Optimized real-time and endpoint RT-LAMP for SARS-CoV-2 using *Bsm* DNA Polymerase and SuperScript IV Reverse Transcriptase

Introduction

Loop-mediated isothermal amplification (LAMP) is a technique that utilizes a DNA polymerase with a strong strand displacement activity to amplify DNA under isothermal conditions. Specially designed primer sets recognize distinct target DNA regions to form loop structures that facilitate amplification. To detect RNA targets, a reverse transcriptase is included in the reaction for reverse transcription (RT) of RNA to cDNA.

The current RT-LAMP protocol utilizes Thermo Scientific™ *Bsm* DNA Polymerase and Invitrogen™ SuperScript™ IV Reverse Transcriptase for faster detection of viral RNA in purified samples. This application note presents potential optimization steps for an **RT-LAMP protocol** and demonstrates detection of a synthetic SARS-CoV-2 viral RNA target in under 30 min. Results can be analyzed immediately using several methods, including visual assessment with Invitrogen™ SYBR™ Green I Nucleic Acid Gel Stain (which induces a color change visible to the naked eye), gel electrophoresis using Invitrogen™ E-Gel™ precast agarose gels, or real-time detection with Invitrogen™ SYTO™ 9 Green Fluorescent Nucleic Acid Stain.

Important notes

- To minimize the risk of environment-borne contamination, clean the laboratory workspace and all equipment thoroughly, using the following reagents in the given order. The cleaning protocol needs to be applied prior to and after each experiment:

1. Invitrogen™ DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890)
2. Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977023)
3. 70% ethanol solution
4. Invitrogen™ RNase*Zap*™ RNase Decontamination Solution (Cat. No. AM9784)
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).
- RNA sample preparation, assay setup, and visualization should be performed in separate areas.
- Negative control reactions are recommended (e.g., no-template control, nontarget template control) to ensure amplification specificity.
- Prior to each experiment, prepare a 50 µM solution of SYTO 9 Green Fluorescent Nucleic Acid Stain in nuclease-free water in an amber microcentrifuge tube to protect it from light.
- For analysis in E-Gel agarose gels, 4-fold dilution of RT-LAMP samples is recommended for optimal visualization.

Materials

- *Bsm* DNA Polymerase, Large Fragment (Cat. No. EP0691)
- SuperScript IV Reverse Transcriptase (Cat. No. 18090050)
- Primer set (from Park et al. [1])
- 10X *Bsm* Buffer (included in Cat. No. EP0691)
- Thermo Scientific™ dNTP Mix (10 mM each, Cat. No. R0191)
- Thermo Scientific™ $MgCl_2$ (25 mM, Cat. No. R0971)
- SYTO 9 Green Fluorescent Nucleic Acid Stain (Cat. No. S34854)
- SYBR Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO (Cat. No. S7567)
- Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor (Cat. No. 10777019)
- DNA*Zap* PCR DNA Degradation Solutions (Cat. No. AM9890)
- RNase*Zap* RNase Decontamination Solution (Cat. No. AM9784)
- E-Gel Agarose Gels with SYBR Safe DNA Gel Stain, 2% (Cat. No. A42135)
- E-Gel 1 Kb Plus Express DNA Ladder (Cat. No. 10488091)

Purification of total RNA

- For sample preparation, use the Applied Biosystems™ MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Cat. No. A42352) and follow the recommended protocol.

Methods

The reaction mix was set up on ice in the order listed in Table 1, using Eppendorf™ DNA Lo-Bind 1.5 mL nuclease-free tubes and filter tips to minimize RNA degradation.

Real-time RT-LAMP was done on an Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System using a 1-step cycling protocol (Table 2). Applied Biosystems™ QuantStudio™ Real-Time PCR Software was used for data analysis. Visual evaluation and gel analysis of RT-LAMP products were performed using the same reaction setup, except that STYO 9 stain was replaced with water. After thermal cycling, reaction mixes were evaluated by the naked eye after addition of SYBR Green I nucleic acid stain, or by electrophoresis on E-Gel agarose gels containing SYBR Safe stain.

Table 2. Cycling protocol.

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

Table 1. Reaction setup.

Component	Volume	Final concentration
RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/µL stock)	1 µL	1.6 U/µL
10X *Bsm* Buffer	2.5 µL	1X
25 mM $MgCl_2$	4 µL	4 mM*
10 mM dNTP mix	3.5 µL	1.4 mM each
40 µM FIP/BIP primers	1 µL each	1.6 µM
10 µM F3/B3 primers	0.5 µL each	0.2 µM
10 µM LoopF/B primers	1 µL each	0.4 µM
Nuclease-free water	4.25 µL	–
50 µM SYTO 9 stain	2.5 µL	5 µM
8 U/µL *Bsm* DNA Polymerase	1 µL	0.32 U/µL
200 U/µL SuperScript IV Reverse Transcriptase	0.25 µL	2 U/µL
RNA template	1 µL	Variable
Total reaction volume	**25 µL**	–

* An additional 2 mM of Mg^{2+} comes from the 10X *Bsm* Buffer. The final concentration of Mg^{2+} is 6 mM.

Results

Real-time detection of RT-LAMP products

To assess whether the RT-LAMP protocol could be used for viral RNA detection, a synthetic truncated SARS-CoV-2 RNA fragment (a part of the coding region in the spike gene) was used along with verified LAMP primers [1]. The QuantStudio 7 Flex Real-Time PCR System was used to determine reaction kinetics. Reaction speed was evaluated using serially diluted samples of the template RNA, ranging from 10^3 to 10^5 copies per reaction.

The SARS-CoV-2 RNA target was amplified to near completion in 25–30 min, depending on copy number input (Figure 1). As expected, amplification was initiated faster when the template input was higher (10^5 copies: 19 min; 10^4 copies: 21 min; 10^3 copies: 23 min).

The RT-LAMP protocol was tested using lower concentrations of SuperScript IV Reverse Transcriptase (total reaction volume remained at 25 µL). Lowering the final concentration of the reverse transcriptase from 2 U/µL (50 U/reaction) to 0.4 U/µL (10 U/reaction) or 0.2 U/µL (5 U/reaction) had little effect on detection time (22 min for both concentrations, vs. 19 min; Figure 2), and the results did not show a statistical difference. Therefore, using lower concentrations of SuperScript IV Reverse Transcriptase (10 or 5 U/reaction) is recommended when preparing a sample set of more than 20 test samples, and when reaction setup conditions involve greater risk of contamination. Lower concentrations of SuperScript IV Reverse Transcriptase minimize the risk of nonspecific amplification occurring in negative control reactions.

Visual evaluation and gel analysis of RT-LAMP products

The RT-LAMP protocol was tested using SYBR Green I nucleic acid stain to induce a color change for visual evaluation by the naked eye. RT-LAMP endpoint analysis was performed using the Invitrogen™ E-Gel™ Power Snap Electrophoresis System to visualize the ladder-like profile of RT-LAMP amplicons. The RT-LAMP reaction was performed as described previously (without SYTO 9 stain). A 24 µL reaction mixture was aliquoted into a 96-well PCR plate, and 1 µL of diluted sample or control was added. After the reaction, 1 µL of diluted (1:10) SYBR Green I stain was added to each well, and a color change was observed for the sample. To minimize the risk of cross-contamination, the stain was first added to the negative control and then to the rest of the samples. An orange color indicated no amplification, while a bright green color indicated the presence of RT-LAMP product (Figure 3, upper panel). Additionally, samples were analyzed by agarose gel electrophoresis. RT-LAMP products were diluted 4-fold by adding 5 µL of sample to 15 µL of nuclease-free water in 0.2 mL RNase-free PCR tubes. Samples were briefly mixed by flicking and loaded into wells of a 2% E-Gel agarose gel with SYBR Safe DNA gel stain. A ladder-like band pattern in the gel represented RT-LAMP products (Figure 3, lower panel).

Summary

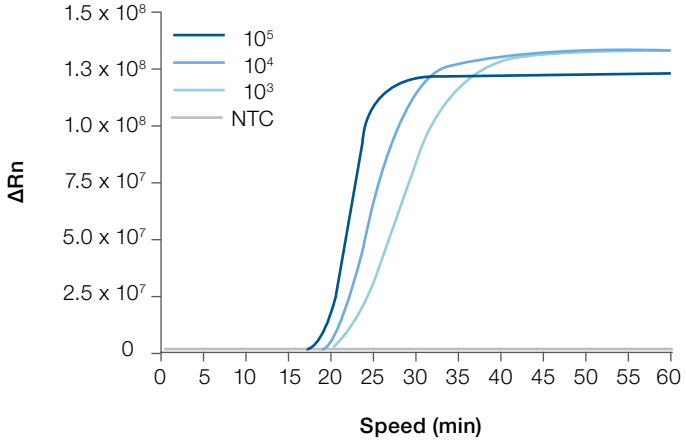

Figure 1. SARS-CoV-2 RNA amplification via real-time RT-LAMP using SYTO 9 stain. Amplification kinetics were demonstrated using 10^5, 10^4, and 10^3 copies of synthetic template RNA per reaction. Multiple technical repeats (n = 4) were performed for each RNA copy number, and data were plotted as average values (blue curves). No-template control (NTC) amplification signal was not detected (gray curve).

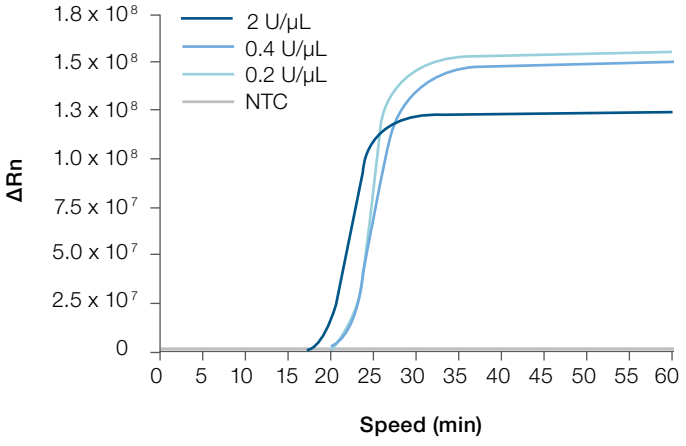

Figure 2. Detection of SARS-CoV-2 RNA using reduced concentrations of SuperScript IV Reverse Transcriptase. When testing samples containing 10^5 copies of synthetic SARS-CoV-2 RNA, decreasing the reverse transcriptase concentration to 0.4 U/µL or 0.2 U/µL had minimal effect on detection time, compared to 2 U/µL.

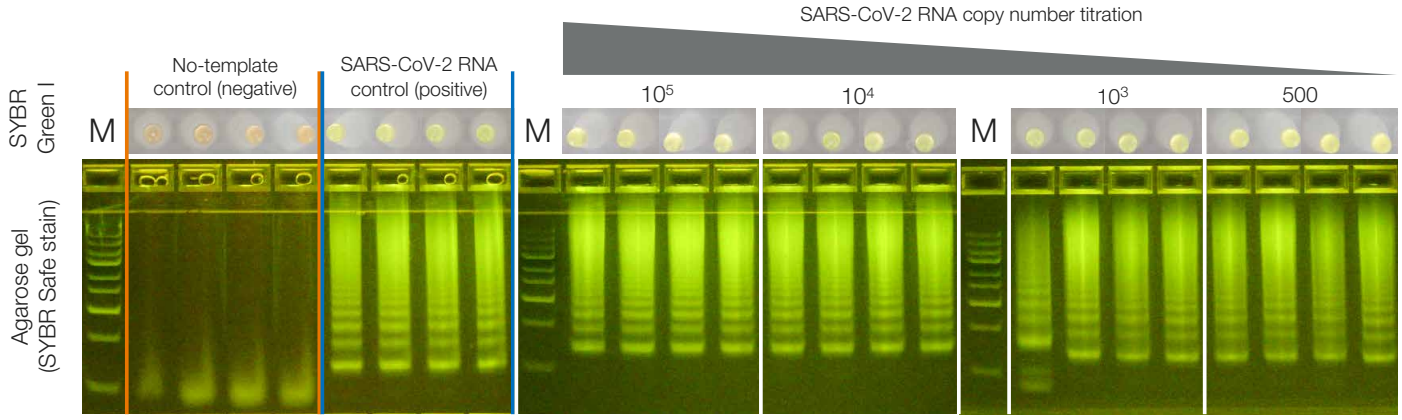

Figure 3. Endpoint analysis of RT-LAMP results using SYBR Green I nucleic acid stain for naked-eye detection via color change as well as ladder-pattern visualization on agarose gels with the E-Gel Power Snap Electrophoresis System. RT-LAMP reactions with 500 to 10^5 copies of the RNA template were used. The SARS-CoV-2 RNA control (positive) is available from Twist Bioscience. **Upper row:** Color changes indicated by SYBR Green I stain after addition of 1 µL of diluted stain to each well (orange: no amplification; green: presence of RT-LAMP product). **Lower row:** Verification of successful RT-LAMP profiles by electrophoresis of 4-fold diluted samples on an E-Gel Agarose Gel with SYBR Safe DNA Gel Stain, and detection of signature ladder-like band patterns of LAMP products. The E-Gel 1 Kb Plus Express DNA Ladder was used as a size standard (M).

RT-LAMP using *Bsm* DNA Polymerase and SuperScript IV Reverse Transcriptase enables detection of SARS-CoV-2 RNA in 25–30 min when using purified RNA samples containing as few as 500 copies. SYBR Green I nucleic acid stain allows for quick and easy endpoint analysis of RT-LAMP reactions by the naked eye, via observation of an immediate color change. RT-LAMP amplicon profiles can be verified using the E-Gel Power Snap Electrophoresis System. The compatibility of RT-LAMP with real-time analysis on the QuantStudio 7 Flex Real-Time PCR System using SYTO 9 nucleic acid stain was also demonstrated.

Additional notes

Results demonstrated in this application note were achieved under ideal conditions for experiment setup. Special care must be taken to prepare and clean the workspace to make sure cross-contamination of negative control samples, or carryover contamination of test samples, is completely avoided. Reactions should be set up in a clean laminar flow chamber, using clean pipettors and filter tips. Negative control samples (e.g., NTCs) must be prepared first and tubes tightly closed before handling any test RNA samples. Otherwise, the amplification signal in negative control samples may be detected after >30 minutes via real-time RT-LAMP analysis.

Ordering information

Product	Cat. No.
Bsm DNA Polymerase, large fragment (8 U/µL)	EP0691
SuperScript IV Reverse Transcriptase	18090050
$MgCl_2$ (magnesium chloride) (25 mM)	R0971
dNTP Mix (10 mM each)	R0191
UltraPure DNase/RNase-Free Distilled Water	10977023
DNA*Zap* PCR DNA Degradation Solutions	AM9890
RNase*Zap* RNase Decontamination Solution	AM9784
RNase*Zap* RNase Decontamination Wipes	AM9786
RNaseOUT Recombinant Ribonuclease Inhibitor	10777019
SYBR Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO	S7567
SYTO 9 Green Fluorescent Nucleic Acid Stain	S34854
E-Gel Agarose Gels with SYBR Safe DNA Gel Stain, 2%	A42135
E-Gel 1 Kb Plus Express DNA Ladder	10488091
MagMAX Viral/Pathogen Nucleic Acid Isolation Kit	A42352

Custom DNA primers are available in several formats, and are synthesized using our state-of-the-art platforms with rigorous quality control. For more information on designing and ordering primers, go to **thermofisher.com/primers**.

Reference

1. Park GS et al. (2020) Development of reverse transcription loop-mediated isothermal amplification assays targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *J Mol Diagn* 22:729–735.

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

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