Lucigen®
Simplifying Genomics

LavaLAMP™ RNA Component Kit

Please read carefully and thoroughly before beginning

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the user supplied reagents are of high quality. Please follow the instructions carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

Lucigen Technical Support:
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Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment. Please avoid using reagents greater than one year from date of receipt.

Product Description

The LavaLAMP™ RNA Component Kit is intended to simplify development and optimization of RNA loop-mediated isothermal amplification (LAMP) reactions. This kit is for research purposes only, and available under the limited-use license described at the end of this document.

LAMP commonly employs a set of six primers (F3, B3, Loop-F, Loop-B, FIP, and BIP), which must be supplied by the user. Previously-established primer designs may be used. Alternatively, Lucigen recommends use of the free Eiken web utility or the commercially available LAMP Design Software from Premier BioSoft to design new primer sets (see Appendix A). Not all primer sets identified by these programs are guaranteed to work with LavaLAMP™ RNA Component Kit or any other LAMP system. We strongly encourage designing multiple primer sets to identify the best performing set. We also highly recommend inclusion of loop primers (Loop-F and Loop-B; Nagamine, 2002) to improve assay performance.

LavaLAMP™ RNA amplification products may be detected by agarose gel electrophoresis or by real-time/end-point monitoring with fluorescent double-stranded DNA-binding dyes, such as the Lucigen Green Fluorescent Dye (Cat. No. 30078-1). Turbidity may also be monitored to assess amplification (Mori, 2001), but this method is less sensitive.

Product Designations and Kit Components

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume
LavaLAMP™ RNA Component Kit	500 Reactions	30096-1	10X LavaLAMP™ RNA Buffer	F824299-1	1.25 mL
			LavaLAMP™ RNA Enzyme	F834300-1	500 µL
			Magnesium Sulfate, 100 mM	F88695-2	1.5 mL
			RNA Positive Control LAMP Primer Mix	F814233-1	25 µL
			RNA Positive Control	F824232-1	10 µL

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume
LavaLAMP™ RNA Component Kit with Dye	500 Reactions	30097-1	10X LavaLAMP™ RNA Buffer	F824299-1	1.25 mL
			LavaLAMP™ RNA Enzyme	F834300-1	500 µL
			Magnesium Sulfate, 100 mM	F88695-2	1.5 mL
			RNA Positive Control LAMP Primer Mix	F814233-1	25 µL
			RNA Positive Control	F824232-1	10 µL
			Green Fluorescent Dye	F883827-2	500 µL

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume
Green Fluorescent Dye	500 Reactions	30078-1	Green Fluorescent Dye	F883827-2	500 µL

Components and Storage

Store all kits and components at -20 °C

Materials Supplied by the User

- 10X target-specific LAMP primer mix
  - 2 µM each F3 and B3 primers
  - 8 µM each Loop-F and Loop-B primers
  - 16 µM each FIP and BIP primers

  ***Note**: See RNA LAMP Reaction Optimization section for additional details on primers*
- dNTP Mix, 25 mM each
- Target RNA
- Thermocycler or heat block (Lucigen recommends using calibrated instruments.)

  Notes:
  - *Recommend using instruments with heated lids*
  - *Recommend using calibrated instruments*
- **Optional**: Use of the Green Fluorescent Dye (Cat. No. 30078-1) for detection of amplified DNA requires a real-time amplification instrument or a fluorometer for end-point analysis; both capable of measuring fluorescence at 520nm.

  Lucigen has used the following instruments successfully with the Green Fluorescent Dye: AmpliFire (Douglas Scientific), CFX96 and iQ5 Thermocyclers (Bio-Rad), ESEQuant TS2 (Qiagen), Genie II (OptiGene), and the ABI 7500 Real-Time PCR System (Thermo Fisher Scientific).

  Fluorescent dyes such as EvaGreen, V13-01184, and SYTO-13 have also been used successfully with the LavaLAMP RNA Component Kit, but optimization of dye concentration is necessary to produce the fastest times to results.

Before You Start

1. For new targets, please refer to the **RNA LAMP Reaction Optimization** section (p.6) to design primer sets, test reaction temperatures, etc.
2. Always wear gloves while handling components. Set up reactions using good laboratory techniques to minimize cross contamination.
3. Thaw and hold reagents on ice and set up reactions on ice to avoid background amplification.
4. Calculate the total volume of each reagent required for the planned experiment and verify that enough reagent is available before proceeding to reaction setup.
5. Decide on the method you will use to detect amplified products. Here are the three most common methods:
   a. **Fluorescent Assays in Real-Time Detection Instruments:** Monitor reaction fluorescence using the FAM or SYBR Green channel to detect amplified product.
   b. **End-point Fluorescent Assays:** Measure fluorescence in a fluorometer using the FAM or SYBR Green channel to detect amplified product.
   c. **Non-Fluorescent End-point Assays:** Agarose gel (visual), spectrophotometer (turbidity, OD_{600})
6. Set a thermocycler or heat block to the desired temperature. If using a heat block, we recommended using 0.2 mL PCR tubes and monitoring the temperature closely.
7. Lucigen encourages all users to perform a No Target Control (NTC) reaction with each primer set. A Positive Control reaction is recommended to confirm proper setup and aid in troubleshooting.

Reaction Setup

For most targets, obtaining a faster time to result and minimal background amplification requires screening of multiple primer sets, optimization of (i) reaction temperature, (ii) magnesium sulfate concentration, (iii) LavaLAMP™ RNA Enzyme amount, and (iv) primer set concentration (see RNA LAMP Reaction Optimization section for guidelines).

1. Thaw all kit components on ice and keep them on ice as you set up the reactions.
2. **Mix each component thoroughly before use by vortexing for three to ten seconds.** Centrifuge briefly to collect contents.

   Note: *LavaLAMP™ RNA Enzyme is provided glycerol-free to allow for lyophilization. LavaLAMP™ RNA Enzyme will freeze during storage at -15°C to -25°C and should be thoroughly mixed prior to use.*

3. Prepare initial reaction mix(es) in a single tube in the order listed below (Table 1). Keep the reaction mixes and all reaction tubes or plates on ice to reduce non-specific background amplification. The No Target Control (NTC) reaction using the target-specific primer set(s) is strongly recommended to demonstrate a lack of background amplification within the reaction time(s) tested.

   Notes:

   - ***LavaLAMP™ RNA Component Kit cannot be used with PCR or Bst DNA Polymerase reaction conditions.***
   - ***Add Green Fluorescent Dye fresh when preparing reactions, do not store the dye with the remaining reaction mix components.***
   - *The LavaLAMP™ RNA Enzyme must be used with the supplied 10X buffer.*
   - *The recommended reaction conditions (Table 1) are for use with Green Fluorescent Dye and 1 µL of Target RNA Sample. Adjust the volume of nuclease-free H_2O when using other dye or target RNA sample amounts.*
   - *Table 1 provides volumes for a single reaction, if multiple reactions are required, increase volumes proportionately. Prepare enough reaction mix cocktail(s) for the number of amplification reactions being performed plus an additional 10% to accommodate slight pipetting errors.*

Table 1. Recommended Setup of Control and Experimental (Default) Reactions

	Positive Control	No Target Control (NTC)	Experimental
Component	**Amount (µL)**	**Amount (µL)**	**Amount (µL)**
Nuclease-free H_2O	14.95	14.95	14.95
10X LavaLAMP™ RNA Buffer	2.5	2.5	2.5
LavaLAMP™ RNA Enzyme	1.0	1.0	1.0
dNTP Mix, 25 mM	0.8	0.8	0.8
Magnesium Sulfate, 100 mM	1.25	1.25	1.25
Target-Specific Primer Mix, 10X	--	2.5	2.5
RNA Positive Control LAMP Primer Mix	2.5	--	--
Green Fluorescent Dye **(optional)**	1.0	1.0	1.0
Total Volume	24.0	24.0	24.0

4. After addition of all reagents, mix the reaction completely by pipetting several times.
5. If more than one reaction is being run, dispense 24 µL of the reaction mix for each reaction into PCR tubes or a 96-well PCR plate.

   ***Note**: To minimize cross-contamination, perform steps 6 - 8 in an area separate from that used to assemble the reaction mix.*
6. Add 1 µL of Target RNA or Positive Control RNA to the appropriate reaction tubes or wells. Add 1 µL of nuclease-free water to the NTC reaction tubes or wells. Mix completely by pipetting.
7. Cap tubes or seal plate wells. Centrifuge briefly to collect contents prior to incubation.
8. Depending on the detection method chosen, incubate the reactions as follows in a real-time detection instrument, thermocycler, or heat block.

Step	Temperature	Time
1. Amplification	**Experimental and NTC**: 68˚C – 74˚C **Positive Control**: 68˚C	30 - 60 minutes
2. Hold (Optional)	4˚C	∞

9. Detect amplified products using your method of choice.
   a. For fluorescent assays in real-time detection instruments: Monitor fluorescence using the FAM or SYBR Green channels at 15-30 second intervals for 30-60 minutes.
   b. For fluorescent or non-fluorescent end-point assays: Immediately stop enzyme activity by one of the following methods and then assay using your detection method of choice:
      i. Place on ice or at 4°C.
      ii. Add gel loading dye that yields a final concentration of 10 mM EDTA.
      iii. Heat-kill the reaction at 95°C for 5 minutes in a thermocycler or heat block.

Note: Amplified reactions may be kept at -20°C for long-term storage.

RNA LAMP Reaction Optimization

Target-specific LAMP Primer Mix Design, Quality, and Concentration

Lucigen strongly recommends design and testing of multiple LAMP primer sets because primer design is extremely important in optimizing LAMP results. For details on primer design, please see Appendix A and Appendix B.

We recommend HPLC purification for the FIP and BIP primers. Standard desalting may be used for the Loop-F, Loop-B, F3, and B3 primers, but HPLC purification of all primers produces optimal results. Resuspend primers in nuclease-free water or low TE (10 mM Tris-Cl, 0.1 mM EDTA, pH 8.0).

The recommended starting 10X Target-specific LAMP Primer Mix is:

- 2 µM each F3 and B3 primers
- 8 µM each Loop-F and Loop-B primers
- 16 µM each FIP and BIP primers

Target RNA and Dilution Buffer

We recommend the use of purified target RNA for RNA LAMP assay development. Testing of experimental samples is recommended only after optimization of conditions with control RNA. The minimum amount of required target RNA is assay specific. We recommend using at least $1x10^3$ copies per reaction.

Instrumentation

We recommend the use of real-time detection instrumentation for optimization of the RNA LAMP reaction. Real-time instrumentation enables for more precise monitoring of the resolution between

the positive reactions and potential background amplification. If real-time instrumentation is not available, please refer to Appendix C for the use of end-point detection for optimization.

Lyophilization

Lucigen provides the LavaLAMP™ RNA Enzyme without glycerol to allow for lyophilization. If lyophilization will be the final assay format, optimization of the reaction must be performed without additives that interfere with lyophilization (e.g., Triton™ X-100, glycerol, betaine, etc.).

Recommended Optimization Plan (in order of priority)

1. Primer Set Selection and Temperature Optimization
2. Magnesium Sulfate Titration
3. Enzyme Titration (optional)
4. Primer Titration (optional)

1. Primer Set Selection and Temperature Optimization

Individual primer sets have optimal reaction temperatures. The combination of primer set and temperature have significant impact on the speed of the reaction and background amplification. The suggested range of reaction temperatures to test with each primer set is 68°C – 74°C.

Recommendations

- Set target input at a moderate level, e.g. $1x10^3$ copies per reaction
- Screen at least three different primer designs at the default 1X reaction conditions (Table 1, p.5).
- Run each primer set in replicate across the reaction temperature range of 68°C – 74°C
- Run a known positive sample and NTC for each primer set

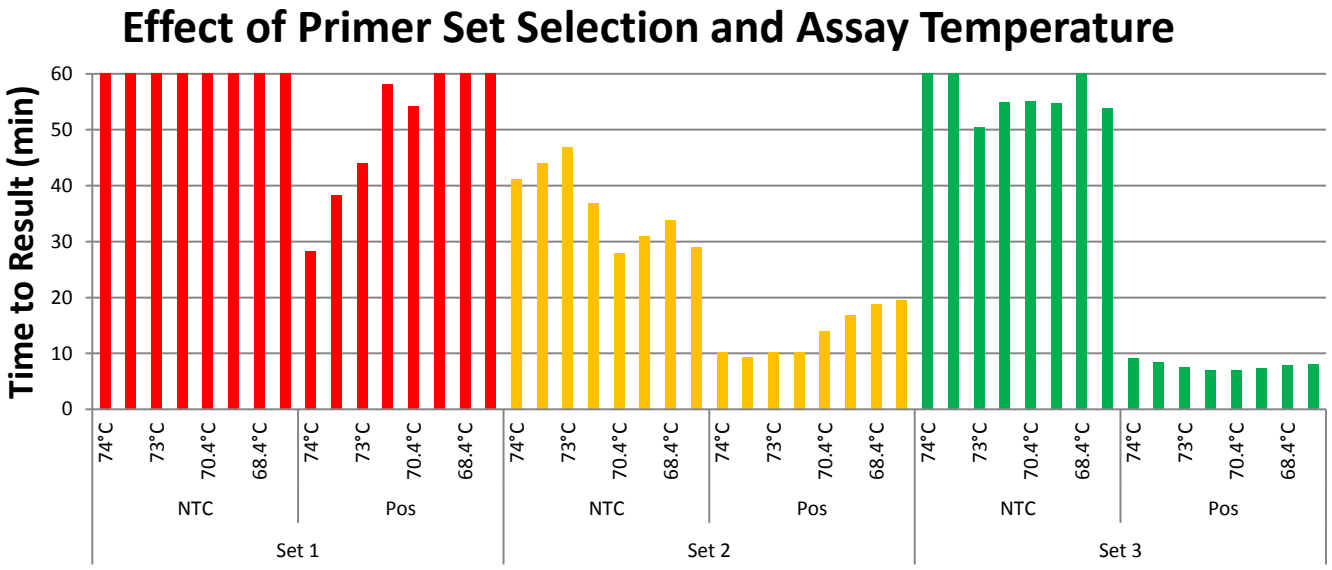

Figure 1. Effect of primer set and temperature on RNA LAMP results. Three primer sets were screened across a temperature range of 68°C – 74°C with recommended 1X, default reaction conditions. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) using Green Fluorescent Dye (Lucigen). Primer Set 3 provided the fastest positive Time to Result with the least amount of background amplification. Within Primer Set 3, 74°C provided the best resolution between the positive and negative samples**.**
Note: *Exact temperatures were set by the CFX96 Thermal Cycler instrument software when the range was selected. Specific temperatures tested will depend on the real-time instrument used.*

2. Magnesium Sulfate ($MgSO_4$)

The suggested reaction concentration of magnesium sulfate is 5 mM. However, some RNA LAMP reaction designs will tolerate other concentrations, which may improve background without adversely affecting positive signal. If undesired background amplification is observed, titrate $MgSO_4$ from 4 mM to 7 mM.

Recommendations

- Set target input at a moderate level, e.g. $1x10^3$ copies per reaction
- Screen at least four concentrations of $MgSO_4$ (4-7 mM) in replicate using the predetermined primer set and optimum temperature keeping other reaction components at 1X.
- Run a known positive sample and NTC for each $MgSO_4$ level tested.

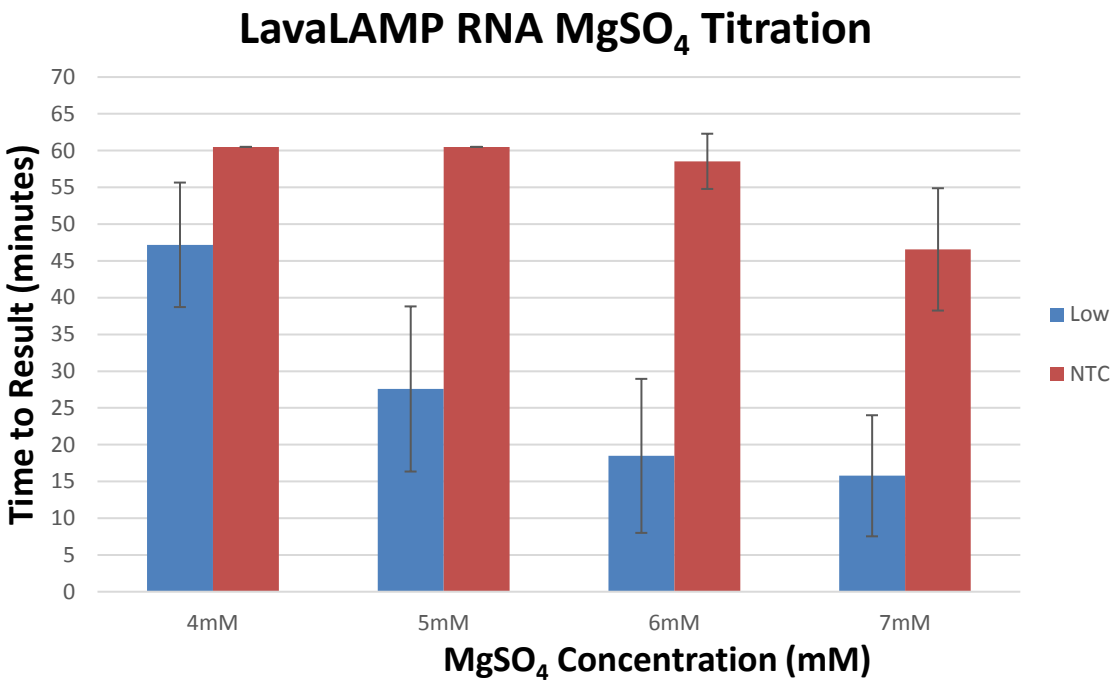

Figure 2. Magnesium sulfate titration using real-time fluorescent detection of amplified products. Four $MgSO_4$ concentrations were titrated with all other reaction components at 1X, default conditions. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) with Green Fluorescent Dye. In this example, 5 mM $MgSO_4$ provided the lowest background amplification; however, increasing $MgSO_4$ allowed for faster positive sample Time to Result.

3. Enzyme Concentration Titration (optional)

The LavaLAMP RNA enzyme concentration may affect RNA LAMP sensitivity and time to result and level of background amplification for some assays. If desired, an enzyme titration from 0.5 µL to 1.5 µL may be performed to decrease background amplification or obtain a faster Time to Result.

Recommendations

- Set target input at a moderate level, e.g. $1x10^3$ copies per reaction.
- Screen at least four different levels of enzyme in replicate at the optimal temperature and $MgSO_4$ concentration.
- Run a known positive sample and NTC for each enzyme level tested.

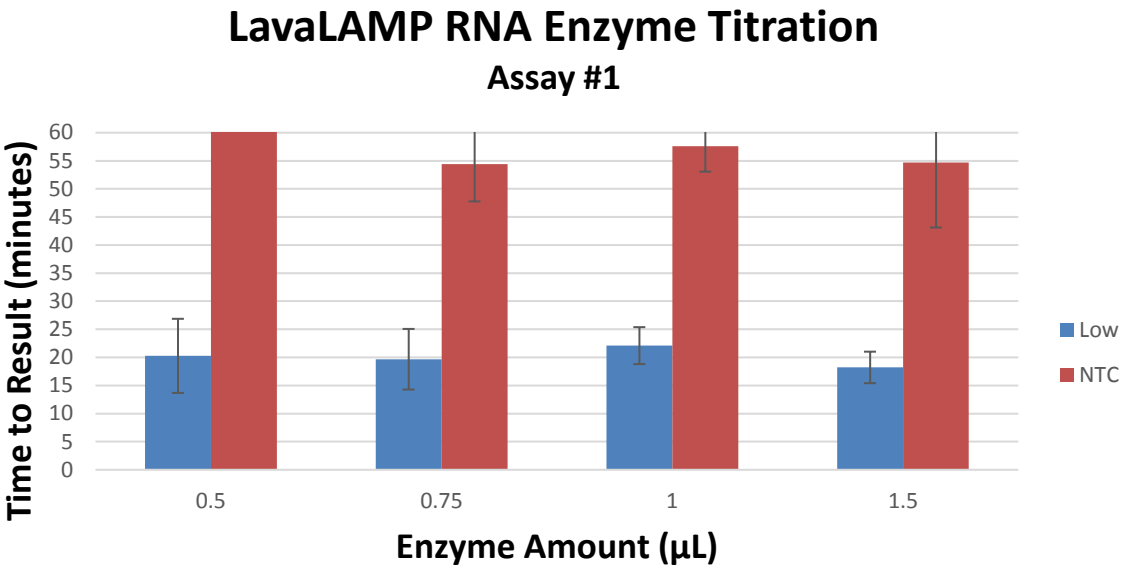

Figure 3. Enzyme titration using real-time fluorescent detection of amplified products – Assay #1. Four enzyme concentrations were titrated. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) in conjunction with Green Fluorescent Dye. In this example, 0.5 µL of LavaLAMP RNA Enzyme provided the lowest background amplification without negatively affecting the positive Time to Result**.**

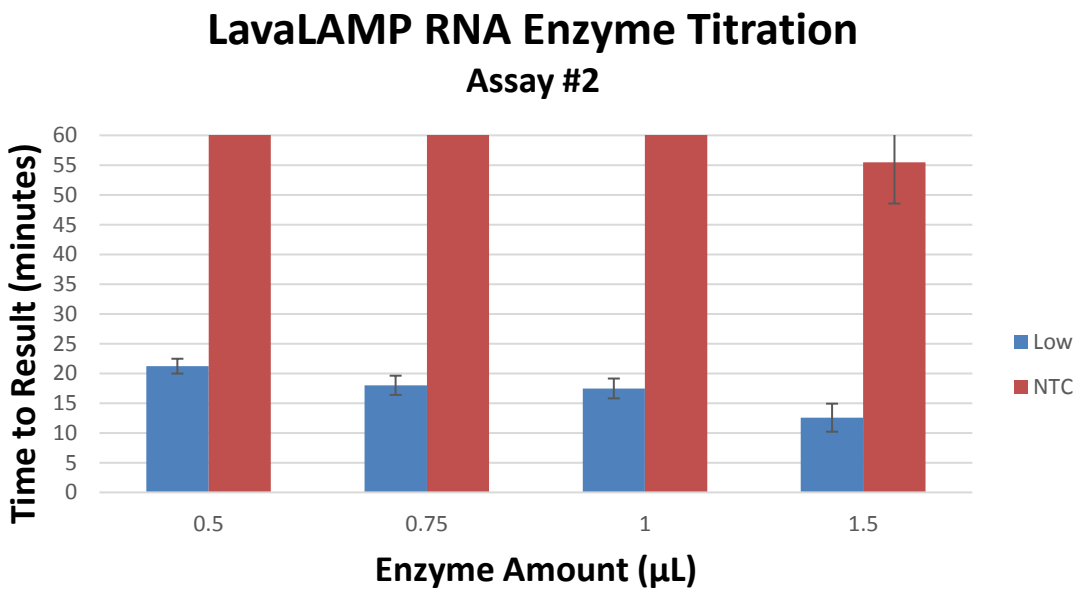

Figure 4: Enzyme titration using real-time fluorescent detection of amplified products – Assay #2. Four enzyme concentrations were titrated. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) in conjunction with Green Fluorescent dye. In this example, 1.5 µL LavaLAMP RNA Enzyme improved the positive Time to Result while slightly increasing background amplification**.**

4. Primer Concentration Titration (optional)

Depending on the primer-template combination, it may be necessary to optimize primer concentration after the optimal reaction temperature is identified. Certain primer sets may be prone to background amplification at or near the commonly used LAMP primer concentrations. If undesired background amplification is still observed after other optimizations, the primer concentration should be titrated from 0.25X – 1X. The concentration of all primers may be adjusted in unison by varying the amounts of Target-specific LAMP Primer Mix added. Reducing the primer concentration may reduce sensitivity and reaction yield, or it may increase the time required to amplify your target. Lucigen does not recommend increasing primer concentration above the recommended levels.

Recommendations

- Set target input at a moderate level, e.g. $1x10^3$ copies per reaction.
- Screen at least four different levels of Primer Mix in replicate at the optimum temperature, $MgSO_4$ concentration, and Enzyme level.
- Run a positive sample and NTC for each primer concentration tested.

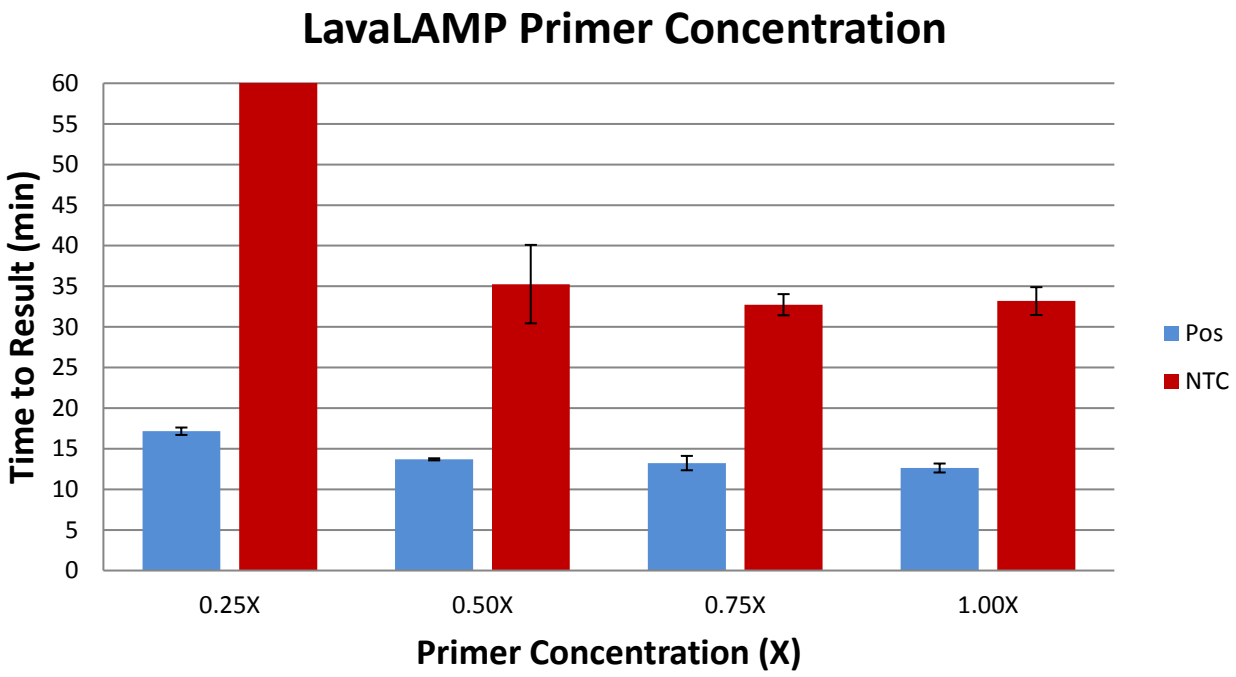

Figure 5. Primer concentration effects using real-time fluorescence detection of amplified products. Four primer concentrations were tested from 0.25X to 1.0X. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) in conjunction with Green Fluorescent Dye. In this example, lower primer concentration significantly decreased background amplification without negatively affecting the positive sample Time to Results.

Typical RNA LAMP Results

Agarose Gel Analysis of RNA LAMP Reaction Products

LavaLAMP™ RNA amplification products may be analyzed by agarose gel electrophoresis to confirm the validity of the reaction. A positive RNA LAMP reaction should generate a ladder of products, typically consisting of 1-10 visible concatemers of the target. In contrast, non-specific amplification typically generates an undefined smear of DNA, with no distinct banding pattern.

Correct Target-specific Amplification

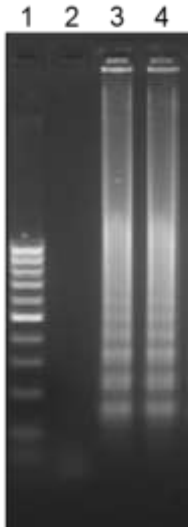

Figure 6. Positive LAMP reaction products from a positive control target. Lane 1: 100 bp Marker, Lane 2: No Target Control reaction. Lanes 3 and 4: A distinct banding pattern is seen among the smear, which is indicative of a positive LAMP reaction.

Spurious Background Amplification

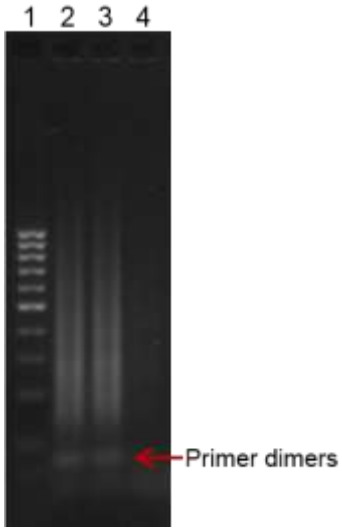

Figure 7. Typical background amplification in a LAMP reaction. Lane 1: 100 bp ladder, Lanes 2 and 3: Non-specific or background amplification appears as a smear of DNA fragments with no visible or distinct bands. A prominent primer dimer band is also characteristic of non-specific amplification. Lane 4: Absence of non-specific amplification (no products).

Fluorescent Signals from Different RNA LAMP Reactions

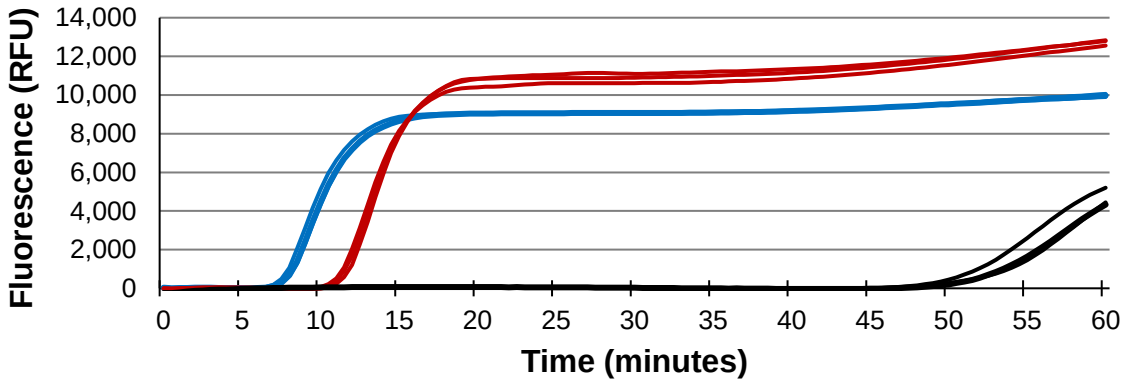

Figure 8. Early Fluorescent signals from positive RNA LAMP reactions and late background signals from No Target Controls after extended incubation. RNA LAMP reactions were run in a real-time thermal cycler. The fluorescent signal from each reaction was captured over a 60-minute reaction time. The red and blue lines represent the fluorescent signals from Positive Control reactions with varying amounts of target. The black lines represent the non-specific background amplification signals that arise later in No Target Control reactions.

Additional Amplification Guidelines

Prevent Target RNA Contamination

RNA LAMP reactions are very sensitive to target RNA or amplicon carryover which can result in false positive amplification. To prevent contamination of RNA LAMP reactions with target RNA or target amplicons, designate and use an area for reaction setup that has never been exposed to the target RNA or amplified products. Use a second area that has never been exposed to amplified material to add your target RNA to your reactions. Finally, designate a third area to analyze RNA LAMP reaction products.

Cold Reaction Setup

The LavaLAMP™ RNA Enzyme exhibits residual activity at temperatures above 4°C, which can cause non-specific background amplification in the subsequent reaction. The following steps can minimize this source of background.

- All LavaLAMP™ RNA reactions should be set up on ice and maintained at 4°C prior to amplification.
- Primers should be added just prior to target addition and incubation.
- To start amplification, directly transfer the reactions from ice to a pre-heated heat block or thermal cycler at the correct reaction temperature.

Target Preparation

Most routine methods of RNA target purification are sufficient (e.g. phenol/chloroform or guanidine/silica-based binding), and RNA LAMP reactions are generally tolerant to some contaminants in the RNA sample. However, trace amounts of purification reagents (e.g. phenol, Proteinase K, ethanol, etc.) may inhibit amplification. In addition, EDTA can inhibit amplification, so it is preferable to dissolve nucleic acid target in water or EDTA-free buffer rather than standard TE (10 mM Tris, 1 mM EDTA). If TE must be used, we recommend using low TE (10mM Tris, 0.1 mM EDTA).

RNA LAMP Reaction Timing

The amplification threshold is usually reached in 8-20 minutes. Therefore, 30 minutes is the recommended incubation time for end-point reactions. Longer incubation times may lead to the appearance of undesired background (see Fig. 7).

Reaction Overlay

A thermal cycler with a heated lid is recommended to prevent evaporation of the reaction mix. If such an instrument is not available, the reaction mixture can be overlaid with one-half reaction volume of PCR-grade mineral oil, but mineral oil may slow the reaction.

Appendix

A: Primer Design Software

We recommend designing multiple LAMP Primer sets and testing them to identify the best performing set. When using these programs, enter your target RNA regions as if they were double-stranded DNA targets.The following software is available to help design primer sets:

The Eiken PrimerExplorer is a free online application that can be accessed at: https://primerexplorer.jp/e/.

The LAMP Designer by Premier Biosoft, is available for purchase at: http://www.premierbiosoft.com/isothermal/lamp.html.

B: Tm Setpoints

LAMP primer design software (including Eiken PrimerExplorer and LAMP Designer by Premier Biosoft) were optimized for Bst polymerase which has an optimal temperature of 63°C - 65°C. Because the LavaLAMP™ RNA Enzyme has an optimal temperature range of 68°C - 74°C, it is necessary to increase the default Tm setpoints in the software as follows:

	Default Tm Settings	Suggested LavaLAMP™ RNA Settings
FIP and BIP	65°C	71°C
F3 andB3	60°C	66°C
FL and BL	62°C	68°C

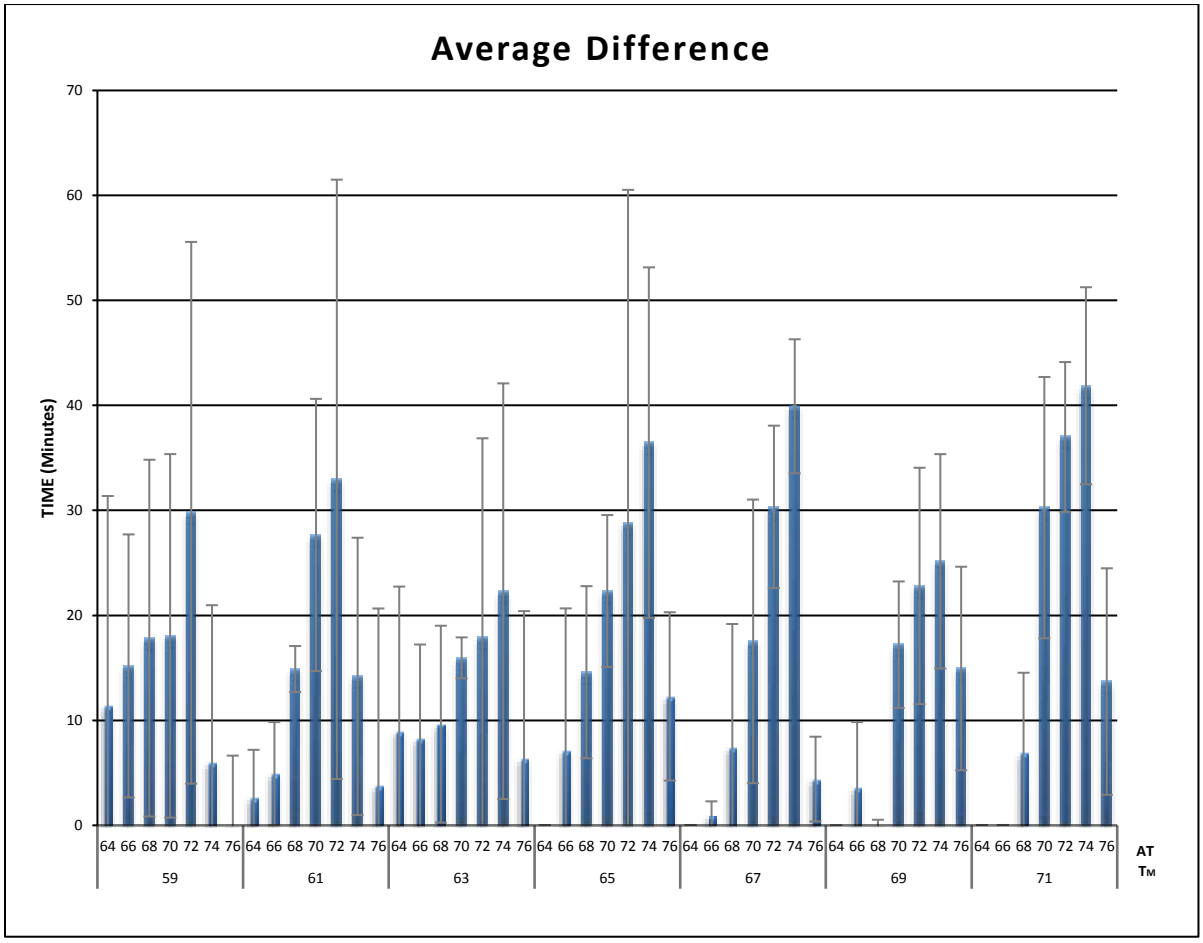

Figure 9. Effect of Tm setpoints in primer design software. The difference between the Time to Result of negative and positive samples for various assay temperature (AT) for individual reactions and primer Tm setpoints ("TM") for FIP and BIP. In this example, the 71°C setpoint provided the largest difference between positive and negative and the largest assay temperature operating window (70°C - 74°C).

C: End-Point Optimization

The preferred method of detection for developing and optimizing RNA LAMP assays is real-time fluorescence with a DNA binding dye; however, it is also possible to use endpoint analysis. Endpoint RNA LAMP assay optimization requires product measurement at three different time points in order to follow the amount of product generated versus time. Both Positive Target and No Target Control RNA LAMP reactions must be run to compare positive signal versus background signal over time. Once the RNA LAMP product starts to increase over background, it typically reaches maximum product (saturation) within 10 minutes. Measuring the RNA LAMP product at 10, 20, and 30 minutes, will indicate if the RNA LAMP product is below detection, between minimum detection and saturation, or at maximum (saturated, see Example below). With optimization of the RNA LAMP conditions, the product will reach significant or maximum levels at an earlier endpoint time.

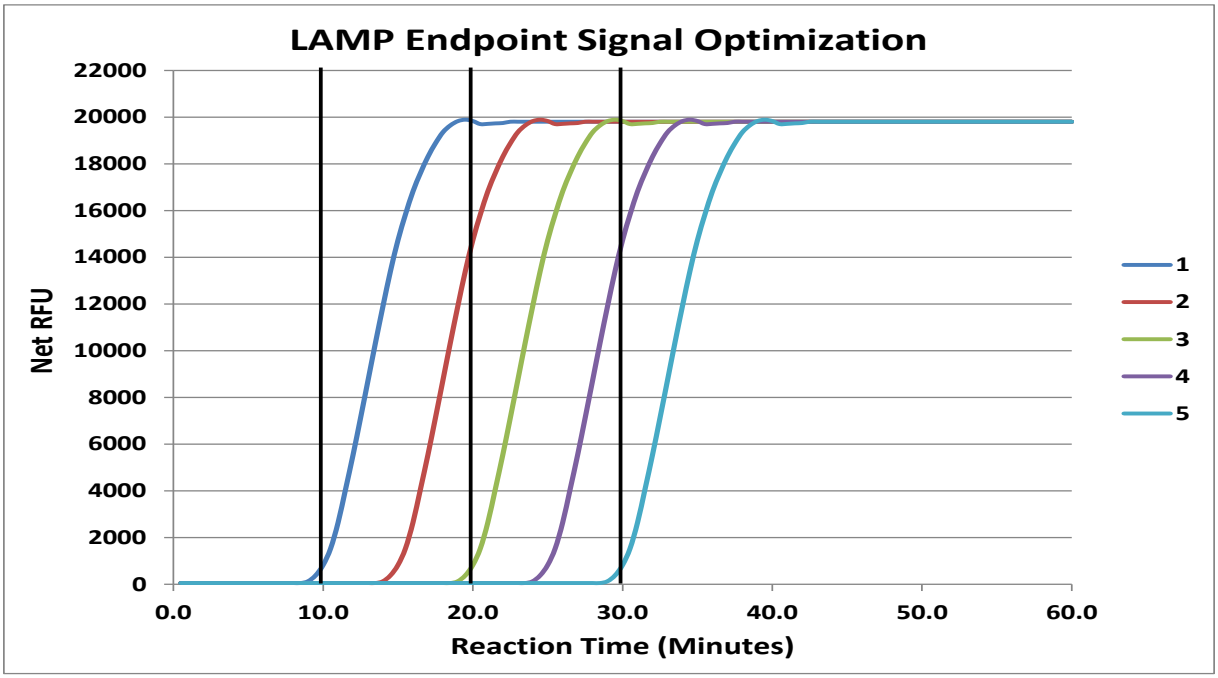

Figure 10. RNA LAMP optimization by endpoint analysis. Theoretical product levels from 5 different RNA LAMP Reactions are shown. Measuring the RNA LAMP product (Net RFU) at the suggested endpoints (vertical black lines) allows detection of positive results. With optimization of the RNA LAMP conditions, the RNA LAMP product will reach maximum levels at earlier endpoint time points

D: Quality Control Assays

Absence of Endonuclease

LavaLAMP™ RNA Enzyme is determined to be free of detectable endonuclease or nicking activity. One µg of supercoiled plasmid DNA is incubated with master mix for 16 hours at 70°C. Reactions are analyzed by agarose gel electrophoresis. The master mix is deemed to be free of endonuclease or nicking activity if there is no alteration in mobility.

Absence of Exonuclease

LavaLAMP™ RNA Enzyme is tested to be free of contaminating exonuclease activity by incubating 1 µg of Hind III-digested lambda DNA with master mix at 70°C for 16 hours. Reactions are analyzed by agarose gel electrophoresis, and the enzyme is deemed to be free of exonuclease activity if there is no alteration in mobility.

Functional Assays

LavaLAMP™ RNA Amplification system is tested for performance by isothermal amplification of a target region within the MS2 genome.

E: References and Additional Reading

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000 28(12):E63.

Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes. 2002 16(3):223-9.

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Mori Y, Hirano T, Notomi T. Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnol. 2006 6:3.

Tomita N, Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc. 2008 3(5):877-82.

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