ARTICLE

Molecular Characterization of Increased Amplicon Lengths in SARS-CoV-2 Reverse Transcription Loop-Mediated Isothermal Amplification Assays

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Loop-mediated isothermal amplification (LAMP) is a power tool for the amplification of specific RNA and DNA targets. Much like PCR, LAMP requires primers that surround a target amplification region and generates exponential product through a unique highly specific daisy-chain single-temperature amplification reaction. However, until recently, attempts to amplify targets of greater than 200 base pairs (bp) have been mostly unsuccessful and limited to short amplicon targets of less than 150 bp. Although short amplicons have the benefit of a rapid detection (<40 min), they do not allow for the prediction of RNA integrity based on RNA length and possible intactness. In this study, 8 primer sets were developed using 2 LAMP primer–specific software packages against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid gene with insert lengths ranging from 262 to 945 bp in order to amplify and infer the integrity of viral RNA. Because these amplification lengths are greater than the current methods that use an insert length of 130 or less, they require a longer incubation, modified primer and temperature strategies, and the addition of specific adjuncts to prevent nonspecific amplification. This proof of concept study resulted in successful reverse transcription LAMP reactions for amplicon targets of 262, 687, 693, and 945 bp using a clinical nasopharyngeal NP sample, purified SARS-CoV-2 RNA, and crude lysate containing inactivated virus.

INTRODUCTION

Loop-mediated isothermal amplification (LAMP) was first described by Notomi *et al.* in 2000 as a novel method to amplify target DNA.[1] Although similar to PCR in exponential synthesis of an amplicon product, the LAMP process varies in its reliance on an autocycling strand displacement DNA synthesis to generate large amounts of daisy-chained concatemers that are synthesized at one temperature[1,2] and are detected using a variety of methods, including visual colorimetric,[3] isothermal quantitative PCR,[4,5] molecular beacons,[6] and electronic.[7–9] LAMP is most often used as a diagnostic tool due to its speed, specificity, and ease of use. It is highly specific because of the simultaneous interactions between 4 and 6 primers for one nucleic acid target.[2] It is well recognized for both RNA and DNA detection[10,11] with a recent focus on detecting RNA targets such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) using reverse transcription LAMP (RT-LAMP; see Dao Thi *et al.*[3]; Rabe and Cepko[12]; Smyrlaki *et al.*[13]; Kellner MJ, Ross JJ, Schnabl J, et al., unpublished results; https://doi.org/10.1101/2020.06.23.166397; Yang W, Dang X, Wang Q, et al., unpublished results; https://doi.org/10.21203/rs.3.rs-28070/v1).

The standard LAMP primer design enables detection of sequence targets 200 base pairs (bp) or less in size. Although this has been useful in enabling detection of pathogens in recent times, we realize that detection of longer amplicons is valuable to understand RNA integrity in both clinical and environmental samples. This is especially true for environmental surveillance studies where verifying surface decontamination procedures is important. In this study, we designed and evaluated the performance of RT-LAMP to successfully amplify regions of the N gene of SARS-CoV-2 by pairing a single reverse primer set to primers at increasing lengths, from 262 to 945 bp.

MATERIALS AND METHODS

Overview of LAMP and Primer Design

The current LAMP analysis method was adapted from well-established protocols including those from New England BioLabs (Ipswich, MA, USA)[14] and Zhang *et al.*[15] LAMP primer design included using a single backward primer set described by Zhang *et al.* [loop backward

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(LB), backward inner primer (BIP), and backward outer primer (B3)], paired with a single forward primer set designed using either Primer Explorer 5.0 (Eiken Chemical Co., Tochigi, Japan) or LAMP primer design tool version 1.0.1 (New England Biolabs) to generate amplicon sizes up to 945 bp (Figure and Table 1). Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA) and were purified using standard desalting or HPLC for forward inner primer FIP and BIP.

All primers were prepared into standard stock concentration of 100 μM in RNA/DNA-free water and further diluted to a standard working 10× primer pool for each primer set as follows: FIP/BIP 16 μM, forward outer primer (F3)/B3 2 μM, and loop forward (LF)/LB at 4 μM. Additional 10× primer pools without the LF primer were also evaluated to prevent false positive colorimetric changes during the LAMP incubation. All primer pools contained the full set of BIP, B3, and BL. LAMP primers for 693 and 945 amplicons were also tested at 0.5× and 0.25× concentration (0.5–1.0 μM B3/F3, 1–2 μM LF/LB, and 4–8 μM FIP/BIP).

LAMPs were performed using the WarmStart Colorimetric LAMP 2× Master Mix (M1800) obtained from New England Biolabs using a 25 μL reaction volume as described by the manufacturer's protocol for the E2019 reagent kit with the following considerations: (1) primers' input volumes adjusted as described above, (2) use of stringency or enhancement reagents in place of the guanidine hydrochloride (GCN), or (3) substituting water for stringency or enhancement reagent. Stringency or enhancement reagents were used to enhance the reaction time and/or prevent positive colorimetric changes of the no-template controls (NTCs). These 4 enhancement reagents were evaluated: GCN[15] at 15–40 mM (included with kit), GC-Melt 5% final volume (S4854; Takara Clontech, Kusatsu, Japan), 5% DMSO, and 150 μg/mL final concentration of polyvinylsulfonic acid (PVSA; 278424; Sigma Aldrich, St. Louis, MO, USA) (Table 2).[13,16] Reactions were prepared on ice using standard thin-walled PCR tubes (0.2 or 0.5 ml) including negative NTCs and incubated at temperatures ranging from 59 to 70°C for 45 to 300 min depending on the primer set and NTC color reaction. Reactions were incubated in standard thermocyclers with heated lid 5°C above the incubation temperature (Figure 2). Initial trial runs were set up as single reactions and inspected every 15 min during incubations followed by further analysis at 2 different core facilities at either duplicate or triplicate reactions.

Reaction Conditions

Multiple reaction conditions varied for each primer evaluation depending on success. Reaction conditions for

TABLE 1

LAMP primer specifications including location, Thermal melting temperature (Tm), percent Guanine-Cytosine (GC), and sequences used in this study.

Primer Name	Type	Start position (NC_045512.2)	Tm (50 mM NaCl)	% GC	Sequence 5'–3'
B3-Rev	B3	29,299	51.8	32	AGATCCAAATTTCAAAGATCAAGTC
LB-Rev	LB	29,250	58.5	60	CTTCGGGAACGTGGTTGACC
262-F3-p	F3	28,951	54.5	45	ATTGAACCAGCTTGAGAGCA
262-LF-p	LF	29,007	55.8	45.8	CTGTCACTAAGAAATCTGCTGCTG
517-F3-n	F3	28,702	55.3	55.6	AGATCACATTGGCACCCG
517-LF-n	LF	28,752	56.1	41.7	AACTTCCTCAAGGAACAACATTGC
689-F3-n	F3	28,525	55.1	52.6	TGGCTACTACCGAAGAGCT
689-LF-n	LF	28,580	53	41.7	GATCTCAGTCCAAGATGGTATTTC
693-F3-p	F3	28,525	55.1	52.6	TGGCTACTACCGAAGAGCT
693-FL-p	LF	28,576	52.8	45.5	GAAAGATCTCAGTCCAAGATGG
943-F3-n	F3	28,285	55.6	55.6	TGGACCCCAAAATCAGCG
943-LF-n	LF	28,325	54.2	52.6	GGTGGACCCTCAGATTCAA
945-F3-p	F3	28,285	55.6	55.6	TGGACCCCAAAATCAGCG
945-FL-p	FL	28,323	57	50	TTGGTGGACCCTCAGATTCAAC
BIP-Rev	BIP		67.1	47.5	CGCATTGGCATGGAAGTCACAATTTGATGGCACCTGTGTA
262-FIP-p	FIP		70	53.7	TTGCCGAGGCTTCTTAGAAGCCAGGCCAACAACAACAAGGC
517-FIP-n	FIP		69.4	53.7	TGCTCCCTTCTGCGTAGAAGCCAATGCTGCAATCGTGCTAC
689-FIP-n	FIP		69.2	53.7	TCTGGCCCAGTTCCTAGGTAGTGACGAATTCGTGGTGGTGA
693-FIP-p	FIP		69.3	56.1	TCTGGCCCAGTTCCTAGGTAGTCCAGACGAATTCGTGGTGG
943-FIP-n	FIP		68.7	53.7	CCACTGCGTTCTCCATTCTGGTAAATGCACCCCGCATTACG
945-FIP-p	FIP		68	52.5	CCACTGCGTTCTCCATTCTGGTAAATGCACCCCGCATTAC

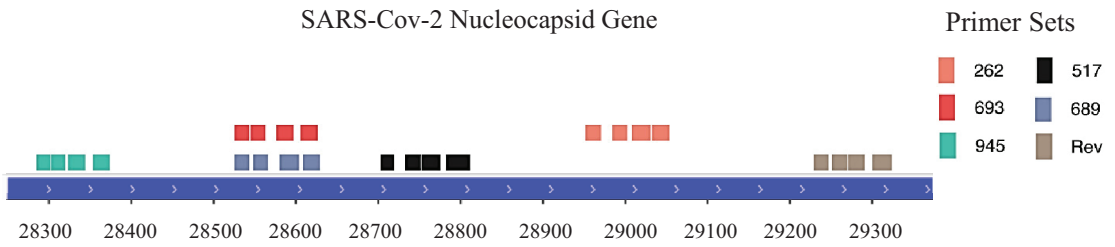


FIGURE 1

Primer binding locations on the Wuhan-Hu-1 (NC_045512.2) SARS-CoV-2 nucleocapsid gene.

primer set 262 bp consisted of 3 primer concentrations (0.25×, 0.6×, and 1×), 4 incubation temperatures (60, 62, 65, and 66°C), 2 additives (5% DMSO; 15 and 40 mM GCN), omitting LF primer, and incubation times of 30–60 min. Test conditions for primer set 517 bp included 2 primer concentrations (0.5× and 1×), 4 incubation temperatures (65, 66, 67.5, and 70°C), 2 additives (5% DMSO; 15 and 40 nM GCN), omitting LF primer, and incubation times of 40–60 min. Primer set 689 bp included 3 primer concentrations (0.5×, 0.7×, and 1×), 5 incubation temperatures (61, 62, 65, 67.5, and 70°C), 3 additives (5% DMSO; 15 and 40 mM GCN; 5% GC-Melt), omitting the LF primer, and incubation times of 45–90 min. Primer set 693 bp included 2 primer concentrations (0.7× and 1×), 6 incubation temperatures (59, 61, 62, 65, 67.5, and 70°C), 4 additives (5% DMSO; 15 and 40 mM GCN; 5% GC-Melt; 150 µg/ml PVSA), omitting the LF primer, and incubation times of 45–180 min. Primer set 945 bp included 3 primer concentrations (0.5×, 0.7×, and 1×), 6 incubation temperatures (58, 61, 61.5, 62, 65, and 66°C), 3 additives (5% DMSO; 15 and 40 mM GCN; 150 µg/ml PVSA), omitting the LF primer, and incubation times of 45–300 min.

Controls

Two positive SARS-CoV-2 controls were employed at 2 separate core facilities, including the heat-inactivated cell lysate USA-WA1/2020 from BEI Resources (NR-52286; Manassas, VA, USA) at 3750 and 1000 copies per reaction and the Twist synthetic SARS-CoV-2 RNA control 2 (MN908947; Twist Bioscience, South San Francisco, CA, USA) at 3.5×10^3 and 3.5×10^4 copies per reaction determined by droplet digital PCR (QX200; Bio-Rad, Hercules, CA, USA).

Sequence Verification

Representative positive samples (yellow colorimetric) from each primer set were subject to verification using several techniques including quantification, gel imaging, secondary PCR followed by Sanger sequencing, Illumina paired end sequencing (Illumina, San Diego, CA, USA), and Oxford Nanopore sequencing (Oxford Nanopore Technologies, Oxford, United Kingdom). All open tube reactions were handled in a biosafety level 2 (BSL-2) amplicon enhanced containment facility designed for handling highly amplified DNA products. Five microliters of each sample was purified using QIAquick PCR purification kit (P28104; Qiagen, Germantown, MD, USA) and eluted in 25 µL of elution buffer. Samples were checked for positive DNA amplification by quantifying with a Qubit spectrofluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the DNA BR and HS reagent kits (Q32850 and Q33230). Fragment analysis was determined by running samples on a fully contained 2% agarose E-GEL (G501802; Thermo Fisher Scientific) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) equipped with the DNA high-sensitivity chip (5067-4626; Agilent Technologies). PCR of diluted LAMP amplicons was performed using corresponding LF and LB primers only to generate nonconcatamer single fragment amplicons for Sanger sequencing verification. PCR was done using KAPA HiFi master mix (KK2601; Roche, Wilmington, MA, USA) according to the manufacturer's protocol with the following 3-step protocol: 95°C for 3 min; 35 cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 60 s. Resulting PCR products were evaluated on a 2% E-GEL and further verified by Sanger sequencing. Sanger sequencing was carried out using LF and LB primers using 2 approaches including 5 ng of purified amplicon RT-LAMP and ExoSAP-IT–treated single amplicon PCR products (78200; Thermo Fisher Scientific) generated from either the paired loop oligos or the B3/F3 primer pairs. Protocol for the cycle sequencing reactions included Big Dye version 3.1 and Big Dye Xterminator (ABI #4337455 and 376484) sequenced on an ABI 3130XL (Applied Biosystems, Foster City, CA, USA). Sequence contigs were assembled using ChromasPro version 2.1.10 (Technelysium, Brisbane, Australia).

Next-generation high-throughput sequencing was performed on the purified LAMP products using the Illumina

MiSeq and Oxford Nanopore GridION×5 to characterize the LAMP amplification dynamics and verify full length of cover of the higher molecule weight targets. Libraries were prepared from intentionally fragmented LAMP products to relax secondary structure from loop-generated concatemers using a Covaris S2 System (Waltham, MA, USA). Illumina MiSeq libraries were synthesized from the RhinoSeq library reagents (24-D5-D7; SeqOnce Inc., Pasadena, CA, USA) and sequenced using a MiSeq Reagent Nano Kit version 2 PE2x150 (MS-103-1001; Illumina). Oxford Nanopore libraries were generated using both the ligation library reagent and rapid DNA sequencing kits (LSK109 and RAD004). Libraries were sequenced on the GridION×5 sequencing system until $>2.0 \times 10^4$ reads were obtained for each sample (Oxford Nanopore Technologies).

Illumina and Oxford Nanopore sequences were mapped to the Wuhan-Hu-1 genome (NC_045512.2) using the Galaxy server (Penn State University, State College, PA, USA) and Integrated Genome Viewer 2.9.4 (Broad Institute, Cambridge, MA, USA) using 3 approaches: Burrows-Wheeler alignment, Minimapper2, and Bowtie.

RESULTS

Successful reactions, *i.e.*, color change seen only with positive samples and not in NTC samples, were observed for 3 of the 5 primer sets when used in combination with the colorimetric M1800 LAMP master mix. Successful primers included 262, 693, and 945 bp with different reaction protocols. Primer set 517 had no successful LAMP reactions [positive spike control turning yellow (positive) and the NTC remaining pink (negative)]. Regardless of protocol, primer set 517 generated a positive NTC reaction. Primer set 689 had 2 successful reactions out of 13 total. Although it is likely that these conditions could be optimized, this was not pursued because primer set 693 gave more favorable results. Therefore, these primer sets were omitted from further study.

Primer set 262 revealed a successful LAMP colorimetric reaction for a standard 65°C, 45 min incubation but also responded over a range of temperatures (62–66°C) and primer concentrations (0.5–1×), and with and without 40 mM GCN, the LF primer, and 5% DMSO. Primer set 689 revealed a single successful reaction condition of 61°C for 45–70 min after extensive testing and was not included in further analysis. Primer set 693 required more optimization than 262 and favored lower incubation temperatures to reduce the false positive result of the NTC. Incubation times for the lower temperatures (59–62°C) required much lower incubation times (≥150 min) but resulted in overall acceptable performance. Addition of PVSA was successfully used to prevent the a false positive color change of the NTC but also required an increased incubation time of 180 min when incubated at 61°C. Results for primer set 945 showed consistent performance at lower temperature without the use of the LF primer (prime set 945-LF) for incubations of 58–62°C for 120–130 min with the primer concentration at 0.67×. Primer concentrations and incubation temperature of 1× and 66–70°C, respectively, indicated less consistent results with false positive changes with the NTC. When 15 mM GCN was included in the reaction mix, results were achieved in 60–90 min regardless of primer concentration or presence of the LF oligo. Reaction conditions using 150 μg/ml PVSA and a 0.5× primer mix (without LF) increased the reaction time to 300 min even when incubated at 65°C but generated acceptable colorimetric results.

TABLE 2

Representative conditions from successful LAMP reactions for each primer set. Not all data are included.

LAMP primer set	Primer	Temp (C°)	Time (min)	LF primer	Additives
262 bp	**1×**	**62–66**	**45**	**+**	**—**
		65	**40**	**−**	**GCN 40 mM + 5% DMSO**
	0.5–0.7×	**62–65**	**60–65**	**+**	**—**
689 bp	0.5×	61	45–70	+	—
693 bp	1×	65	52	+	—
	0.7×	62–65	85–120	+	—
		59–61	150–180	+	—
	0.5×	61	180	+	150 μg/mL PVSA
945 bp	1×	65–66	85	−	GCN 15 mM
		58–62	90–130	+	—
	0.7×	62–65	120–130	−	—
		62–65	160–165	+	—
		61.5	130	−	—
	0.5×	65	300	−	150 μg/ml PVSA

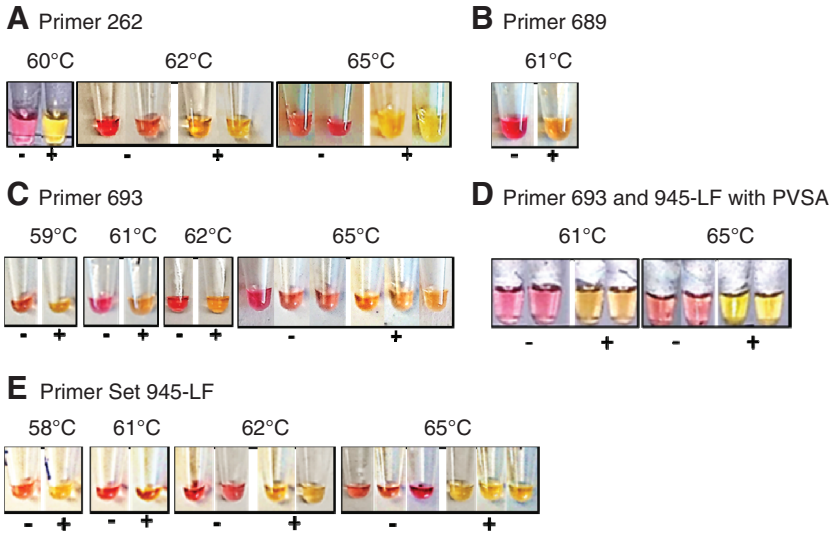


FIGURE 2

Colorimetric reactions for each primer set and additives. Incubation temperatures indicated below. *A*) Primer set 262 incubated for 60–65 min. *B*) Primer set 689 incubated 70 min. *C*) Primer set 693 incubated 105–120 min. *D*) Primer sets 693 (150 min) and 945-LF (300 min) incubated with 3.7×10^3 copies of the BEI Resources standard with 150 µg/ml of PVSA. *E*) Primer set 945 without LF incubated 120–165 min.

Amplicon Data

Positive amplicons were quantified using Qubit spectrofluorometry and evaluated for molecular weight distribution using 2% gel electrophoresis and Agilent Bioanalyzer 2100. Samples were diluted to 3 ng/µl for analysis on a Bioanalyzer DNA high-sensitivity chip. See Fig. 3. Results demonstrated the expected wide distribution of fragment sizes as described by others.[1,2,17,18] Qubit quantification of Qiagen-purified amplicon product exceeded 6 µg total, whereas NTCs were below 100–300 ng (data not shown).

Sanger Sequencing

The RT-LAMP reaction to produce a complete 262-, 693-, or 945-bp amplicon product was verified on over 90 Sanger sequencing reactions using both purified RT-LAMP product and PCR amplicons produced using the LF and LB primers. Successful Sanger sequencing (see Fig. 4) was obtained from primer set 262, which confirmed complete coverage of the 262-bp product, indicating successful RT-LAMP for that length. Results for primer sets 693 and 945-LF were unsuccessful at producing Sanger sequencing to cover the entire LAMP amplicon regardless of input template and only generated short sequences (≤50 bp) for several reactions at the ends of the amplicon near the region of the loop primers. Results for primer sets 693 and 945-LF were unusable for Sanger verification purposes.

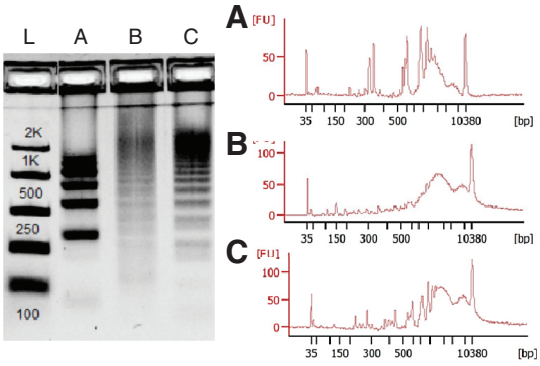


FIGURE 3

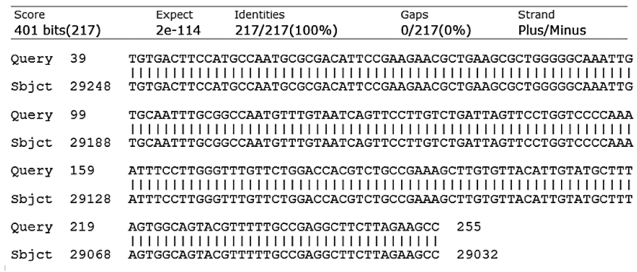
Size distribution of RT-LAMP product by 2% gel electrophoresis (left panel) and Bioanalyzer 2100 DNA high-sensitivity assessment (right panel). *A*) Primer set 262. *B*) Primer set 693. *C*) Primer set 945-LF.

Oxford Nanopore and Illumina Sequencing

Purified RT-LAMP products were sequenced using both Oxford Nanopore and Illumina sequencing to determine coverage depth across the target amplicon. Nanopore sequencing for the 262-bp LAMP product generated 2.3×10^4 reads and showed complete coverage with acceptable read depth throughout the length of the amplicon, indicating a successful RT-LAMP reaction. Sequencing results for the 693-bp LAMP product were generated from 1.0×10^5 Illumina reads and 3.9×10^3 nanopore reads and showed inconsistent but full coverage across the amplicon with most reads mapping to the ends of the amplicon and limited coverage depth between LF and LB primers. Sequencing results for the 945-LF LAMP product were generated from 1.05×10^5 Illumina reads and 3.7×10^4 nanopore reads, which mapped almost exclusively to the 3' and 5' end of the LAMP product. Full coverage across the amplicon product was not observed, with limited reads mapping between the LF and LB primer location, indicating incomplete synthesis and amplification (see Fig. 5).

DISCUSSION

RT-LAMP is a very powerful and fast diagnostic tool with many factors governing its performance and success. In this



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401 bits(217)	2e-114	217/217(100%)	0/217(0%)	Plus/Minus

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Query  99     TGCAATTTGCGGCCAATGTTTGTAATCAGTTCCTTGTCTGATTAGTTCCTGGTCCCCAAA
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Query  219    AGTGGCAGTACGTTTTTGCCGAGGCTTCTTAGAAGCC  255
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Sbjct  29068  AGTGGCAGTACGTTTTTGCCGAGGCTTCTTAGAAGCC  29032
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FIGURE 4

Alignment of Sanger sequence data for amplicons generated with 262-bp LF primer to the National Center for Biotechnology Information accession NC_045512.2 of SARS-CoV-2 Wuhan-Hu-1.

study we characterized the effect of primer design, reaction conditions, and the ability to amplify long RNA fragments (up to 1 kbp) for SARS-CoV-2 detection to provide possible information on RNA integrity using the colorimetric WarmStart RT-LAMP reagent master mix as the base.[14] This reagent contains Bst 2.0 DNA polymerase, RTx reverse transcriptase, and phenol red as the visible pH indicator. Data showed that 3 out of 7 primers sets generated successful colorimetric reactions when challenged with 2 positive controls. Primer sets included 2 designs for each amplicon size of ~250 bp, ~690 bp, and ~945 bp that was used in combination with enhancement reagents and a range of incubation temperatures. Four of the 7 primer sets failed to produce a negative colorimetric reaction for the NTC and were not pursued. The 3 primer sets that produced successful colorimetric results were further characterized using next-generation sequencing to understand the LAMP dynamics and whether the entire amplicon was evenly represented. Sequencing results suggest that larger RT-LAMP amplicons are not amplified evenly and have excessive loop end amplification, as seen with the 693-bp and 945-bp LAMP reactions. This phenomenon is likely

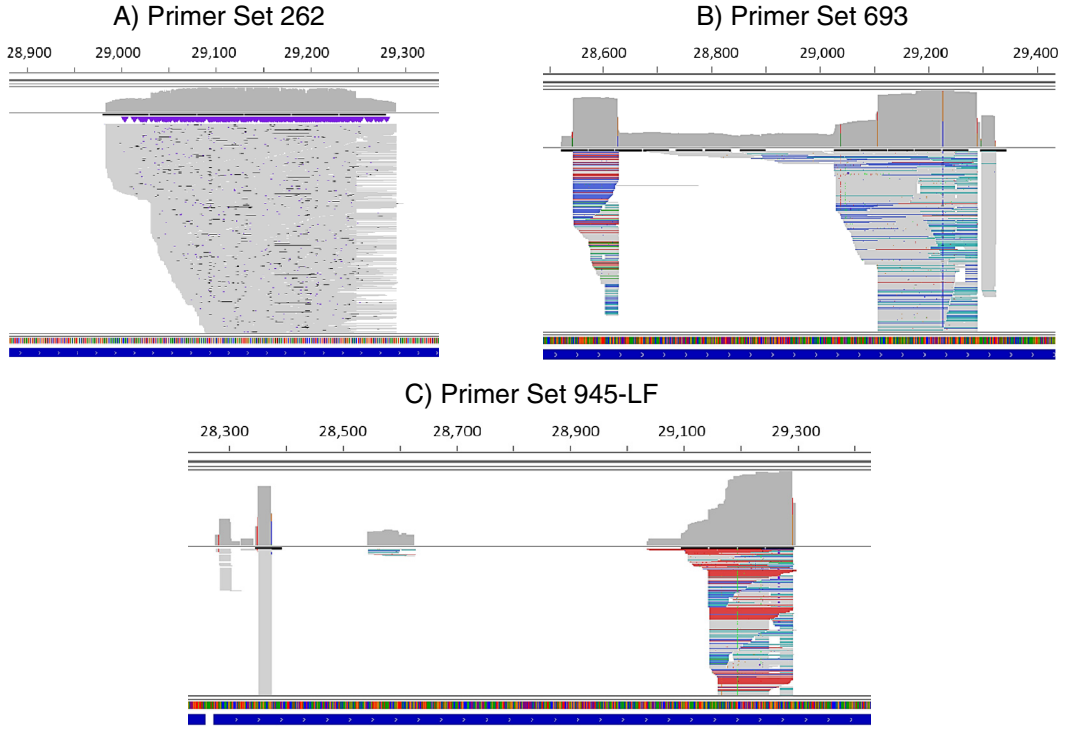

FIGURE 5

Alignment of the 3 extended-length COVID-19 RT-LAMP amplicon products against the SARS-CoV-2 Wuhan-Hu-1 (NC_045512.2) genome. *A*) Illumina sequence mapping for the 262-bp RT-LAMP product. *B*) Nanopore sequence mapping for primer set 693. C) Nanopore sequence mapping for primer set 945-LF.

a function of the LAMP chemistry master mix (reverse transcriptase and DNA polymerase), primer design, and reaction conditions. Although our data indicate that amplicons larger than 250 bp are clearly achievable, further optimization is needed to achieve higher molecular weights. It is currently unknown what the amplicon size limits are, but these data suggest 500–600 bp using the current chemistry and conditions. Although developments in LAMP chemistries continue to be reported (*e.g.*, Rohatensky *et al.*[19]; Paik I, Ngo PHT, Shroff R, *et al.*, unpublished results; https://doi.org/10.1101/2021.04.15.439918), future innovations will be necessary to advance the technology to a point of achieving rapid detection combined with assessing RNA integrity in one simple assay. Such a technique could provide researchers with an additional tool to help discriminate intact viruses from remnant RNA in samples that require additional biomolecular resolution.

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