spurious products, so the inclusion of this enzyme can effectively suppress the detection of spurious products.[10]

Finally, agitation is known to increase the speed and lower the limit of detection of other isothermal amplification reactions.[11,12] We hypothesized that either continuous agitation or one pre-reaction vortex and centrifuge of our RT-LAMP reactions could improve temperature homogeneity and reduce the likelihood of nonspecific primer interactions, particularly due to the larger reaction volume of 50 μL required by the Axxin T8.[13] Axxin molecular diagnostic instruments allow continuous mixing by including a magnet on the optical module, which pulls up and drops a magnetic ball included in the reaction each time the fluorescence reader passes a tube. Therefore, continuous agitation was accomplished in this study by including a magnetic steel ball bearing in each reaction, and a single pre-reaction vortex and centrifuge was investigated as a fourth and final strategy to reduce non-template amplification.

Materials and Methods/Experimental

Instruments and Protocol

Point-of-care instruments for SARS-CoV-2 diagnostic testing should be affordable, portable, and easy to use without specialized training. For real-time detection of fluorescent RT-LAMP reactions, a point-of-care instrument should heat the sample to a predefined temperature, accurately monitor fluorescence vs time, and automatically interpret results as positive or negative. We selected a commercially available instrument — the Axxin T8-ISO (Axxin Pty Ltd, Fairfield, Australia) — that met these criteria off-the-shelf and without customization for this study.

The latest T8-ISO software (v.3.3.00-383) was installed and test protocols were developed with the provided PC desktop software, (Axxin, v.2.8.0.1). Reactions were run at 65º C. The FAM channel was set to 7% PWM and readings taken every 10 seconds; the second HEX channel was

not used. Reactions were considered positive if the relative fluorescence intensity reached 2,500 mV within 20 minutes. Results were compared to those obtained with a Bio-Rad CFX96 Touch thermocycler (Hercules, CA) and its associated CFX Maestro software (v.4.1.2433.1219). All reactions were run at 65º C for 45 minutes in a Bio-Rad CFX96 Touch with or without heated lid set at 105º C. The positive threshold for the Bio-Rad was considered to be a fluorescence increase of 500 RFU from the baseline fluorescence value within 45 minutes.

The oligonucleotide primers (oligos) for the RT-LAMP assay were designed by New England Biolabs, Inc. (NEB) (Ipswich, MA)[14] to target the N2 and E1 genes and Rabe and Cepko[15] to target the Orf1 gene of the SARS-CoV-2 genome. All oligos were ordered from Integrated DNA Technologies, Inc. (IDT) (Coralville, IA) and resuspended in 1X TE buffer at a 1 mM concentration. Oligos were combined to make 1,000 µL of a 25X mix as follows: 40 µL FIP, 40 µL BIP, 5 µL F3, 5 µL B3, 10 µL LF, 10 µL LB, and 890 µL nuclease-free water.

All RT-LAMP reagents were purchased from NEB (E1700). Standard reaction conditions for the thermocycler include 5 µL of sample in a total reaction volume of 25 µL. Each 25 µL reaction contained 12.5 µL of 2X Master Mix, 1 µL of 25X N2 primer mix,[14] 1 µL of 25X E1 primer mix,[14] 1 µL of 25X As1e primer mix,[15] and 0.5 µL of 50X fluorescent dye (NEB B1700). Standard reactions were supplemented to 20 µL with nuclease-free water, and then 5 µL of RNA template were added in low-profile 8-tube strips (Bio-Rad, TLS0801) with flat, ultra-clear caps (Bio-Rad TCS0803). All reactions run on the BioRad CFX96 were performed with these specifications, except for the direct comparison between the two instruments (**Fig. 1**), in which the dye concentration typically used for the Axxin T8-ISO, (0.5 µL 5X dye per reaction) described below was used for BioRad reactions as well.

In reactions run on the Axxin T8-ISO, 0.5 µL of 5X fluorescent dye (NEB B1700) was used.[16] Additionally, due to the instrument specification for reaction volume of a minimum of 30 µL, reaction volumes of 50 µL were used on the Axxin T8-ISO. In these reactions, the volume of each component of the RT-LAMP assay described above was doubled, and reactions were assembled in high-profile 8-tube strips (Axygen PCR-0208-C) with domed caps (Bio-Rad TCS0801). 0.2 µL

of *Tte* UvrD Helicase (New England Biolabs, Inc, M1202S) was included in some reactions on the Axxin T8-ISO.

Molecular biology-grade mineral oil was purchased from Sigma-Aldrich (69794). 2 mm grade 100 AISI 420 stainless steel ball bearings were obtained from SimplyBearings (Leigh, UK). Samples that were vortexed were agitated at maximum speed for approximately 3-5 seconds then quickly spun in a microcentrifuge. Samples mixed by pipetting were pipetted up and down 3-5 times.

Viral RNA sequences used in this study were purified RNA controls from Twist Biosciences (SKU 102019). Upon receipt, Twist Biosciences control RNA was quantified via RT-qPCR against SARS-CoV-2 genomic RNA (ATCC® VR-1991D™), then stored in single-use aliquots of 5 µL each at -80º C until use. Upon use, each aliquot was diluted in nuclease-free water, kept on ice, and used within 4 hours.

Clean reaction setup

All amplification reactions were assembled and sealed prior to amplification in a dedicated pre-amplification room that was regularly decontaminated with 10% bleach prepared daily and had limited personnel access. Once reactions were run, reaction tubes were discarded without opening to prevent post-amplification contamination of future reactions.

Clinical sample collection and lysis

All lysis buffer solutions were made from molecular grade reagents. TCEP/EDTA/NaOH/GuHCl buffer was used at a concentration of 5X. First, a concentrated TCEP/EDTA/NaOH buffer was adapted from Rabe and Cepko as follows: 358 mg of TCEP-HCl (Millipore Sigma 580567) was dissolved in 568 nuclease-free water. Next, 1 mL of 0.5 M EDTA pH 8 (ThermoFisher Scientific AM9260G), and 575 µL 10 N NaOH (Fisher Scientific SS267) were added. 2,009 mg of GuHCl (Promega H5381 or H5383, used interchangeably) was dissolved into 1.5 mL nuclease-free water, yielding 3 mL of 7 M GuHCl. 2,857 µL of 7 M GuHCl were added to the solution containing TCEP-HCl, EDTA, and NaOH, bringing the total concentrated lysis buffer volume to 5.0 mL.

When used with nasopharyngeal swabs, the concentrated lysis buffer was diluted 1:20 in nuclease-free water, yielding the “swab lysis buffer”. 300 µL of swab lysis buffer was aliquoted into each 1.5 mL collection tube (Sarstedt 72.692.405). When used with saliva, 5 mL of concentrated lysis buffer was added to 10 mL of Tris pH 8.0 (Invitrogen AM9855G), yielding the “saliva lysis buffer”. Saliva collection tubes were not pre-loaded with lysis buffer. All solutions were sterile-filtered through a 0.2-µm filter (Pall Life Sciences Acrodisc 4652), then stored at 4º C for up to 7 days.

Nasal and nasopharyngeal swabs were collected under a protocol approved by the Institutional Review Boards at the University of Texas MD Anderson Cancer Center and Rice University. A medical provider collected nasopharyngeal swabs (Fisher Scientific 23-349-822 or MedicoSwab FS-N96000) into 300 µL of diluted swab lysis buffer. The swab was cut with clean scissors at the top of the tube, leaving the swab tip in buffer. Participants self-collected saliva by passively drooling into a sterile 5 mL tube (MTC Bio C2540). Samples were transported to Rice University on ice and were tested on the Axxin T8-ISO and Bio-Rad CFX96 Touch on the day of collection.

To test samples upon receipt, tubes containing swabs and swab lysis buffer were surface decontaminated, vortexed for 20 seconds, briefly spun in a microcentrifuge, and heated at 95º C for 5 minutes in a dry heat block (VWR 10153-348) for lysis. To lyse saliva samples, 255 µL of saliva were combined with 45 µL of saliva lysis buffer; the mixture was vortexed and heated at 95º C for 6 minutes. After heating, tubes were placed on ice for at least one minute prior to adding to RT-LAMP reactions.

RT-LAMP and RT-qPCR assay results were compared for swab and saliva samples. To run samples in RT-qPCR, RNA was extracted from 200 µL of heated swab and saliva samples using the PureLink Viral RNA/DNA extraction kit (Invitrogen 12280050) per manufacturer’s instructions, including the Proteinase K lysis step and with a final elution into 50 µL of nuclease free water. RT-qPCR reactions were assembled using the CDC SARS-CoV-2 assay, including N1, N2, and RPP primers ordered from IDT, and TaqPath RNA-to-Ct RT-qPCR kit (Applied Biosystems 4392653) per manufacturer’s instructions.

Results and Discussion

After initial experiments validating the performance of the RT-LAMP reaction on the BioRad CFX96, attempts to perform the same RT-LAMP reactions on the Axxin T8-ISO resulted in false positive amplification as early as 27 minutes (**Fig. 1**). False positive formation occurs inconsistently on the T8-ISO, indicating spurious non-specific product generation. False positive results were obtained when identical reaction mixes were prepared in the clean reaction room, separated into 2 sealed 8-tube strips, and run concurrently on the two instruments, therefore ruling out the possibility of environmental contamination in only the T8-ISO strips. The presence of a heated lid on the BioRad CFX96 is a major difference from the T8-ISO, and we hypothesized that temperature heterogeneity—as well as local concentration increases at the reaction surface to due to evaporation—in reaction tubes incubated in an instrument without a heated lid may lead to nonspecific amplification.

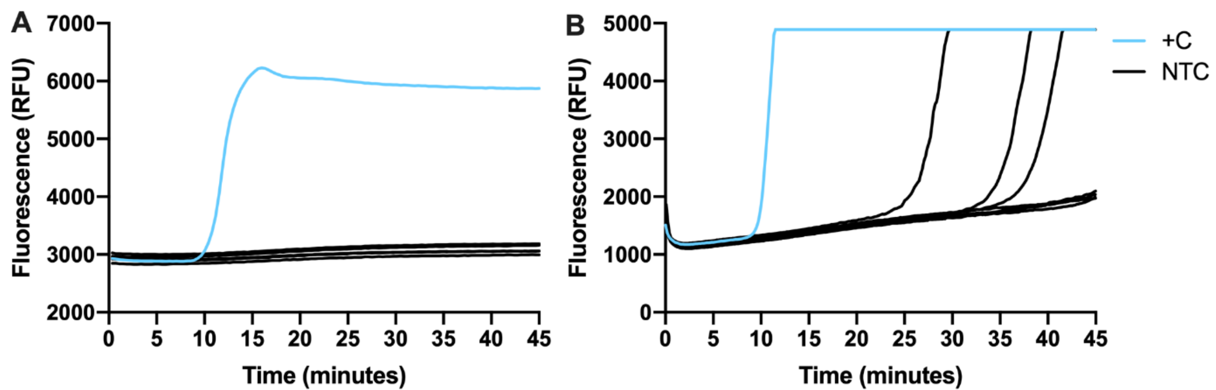

Figure 1. Fluorescence vs. time for seven no-template control (NTC) and one positive control (+C) RT-LAMP reaction prepared from the same master mix and run on (A) the BioRad CFX96 in 25 μL reaction volumes and (B) the Axxin T8-ISO in 50 μL reaction volumes. Reactions contain either water (NTC), or 10 copies/μL of synthetic SARS-CoV-2 RNA.

To investigate the theory that false positive amplification occurs in the absence of a heated lid, we performed RT-LAMP on the BioRad CFX96 with the heated lid set to 105º C and with the heated lid switched off. With the heated lid switched off, amplification was observed in no-template control samples, supporting the hypothesis that temperature heterogeneity within the reaction tube leads to spurious amplification (**Fig. 2A and 2B**). Furthermore, melt curve derivative peaks in the no-template control samples that are distinct from those of true products suggest that the amplification products generated in the absence of lid heating were due to non-template amplification **(Fig. 2D)**. LAMP-based assays have previously been demonstrated without false

positive amplification in instruments without heated lids, potentially indicating that the phenomenon observed in this report is due to a specific combination of primers, tubes, and instrumentation.

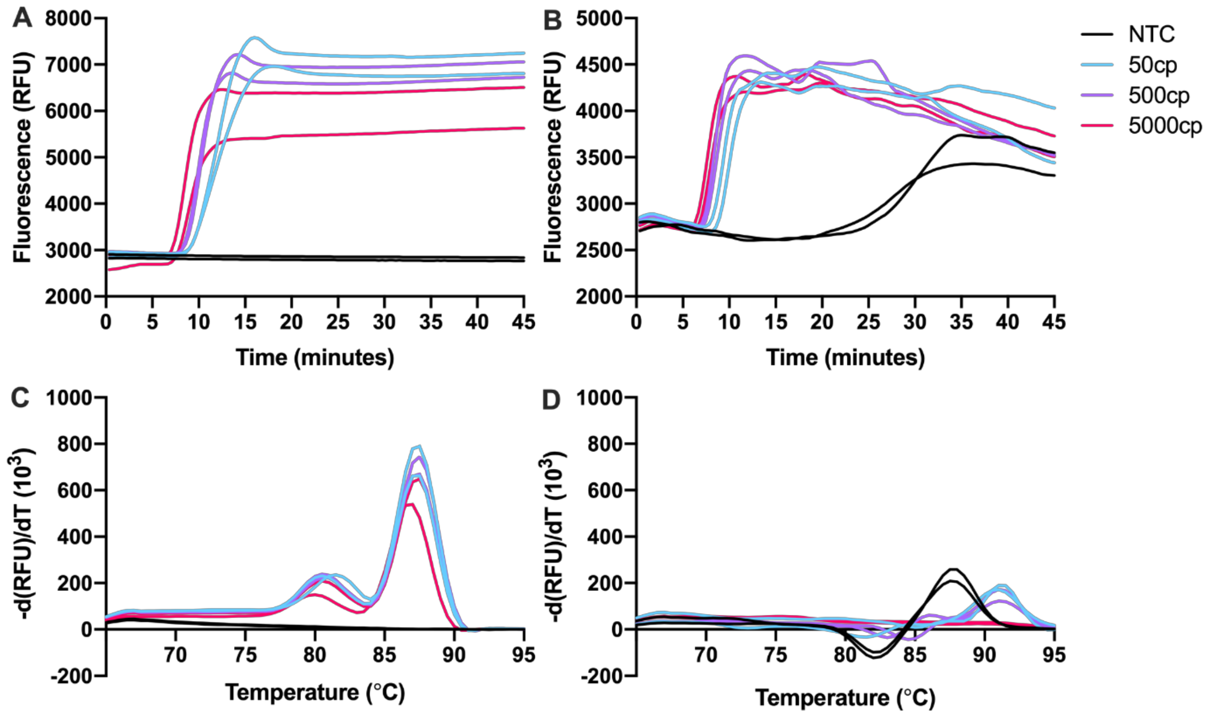

Figure 2. Fluorescence vs. time (A,B) and melt curve derivative data (C,D) for identical RT-LAMP reactions run on the BioRad CFX96, (A,C) with the heated lid set to 105º C, and (B, D) without the heated lid. Reactions contain either water (NTC), 50, 500, or 5,000 copies of synthetic SARS-CoV-2 RNA.

Four strategies were implemented to mitigate non-template amplification observed in the initial reactions on the T8-ISO: (1) overlaying the reaction with mineral oil, (2) including the enzyme *Tte* UvrD Helicase in the reaction, (3) including mixing during the amplification reaction, and (4) performing a brief vortex and centrifuge prior to the reaction rather than relying on mixing by pipetting. These strategies were evaluated alone, and in combination, by running seven no-template control reactions (using nuclease-free water as the sample) and one positive control reaction (50 copies of control RNA) in a strip of 8 tubes. Representative results are shown in **Fig. 3** and results are summarized in **Table 1**.

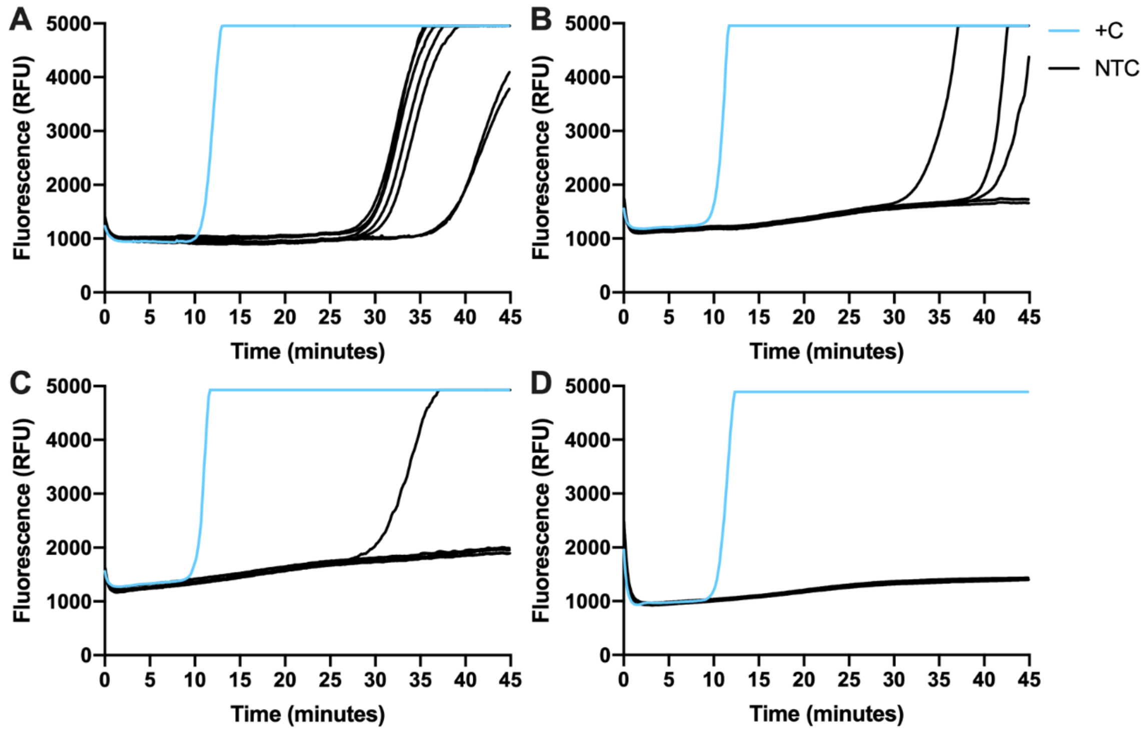

Figure 3. Fluorescence vs. time for three of the strategies studied to eliminate false positives in no-template control samples. RT-LAMP reactions were run on the Axxin T8-ISO and contain either nuclease-free water (NTC) or 50 copies of synthetic SARS-CoV-2 RNA (+C). (A) mixing ball; (B) 25 μL mineral oil overlayed; (C) 0.2 μL helicase per reaction; (D) 25 μL mineral oil + 0.2 μL helicase per reaction.

Mineral oil (μL)	Helicase	Mixing ball	Vortex + centrifuge?	# positives/7 NTCs
0	No	No	Yes	3
25	No	No	Yes	3
50	No	No	Yes	2
0	No	Yes	Yes	7
0	Yes	No	Yes	1
50	Yes	No	Yes	0
50	**Yes**	**No**	**No**	**0**
25	**Yes**	**No**	***No***	**0**

Table 1. Summary of strategies studied to eliminate false positives in no-template control samples and the resulting number of no-template control samples, out of 7, that returned a positive result.

The conditions that most reliably suppressed non-template amplification were: *Tte* UvrD Helicase included at 0.2 μL per 50 μL reaction together with 25 or 50 μL of mineral oil atop the reaction. These results were repeated three times in separate experiments for confirmation (results not shown).

Finally, clinical samples of varying sample type were evaluated on the Axxin T8 and the BioRad CFX96 with the optimized conditions for each instrument, respectively. Results were found to be highly consistent across the two instruments, with clinical samples that were also positive by PCR generally amplifying on both instruments within approximately 20 minutes, and clinical samples that were negative by PCR and no-template controls remaining negative (representative results and controls shown in **Fig. 4**).

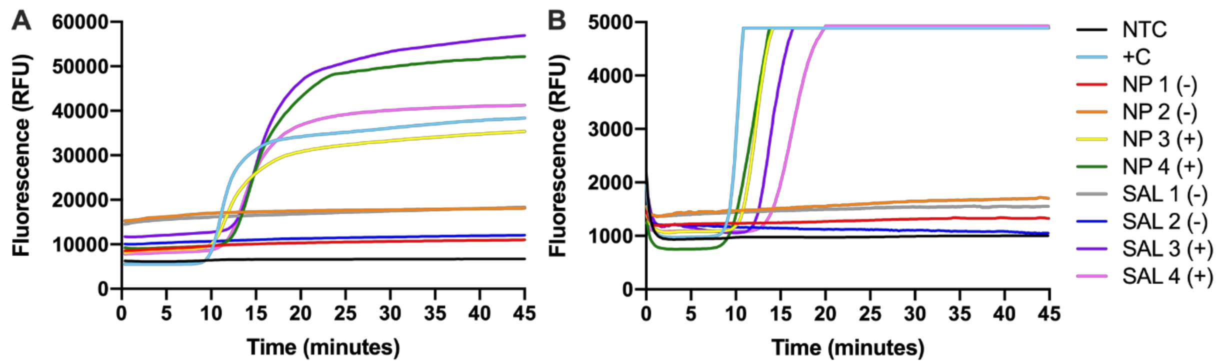

Figure 4. Fluorescence vs. time using eight patient nasopharyngeal or saliva samples following the point-of-care lysis procedure and using (A) the manufacturer-recommended RT-LAMP protocol on the BioRad CFX96, and (B) the RT-LAMP protocol optimized for the Axxin T8-ISO). NP: nasopharyngeal samples; SAL: saliva samples; NTC: no-template control (water); +C: 500 total copies synthetic SARS-CoV-2 RNA. (+) and (-) refers to result obtained by RT-qPCR on the same samples.

While false positive events generally occurred close to or after 30 minutes of incubation, and true positives amplified earlier than 30 minutes in the representative curves shown in Fig. 3, we were unable to define a precise time threshold that could reliably distinguish between true positives and false positives without any of the interventions described. Particularly with low viral loads, sample amplification of RT-qPCR-confirmed positive samples can be delayed to the 30-45 minute range by inhibitors in the clinical matrix that remain in the reaction without an extraction or purification step (results not shown). Additionally, when reactions are performed on a thermocycler, melt curve derivatives can provide additional insight on product specificity for late-amplifying samples, but melt curves cannot be acquired from the Axxin T8-ISO or other field-deployable molecular diagnostic instruments. Therefore, the approaches described here allow for longer incubation times to improve sensitivity without compromising specificity when using low-resource instruments.

Conclusion

Successful implementation of RT-LAMP for SARS-CoV-2 in point-of-care settings will remain useful in the foreseeable future to contain the virus spread as vaccines in development are validated for different populations at varying speeds, and rollout reaches different areas of the globe. After the COVID-19 pandemic is no longer a threat, strategies to adapt LAMP-based assays to point-of-care fluorimeters will remain useful for other disease targets. Here, we have described challenges encountered when adapting an RT-LAMP assay to the Axxin T8-ISO isothermal fluorimeter and strategies used to eliminate false positive events. Further optimization using lyophilized LAMP reagents and alternate strategies to seal reactions, such as low-temperature wax dots, could increase ease of implementation of similar assays and instruments. Using the optimized conditions described in this work, the Axxin T8-ISO could provide a promising alternative to traditional thermocyclers for identification of SARS-CoV-2 infections in settings with limited infrastructure and low throughput needs.

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Figure 1

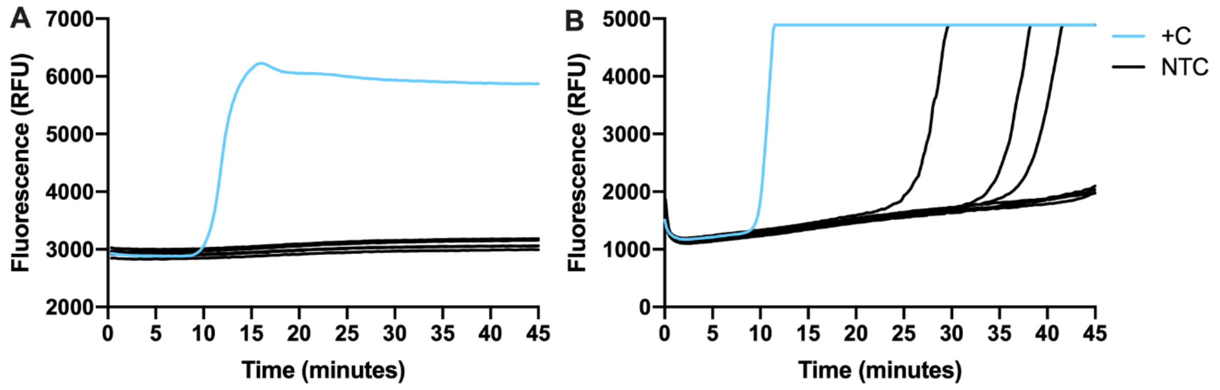

Figure 2

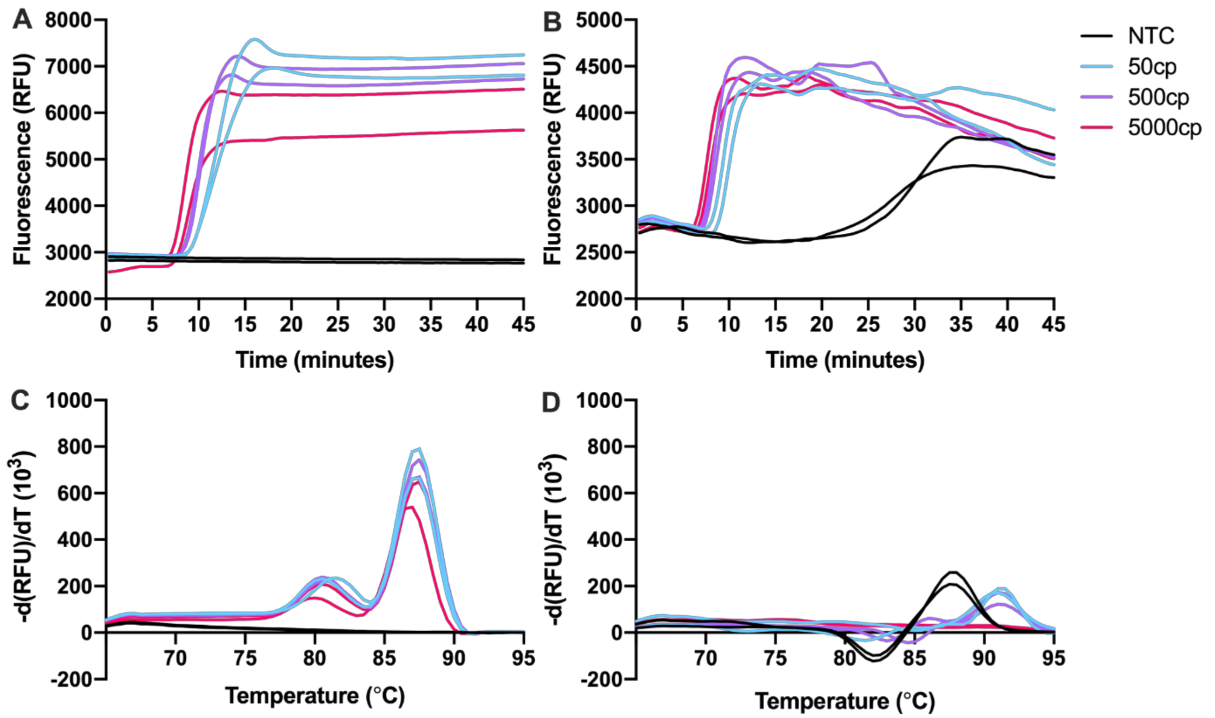

Figure 3

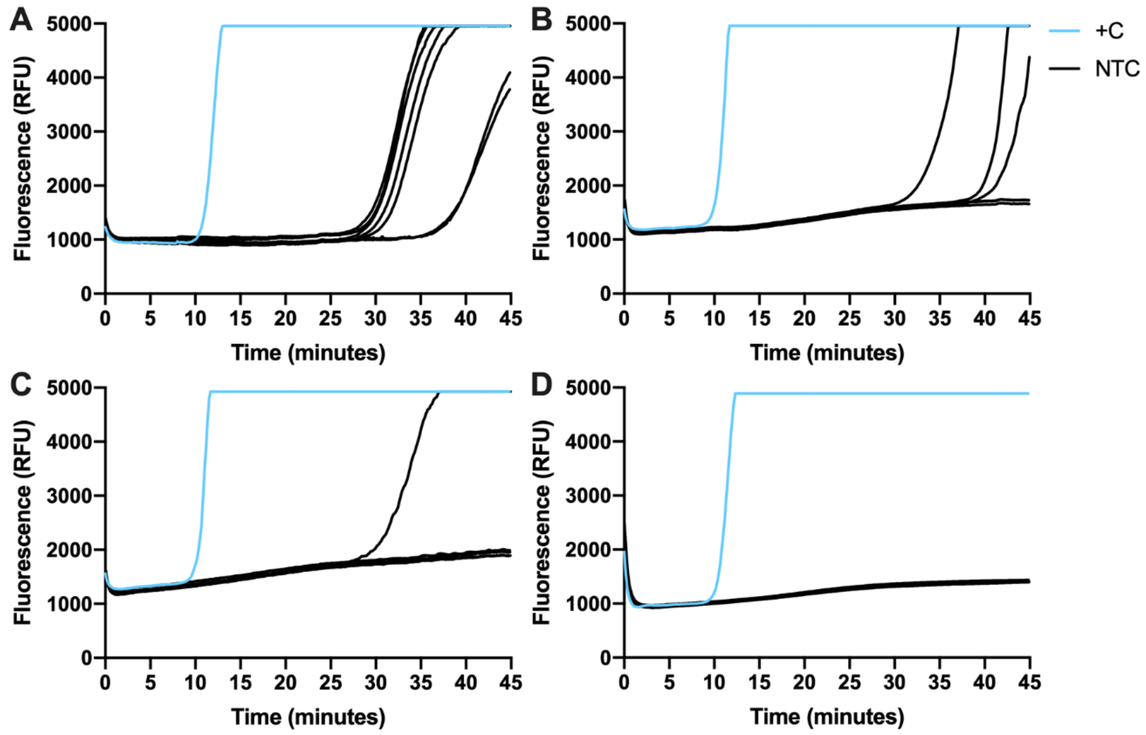

Figure 4