RT-LAMP of clinical SARS-CoV-2 RNA using SuperScript IV RT-LAMP Master Mix

Introduction

The global crisis caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) demonstrated a massive demand for fast pathogen detection and surveillance methods. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a technology that allows fast and specific viral RNA amplification in 5–30 minutes under isothermal conditions. Typical RT-LAMP reactions include 4–6 target-specific primers, which facilitate formation of loop structures and promote faster amplification, a reverse transcriptase for reverse transcription (RT) of RNA to cDNA, and a DNA polymerase with strong strand-displacing activity. Target amplification is performed at a constant temperature (e.g., 65°C), hence simple equipment such as a heat block can be used for incubation of reactions.

Here we demonstrate the use of Invitrogen™ SuperScript™ IV RT-LAMP Master Mix and a custom-designed RT-LAMP primer set for fast detection of SARS-CoV-2 RNA following RNA purification from clinical samples. Average time-to-signal was less than 10 minutes regardless of RNA input amount. The current protocol demonstrates compatibility of SuperScript IV RT-LAMP Master Mix with real-time fluorescence detection using Invitrogen™ SYTO™ 9 stain. Also demonstrated is endpoint detection, which includes visual evaluation of a color change in the reaction mix using Invitrogen™ SYBR™ Green I stain or visual evaluation of RT-LAMP products using Invitrogen™ E-Gel™ precast gels.

Cleaning instructions

To minimize the risk of environment-borne contamination, clean the laboratory workspace and all equipment thoroughly prior to setting up RT-LAMP reactions, using the following reagents in the given order:

1. Invitrogen™ DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890)
2. Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977023 and Cat. No. 10977049)
3. 70% ethanol solution
4. Invitrogen™ RNase*Zap*™ RNase Decontamination Solution (Cat. No. AM9780)
5. 70% ethanol solution

Prior to handling RNA samples, spray RNase*Zap* RNase Decontamination Solution on gloved hands, and wipe down instruments, pipettors, and other surfaces using Invitrogen™ RNase*Zap*™ RNase Decontamination Wipes (Cat. No. AM9786) to avoid liquid contact with sensitive parts (e.g., electronic control modules).

Important notes

- Use separate workspaces as well as specially dedicated equipment and supplies for:
  - RT-LAMP reaction setup and amplification
  - Analysis of RT-LAMP products by endpoint detection
- Opening of tubes or wells after RT-LAMP in the separate post-reaction area helps eliminate risks of environmental contamination.
- No-template control (NTC) and/or other negative control reactions are strongly recommended, in order to confirm absence of background amplification.
- Close tubes with NTC before adding target RNA to minimize risks of sample cross-contamination.
- Prior to each real-time RT-LAMP experiment, prepare a fresh 50 µM solution of SYTO 9 Green Fluorescent Nucleic Acid Stain by 1:100 dilution of 5 mM SYTO 9 stock solution.
- For evaluation of RT-LAMP amplicons by agarose gel electrophoresis, 10-fold dilution of RT-LAMP samples stained with SYBR Green I stain (Cat. No. S7567; 10,000X stock) is recommended. If RT-LAMP reactions are not stained, a 5-fold dilution is enough to visualize a ladder-like pattern on an E-Gel precast gel.

Materials

- SuperScript IV RT-LAMP Master Mix (Cat. No. A51801)
- SYTO 9 Green Fluorescent Nucleic Acid Stain (Cat. No. S34854)
- Customized RT-LAMP primer set used from Thermo Scientific™ Colorimetric ReadiLAMP™ Kit, SARS-CoV-2 (Cat. No. A52539)
- Thermo Scientific™ Water, nuclease-free (Cat. No. R0581)
- SYBR Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO (Cat. No. S7567)
- Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder (Cat. No. 10488091)
- Invitrogen™ E-Gel™ Double Comb Agarose Gels with SYBR™ Safe DNA Gel Stain, 2% (Cat. No. A42348)
- 70% ethanol solution
- DNA*Zap* PCR DNA Degradation Solutions (Cat. No. AM9890)
- RNase*Zap* RNase Decontamination Solution (Cat. No. AM9780)
- Twist Synthetic SARS-CoV-2 RNA Control 1 (Twist Bioscience, Cat. No. 102019)

Methods

RT-LAMP reaction setup

The reaction mix was prepared on ice according to Table 1. Total RT-LAMP reaction volume was 25 µL. Usage of Eppendorf™ DNA LoBind™ 1.5 mL nuclease-free, nonstick tubes along with sterile filtered pipette tips helped prevent both RNA degradation and aerosol contamination for maximum performance of the reaction. When real-time RT-LAMP was performed, SYTO 9 Green Fluorescent Nucleic Acid Stain was added to the reaction mix.

Table 1. Reaction setup.

Component of the reaction mix	Detection of target RNA	No-template control (NTC)	Final concentration
SuperScript IV RT-LAMP Master Mix	12.5 µL	12.5 µL	1X
40 µM FIP/BIP primers	1 µL each	1 µL each	1.6 µM
10 µM F3/B3 primers	0.5 µL each	0.5 µL each	0.2 µM
10 µM LoopF/LoopB primers	1 µL each	1 µL each	0.4 µM
SYTO 9 nucleic acid stain solution, 50 µM (freshly made)	2.5 µL	2.5 µL	5 µM
Nuclease-free water	4 µL	5 µL	–
NTC tubes or wells were closed prior to adding target RNA			
Purified clinical or synthetic viral RNA	1 µL	–	–
Total volume	**25 µL**	**25 µL**	**–**

* For real-time fluorescence detection only

Real-time RT-LAMP

Real-Time RT-LAMP detection of SARS-CoV-2 was performed using Applied Biosystems™ QuantStudio™ 5, 6 Flex, and 7 Flex Real-Time PCR Systems, where a 1-step cycling protocol was applied (Table 2). Reaction kinetics and specificity of amplified products were analyzed using appropriate programs, i.e., Applied Biosystems™ QuantStudio™ Real-Time PCR Software for QuantStudio 6 Flex and 7 Flex systems and Applied Biosystems™ Design and Analysis 2 Software for the QuantStudio 5 system. Specificity of the real-time RT-LAMP amplicons was determined by melting curve analysis.

Table 2. One-step cycling protocol.

Number of cycles	Step	Temperature	Time
60	Amplification	65°C	30 sec
1	Inactivation	95°C	2 min
–	Melt curve	60–95°C	–

Endpoint detection

Visual evaluation and gel analysis of RT-LAMP products was performed using the same reaction setup as described in Table 1, except that SYTO 9 stain was replaced with nuclease-free water. Reactions were incubated at 65°C for 15 minutes on the preheated block and subsequently inactivated on a separate preheated block at 95°C for 2 minutes. For the color change evaluation by the naked eye, 1 µL of 10-fold diluted SYBR Green I stain (Cat. No. S7567; 10,000X stock) was added to each reaction tube. To prevent contamination with amplicons from the positive reaction tubes, negative control reactions were stained first. For the same reason, one tube was opened at a time and closed quickly after adding SYBR Green I stain. Bright green color corresponded to successful target amplification, whereas orange color indicated no amplification. In addition, RT-LAMP amplicons were analyzed by agarose gel electrophoresis using E-Gel Double Comb 2% Agarose Gels with SYBR Safe DNA Gel Stain (Cat. No. A42348) and the Invitrogen™ E-Gel™ Power Snap Electrophoresis System to visualize the typical RT-LAMP ladder-like pattern. For the RT-LAMP reaction samples initially stained with SYBR Green I stain, a 10-fold dilution was used (2 µL of the sample mixed with 18 µL of nuclease-free water). E-Gel 1 Kb Plus Express DNA Ladder was diluted by adding 10 µL of the ladder into 10 µL of nuclease-free water. Prepared ladder and samples (20 µL) were transferred into wells of an E-Gel Double Comb 2% Agarose Gel, starting with the negative control reactions. NTC and negative test samples were loaded into separate lanes on the same gel (distanced from positive test samples) to minimize risk of contamination.

Results and discussion

Real-time detection of RT-LAMP products and analysis of reaction kinetics

The sensitivity of SuperScript IV RT-LAMP Master Mix was evaluated by testing purified RNA from 57 clinical samples. Those samples were received from individuals who tested positive for SARS-CoV-2 by reverse transcription quantitative PCR (RT-qPCR). RT-LAMP amplification speed was determined using a real-time RT-LAMP reaction setup that included SYTO 9 stain. Commercial synthetic SARS-CoV-2 RNA (Twist Bioscience) was used as a positive control. The following formula was used to calculate sensitivity:

$$\text{Sensitivity (\%)} = \frac{\text{100 x true positives}}{\text{(true positives + false negatives)}}$$

A tested clinical sample was considered as a true positive if the specific amplification signal occurred under 30 minutes in 75% of all technical repeats. SuperScript IV RT-LAMP Master Mix showed 100% sensitivity and average amplification speeds of less than 10 minutes regardless of RNA input amount (Figure 1).

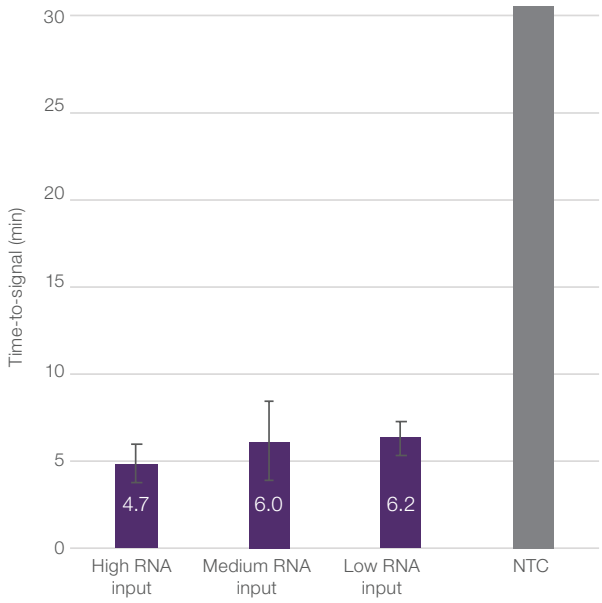

Figure 1. Amplification of SARS-CoV-2 RNA via real-time RT-LAMP using SYTO 9 stain. Amplification kinetics were demonstrated using low (n = 18), medium (n = 18), or high (n = 21) RNA input, where amount of RNA from each clinical sample was determined by RT-qPCR. Error bars represent standard deviation of reaction speed (time-to-signal) values calculated from multiple technical repeats (n = 3). Regardless of RNA input amount, SuperScript IV RT-LAMP Master Mix amplified SARS-CoV-2 RNA in less than 10 minutes. No template control (NTC) signal was not detected under 30 minutes.

Correlation between SuperScript IV RT-LAMP Master Mix reaction speed (time-to-signal) and SARS-CoV-2 RNA copy number in clinical samples was evaluated by plotting RNA copy number determined by RT-qPCR versus RT-LAMP reaction speed (Figure 2). SuperScript IV RT-LAMP Master Mix demonstrated strong correlation between RT-LAMP speed and RNA template copy number (R^2 = 0.970) with *p*-value for the *F* statistic equal to 0.001. However, the variation in RT-LAMP reaction speed between ~300 and ~400,000 RNA copy number is minimal (2 minutes).

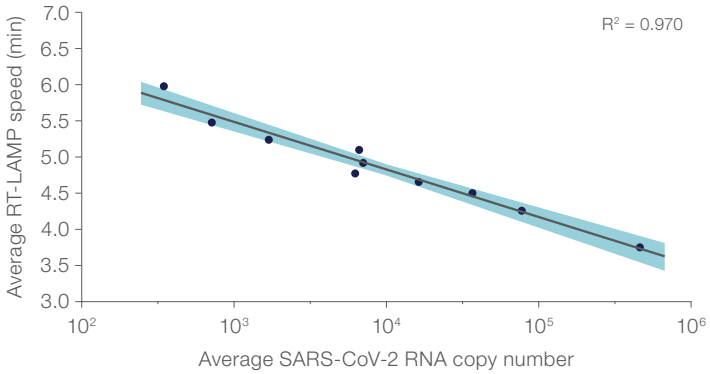

Figure 2. Correlation of RT-LAMP reaction speed and SARS-CoV-2 RNA copy number in clinical samples. Average RT-LAMP reaction speed was calculated from multiple technical repeats (n = 3). Commercial synthetic SARS-CoV-2 RNA (Twist Bioscience) was used to generate a standard curve for quantitating SARS-CoV-2 RNA in clinical samples by RT-qPCR. Average SARS-CoV-2 RNA copy number was calculated from multiple technical repeats (n = 3).

The specificity of SuperScript IV RT-LAMP Master Mix was estimated by performing real-time RT-LAMP with purified RNA from 32 clinical samples, received from individuals who tested negative for SARS-CoV-2 by RT-qPCR. The same custom-designed SARS-CoV-2 primer set mentioned previously was used during the reaction setup. The following formula was used to calculate specificity:

$$\text{Specificity (\%)} = \frac{\text{100 x true negatives}}{\text{(true negatives + false positives)}}$$

SuperScript IV RT-LAMP Master Mix demonstrated 100% specificity with 32/32 true negative results. Late nonspecific amplification signal was observed after 50 minutes, similar to the NTC (Table 3). A tested clinical sample was considered a true negative if the amplification signal occurred after 30 minutes in 75% of all technical repeats.

Table 3. Results of real-time RT-LAMP performed with clinical samples that tested negative for SARS-CoV-2.

Template	Average time-to-signal (min)
Clinical samples (SARS-CoV-2 negative)	56.8
No template control (NTC)	55.4
Commercial SARS-CoV-2 RNA positive control	4.9

Endpoint detection of RT-LAMP products

For endpoint detection, 36 positive and 28 negative SARS-CoV-2 clinical samples were tested. Synthetic SARS-CoV-2 RNA (5,000 copies) was utilized as a positive control. SYBR Green I Nucleic Acid Gel Stain was used to induce a color change for visual evaluation of the reaction outcome. An orange color indicated no amplification, whereas a bright green color demonstrated presence of RT-LAMP amplicons. Additionally, samples were analyzed by agarose gel electrophoresis. A signature ladder-like band pattern showed the presence of RT-LAMP products (Figure 3).

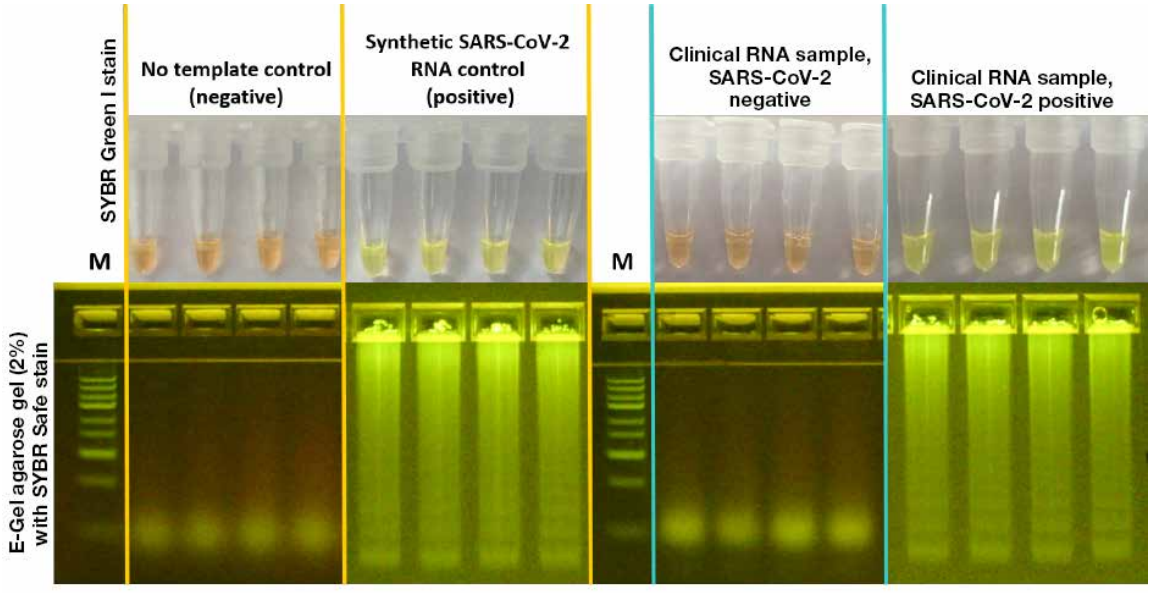

Figure 3. Endpoint analysis of RT-LAMP results using SYBR Green I Nucleic Acid Gel Stain for the naked-eye detection and ladder-like pattern visualization on agarose gels with the E-Gel Power Snap Electrophoresis System. Purified RNA from SARS-CoV-2 negative and positive clinical samples was used along with a no template control and a commercial synthetic SARS-CoV-2 RNA positive control. Representation of one negative and one positive clinical RNA sample is demonstrated. Upper row: Color changes induced by SYBR Green I stain after addition of 1 µL of the diluted stain into each reaction tube (bright green color: RT-LAMP product; orange color: no amplification). Lower row: Successful RT-LAMP verified by visualization of a ladder-like pattern in the E-Gel Double Comb Agarose Gel (2%) with SYBR Safe DNA Gel Stain. Lane M contains E-Gel 1 Kb Plus Express DNA Ladder.

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Conclusions

SuperScript IV RT-LAMP Master Mix demonstrated outstanding reaction speed by amplifying SARS-CoV-2 target RNA purified from clinical samples in less than 10 minutes. It also demonstrated 100% specificity (no false positive results) when clinical samples that were previously confirmed negative for SARS-CoV-2 were tested using target-specific primer sets. Nonspecific amplification signal was detected only after 50 minutes in these reactions, suggesting that specific assays with short total reaction time could be designed using this product. Even though RT-LAMP reaction speed correlates with RNA input amount, the variation between amplification of minimum and maximum copies tested was within 2 minutes. Excellent compatibility with both real-time and endpoint detection methods provides new opportunities for SuperScript IV RT-LAMP Master Mix to be implemented during development of highly accurate assays for detection of viral pathogens.

Ordering information

Product	Cat. No.
DNA*Zap* PCR DNA Degradation Solutions	AM9890
UltraPure DNase/RNase-Free Distilled Water	10977023
RNase*Zap* RNase Decontamination Solution	AM9780
RNase*Zap* RNase Decontamination Wipes	AM9786
SuperScript IV RT-LAMP Master Mix	A51801
SYTO 9 Green Fluorescent Nucleic Acid Stain	S34854
Water, nuclease-free	R0581
SYBR Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO	S7567
E-Gel Double Comb Agarose Gels with SYBR Safe DNA Gel Stain, 2%	A42348
E-Gel 1 Kb Plus Express DNA Ladder	10488091

Find out more at **thermofisher.com/rtlampmastermix**

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