Manual

LavaLAMP DNA Component Kit

For Research Use Only. Not for use in diagnostic procedures.

BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

Manual

LavaLAMP DNA Component Kit

Contents

1. Introduction 3
2. Product designations and kit components 3
3. Product specifications 4
4. General considerations 4
    4.1 Materials supplied by the user 4
    4.2 Positive controls 5
    4.3 Primer design 6
5. Reaction setup 6
    5.1 Before you start 7
    5.2 Step-by-step protocol 7
6. LAMP assay detection 8
    6.1 Green Fluorescent Dye 9
7. Recommended optimisation plan (in order of priority): 9
    7.1 Primer set selection and temperature optimisation 9
    7.2 Magnesium Sulfate ($MgSO_4$) 11
    7.3 Enzyme concentration titration (optional) 12
    7.4 Primer concentration titration (optional) 14
8. Typical LAMP results 15
9. Further support 15

Manual

LavaLAMP DNA Component Kit

1. Introduction

The LavaLAMP™ DNA Component Kit is intended for researchers who wish to further optimise their DNA loop-mediated isothermal amplification (LAMP) reactions. Reaction components are provided individually to allow greater flexibility and control over exact reaction set up.

2. Product designations and kit components

Product	Kit size	Catalogue number	Reagent description	Part number	Volume
LavaLAMP DNA Component Kit	500 reactions	30076-1	10x LavaLAMP DNA Buffer	F834098-1	1.25 mL
			LavaLAMP DNA Enzyme	F832817-1	500 µL
			Magnesium Sulphate 100 mM	F88695-2	1.5 mL
			DNA Positive Control LAMP Primer Mix	F813735-1	25 µL
			DNA Positive Control	F823736-1	10 µL
LavaLAMP DNA Component Kit with Dye	500 reactions	30077-1	10x LavaLAMP DNA Buffer	F834098-1	1.25 mL
			LavaLAMP DNA Enzyme	F832817-1	500 µL
			Magnesium Sulphate 100 mM	F88695-2	1.5 mL
			DNA Positive Control LAMP Primer Mix	F813735-1	25 µL
			DNA Positive Control	F823736-1	10 µL
			Green Fluorescence Dye	F883827-2	500 µL
Green Fluorescence Dye	500 reactions	30078-1	Green Fluorescence Dye	F883827-2	500 µL

Table 1. LavaLAMP DNA Component kits and constituent components.

3. Product specifications

Storage: Store all kits and components at -20 °C.

Quality control:

- **Absence of endonuclease**
  LavaLAMP DNA 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 analysed 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 DNA 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 analysed 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 DNA Amplification system is tested for performance by isothermal amplification of a target region within the M13mp18 ssDNA genome.

4. General considerations

4.1 Materials supplied by the user

- 10x target-specific LAMP primer mix
  - Typical 10x formulation:
    - 2 µM each, forward outer primer (F3) and backward outer primer (B3)
    - 8 µM each, forward loop primer (FL) backward loop primer (BL)
    - 16 µM each, forward inner primer (FIP) and backward inner primer (BIP)
- dNTP Mix, 25 mM each
- Target DNA: Most routine methods of target purification are sufficient (e.g., phenol/chloroform or guanidine/silica-based methods), and LAMP reactions are generally more tolerant to some contaminants in the DNA sample. However, trace amounts of purification reagents (e.g., phenol, Proteinase K, ethanol, etc.) may inhibit amplification. In addition, EDTA can inhibit amplification and, as such, it is preferable to use nucleic acid target that is dissolved 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 with 0.1 mM EDTA.
- Thermal cycler or heat block: A thermal cycler with a heated lid is ideal to prevent evaporation of the reaction mix. If no such lid is available, the reaction mixture can be overlaid with one-half reaction volume of PCR-grade mineral oil, but mineral oil may slow the reaction. We recommend using calibrated instruments.
- Real-time instrument or fluorometer (if using Green Fluorescent Dye for detection – see section 6)

4.2 Positive controls

The positive controls provided with all our LavaLAMP kits are designed to be used for two main purposes. These are i) initial verification of laboratory set up and processes for new users of LAMP, and ii) troubleshooting if you are experiencing any issues with your reactions.

If you are completely new to LAMP, we recommend using the positive control reactions that are provided to set up a trial reaction before you begin working with your own DNA and assays. Each kit includes the necessary DNA template and LavaLAMP assay primers.

A LavaLAMP reaction involving the provided positive control should be set up as detailed in Table 2.

Component	Volume (µL)
Nuclease-free water	13.7
LavaLAMP DNA Buffer	2.5
LavaLAMP DNA Enzyme	1.0
dNTP Mix, 25 mM	0.8
Magnesium Sulphate, 100 mM	2.5
DNA positive Control LAMP Primer Mix	2.5
Green Fluorescent Dye (optional) (If not using, replace volume with nuclease-free water)	1.0
Total reaction volume	24.0

Table 2. Reaction set up for LavaLAMP DNA positive control reaction.

The DNA positive control requires amplification to be performed at 74 °C for 30 to 60 minutes.

The positive control can also be used for troubleshooting issues with your own LavaLAMP assays. Running a positive control reaction will enable you verify that all components other than the designed primers are functioning correctly.

Please note that it may not be possible to run a positive control reaction alongside your own LavaLAMP assays as designed because the control must be amplified at a specific temperature. We do not advise amplifying the positive control reactions at any other temperature.

4.3 Primer design

LAMP commonly employs a set of six primers which must be supplied by the user. LGC Biosearch Technologies™ recommends using previously established designs or designing new primer sets. Below are two tools that you may find helpful for primer design:

- LAMP Designer by Premier Biosoft is available for purchase at: http://www.premierbiosoft.com/isothermal/lamp.html
- PrimerExplorer by Eiken is a free online application that can be accessed at: https://primerexplorer.jp/e/

Detailed guidance on primer design and on performing primer design screens can be found in section 5 and section 6 of our LavaLAMP: technical guide for new users respectively.

5. Reaction setup

For most targets, obtaining a faster time to result and minimal background amplification requires screening of multiple primer sets, optimisation of (i) reaction temperature, (ii) magnesium sulphate concentration, (iii) LavaLAMP DNA Enzyme amount, and (iv) primer set concentration (see Recommended Optimisation Plan section for guidelines).

A no template control (NTC) reaction should be run alongside experimental samples.

Biosearch Technologies recommend preparing sufficient reaction mix for the number of individual reactions that are to be performed, with an additional 10% to accommodate for pipetting inaccuracies.

LAMP reactions are very sensitive to target DNA or amplicon carryover contaminants which can result in false positive amplification results. To prevent contamination of your LAMP reactions with target DNA or target amplicons, designate and use an area for reaction setup that has never been exposed to the target DNA or amplified products. Then use a different area to add your target DNA to your reactions (steps 6-8) that has never been exposed to amplified material. Finally, analyse LAMP reaction products (section 6) in an area separate from both reaction setup areas.

Lyophilisation: The LavaLAMP DNA Enzyme is provided without glycerol to allow for lyophilisation. If lyophilisation will be the final assay format, optimisation of the reaction must be performed without additives that interfere with lyophilisation.

5.1 Before you start

1. Always wear gloves while handling components. Set up reactions using good laboratory techniques that minimise cross contamination.
2. Thaw and hold reagents on ice and set up reactions on ice to avoid background amplification.
3. Calculate the total volume of each reagent required for the planned experiment and verify that sufficient volume is available before proceeding to reaction setup.
4. Set a thermal cycler 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.
5. Biosearch Technologies encourages all users to perform an NTC reaction with each primer set.

5.2 Step-by-step protocol

1. Thaw all kit components on ice. All LavaLAMP DNA Enzyme reactions should be set up on ice and maintained at 4 °C prior to amplification.
2. Mix each component thoroughly before use by vortexing each tube for three to ten seconds and then centrifuge briefly at 4 °C to collect contents.
3. Prepare initial reaction mixes in a single tube in the order listed in table 3. During this step, the reaction mixes and all reaction tubes/plates should be kept on ice to reduce non-specific amplification. Primers should be added just prior to target addition and incubation.

	NTC	Experimental
Component	**Volume (µL)**	**Volume (µL)**
Nuclease-free water	13.7	13.7
10X LavaLAMP DNA Buffer	2.5	2.5
LavaLAMP DNA Enzyme	1.0	1.0
dNTP Mix, 25 mM	0.8	0.8
Magnesium Sulphate, 100 mM	2.5	2.5
Target-Specific Primer Mix, 10X	2.5	2.5
Green Fluorescent Dye (optional)	1.0	1.0
Total volume	24.0	24.0

Table 3. Reaction setup for LavaLAMP DNA Enzyme reactions. Reactions assume Green Fluorescent Dye (supplied with Cat. No. 30067-1) and 1 µL of target DNA sample are being used. If you are not using Green Fluorescent Dye and/or a different volume of target DNA is being used, adjust the volume of nuclease-free water accordingly.

4. After all reagents have been added, mix the combined reaction components by pipetting. This step is required to ensure uniform distribution of all reaction components in the reaction mix.
5. Dispense 24 µL of the prepared reaction mix(es) into the required number of PCR tubes or a 96-well PCR plate.
6. Add 1 µL of target DNA (or positive control template DNA if running positive control reactions) to the appropriate reaction tubes or wells and 1 µL of nuclease-free water to the NTC reaction tube(s) or well(s). Mix completely by pipetting.
7. Cap tubes or seal plate wells. Centrifuge briefly to collect contents prior to incubation.
8. Using a heat block or thermal cycler, incubate the reactions as detailed in table 4. Reactions should be transferred directly from ice to a pre-heated heat block or thermal cycler set and equilibrated to the correct reaction temperature.

Step	Temperature	Time
1. Amplification	**Experimental and NTC**: 68-74 °C **DNA positive control template**: 74 °C	30-60 minutes
2. Hold (optional)	4 °C	∞

Table 4. Temperature requirements for the LavaLAMP DNA reaction. The amplification threshold is usually reached in 8-20 minutes. As a result, 30 minutes is the recommended incubation time for end-point reactions. Longer incubation times may lead to the appearance of undesired background (see figure 3 for an example).

9. Immediately stop amplification reactions using one of the three methods below. This step stops enzyme activity.
   a. Hold on ice or at 4 °C.
   b. Add gel loading dye that produces a final 10 mM EDTA concentration.
   c. Perform a heat-kill step in a thermal cycler or heat block at 95 °C for 5 minutes.
   Note: *Amplified reactions may be kept at -20 °C for long term storage.*
10. Detect amplified product – see section 6 for details.

6. LAMP assay detection

For LAMP assay detection, there are three main methods we would recommend:

- Fluorescent assays in real-time detection instruments: monitor reaction fluorescence using the FAM channel to detect amplified product.
- End-point fluorescent assays: measure fluorescence in a fluorometer using the FAM channel to detect amplified product.
- Non-fluorescent end-point assays: agarose gel (visual) or spectrophotometer (turbidity, OD600). Note: turbidity is a low-cost option and is field deployable but is single-use only and not as sensitive as other methods. Agarose gel is more applicable for diagnostics, and for troubleshooting e.g. determining whether NTC amplification is due to contamination or primer dimerisation.

6.1 Green Fluorescent Dye

While not required, we recommend using Green Fluorescent Dye to detect amplified DNA. Green Fluorescent Dye is packaged separately from the master mix. Do not combine the dye with the master mix until you are setting up the reaction.

Detection of Green Fluorescent Dye requires a real-time amplification instrument or a fluorometer for end-point analysis. This should be capable of excitation at 490 nm and measuring fluorescence at 520 nm.

- The following instruments have been successfully used with Green Fluorescent Dye: AmpliFire (Agdia), CFX96 and iQ5 Thermo Cyclers (Bio-Rad), ESEQuant TS2 (Qiagen), Genie II (OptiGene), and the ABI 7500 Real-Time PCR System (Thermo Fisher Scientific).
- Fluorescent dyes such as V13-01184, EvaGreen and SYTO-13 have also been used successfully with the LavaLAMP DNA Component Kit, but SYBR Green Dye will not work acceptably. Optimisation of dye concentration is necessary to produce the fastest TTR.

7. Recommended optimisation plan (in order of priority):

1. Primer set selection and temperature optimisation
2. Magnesium Sulfate titration
3. Enzyme titration (optional)
4. Primer titration (optional)

7.1 Primer set selection and temperature optimisation

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 68-74 °C.

Recommendations

- Set target input at a moderate level
- Screen at least three different primer designs at the default 1x reaction conditions
- Run each primer set in replicate across the reaction temperature range of 68-74 °C
- Run a known positive sample and NTC for each primer set

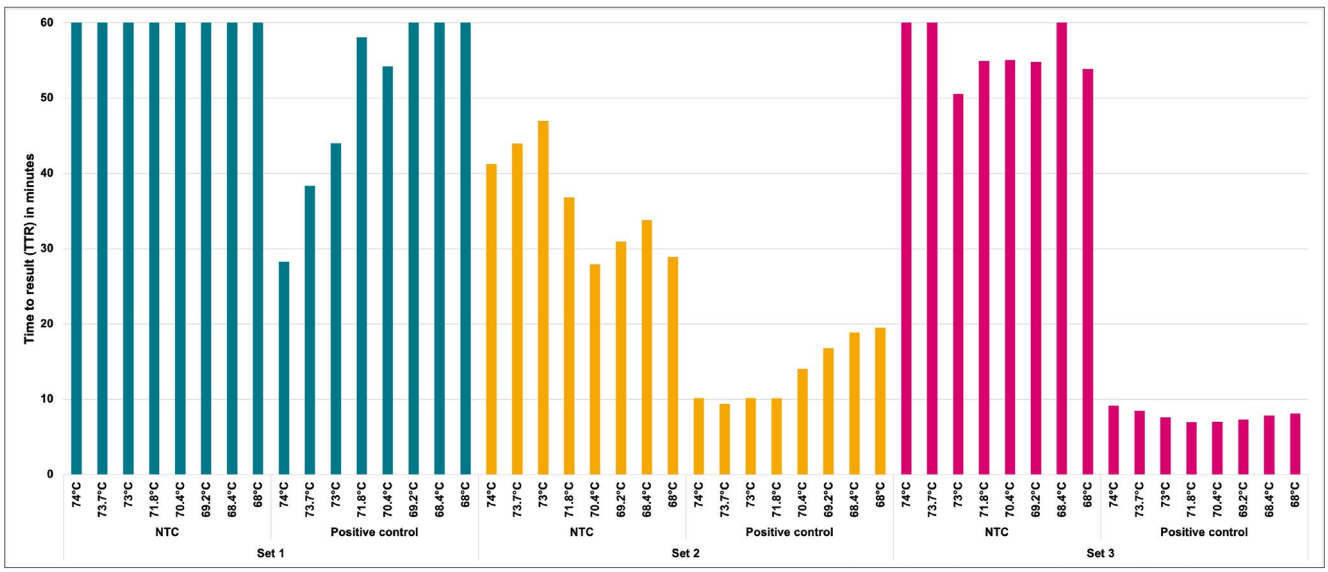

Figure 1. Effect of primer set and temperature on LAMP results. Three primer sets were screened across a temperature range of 68-74 °C with recommended 1x default reaction conditions. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) using Green Fluorescent Dye. Primer set 3 provided 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.

7.2 Magnesium Sulfate ($MgSO_4$)

The suggested reaction concentration of magnesium sulphate is 10 mM. However, some LAMP reaction designs will tolerate lower concentrations, which may improve background without adversely affecting positive signal. If undesired background amplification is observed, titrate $MgSO_4$ from 6 mM to 12 mM.

Recommendations

- Set target input at a moderate level.
- Screen at least four concentrations of $MgSO_4$ (6-12 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.

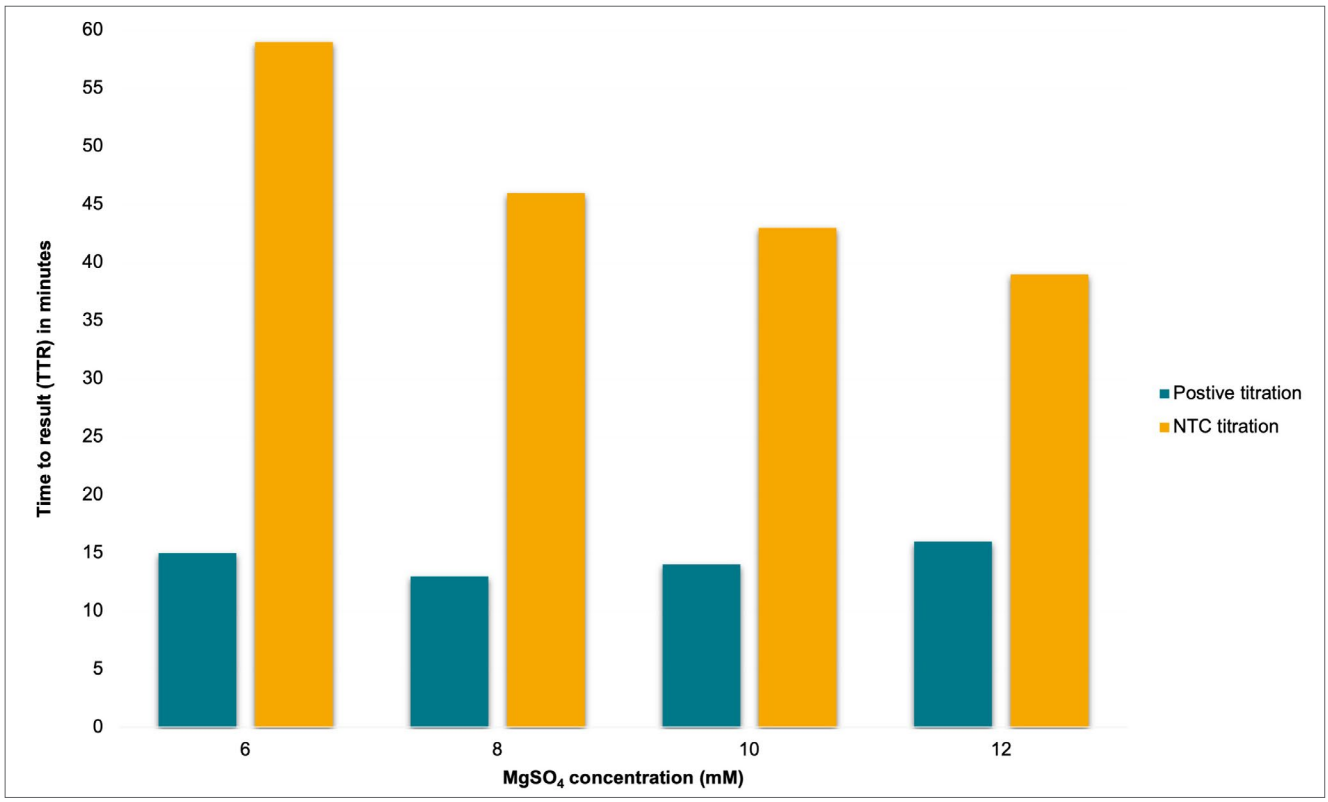

Figure 2. Magnesium sulphate titration using real-time fluorescent detection of amplified products. Four $MgSO_4$ concentrations were titrated with reactions at 1x default conditions. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) with Green Fluorescent Dye. In this example, 6 mM $MgSO_4$ provided the lowest background amplification without negatively affecting the positive sample time to result.

7.3 Enzyme concentration titration (optional)

The LavaLAMP DNA enzyme concentration may affect 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
- 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.

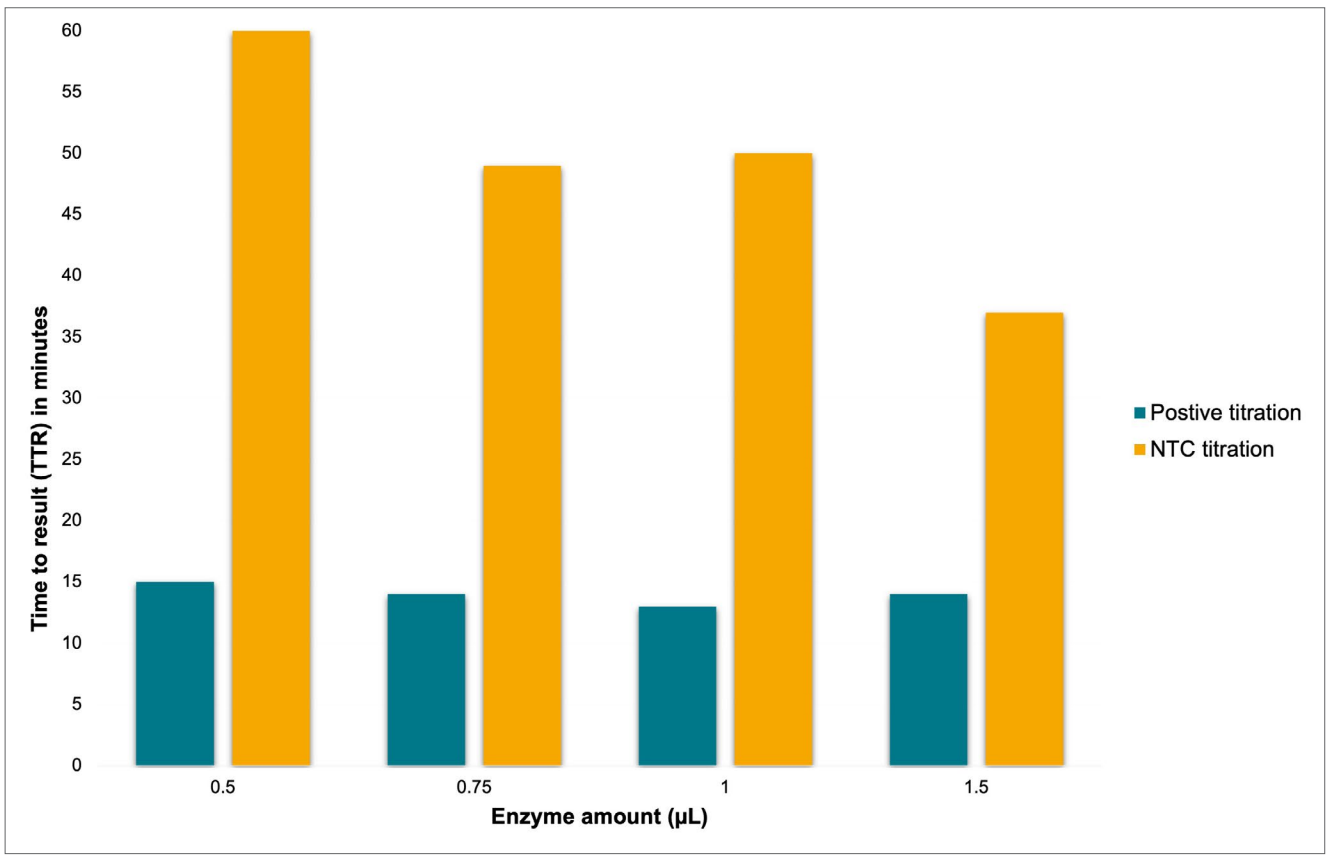

Figure 3. Enzyme titration using real-time fluorescent detection of amplified products. Four enzyme concentrations were titrated. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) in conjunction with Green Fluorescent Dye. In this example, 0.5 µL of LavaLAMP DNA Enzyme provided the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

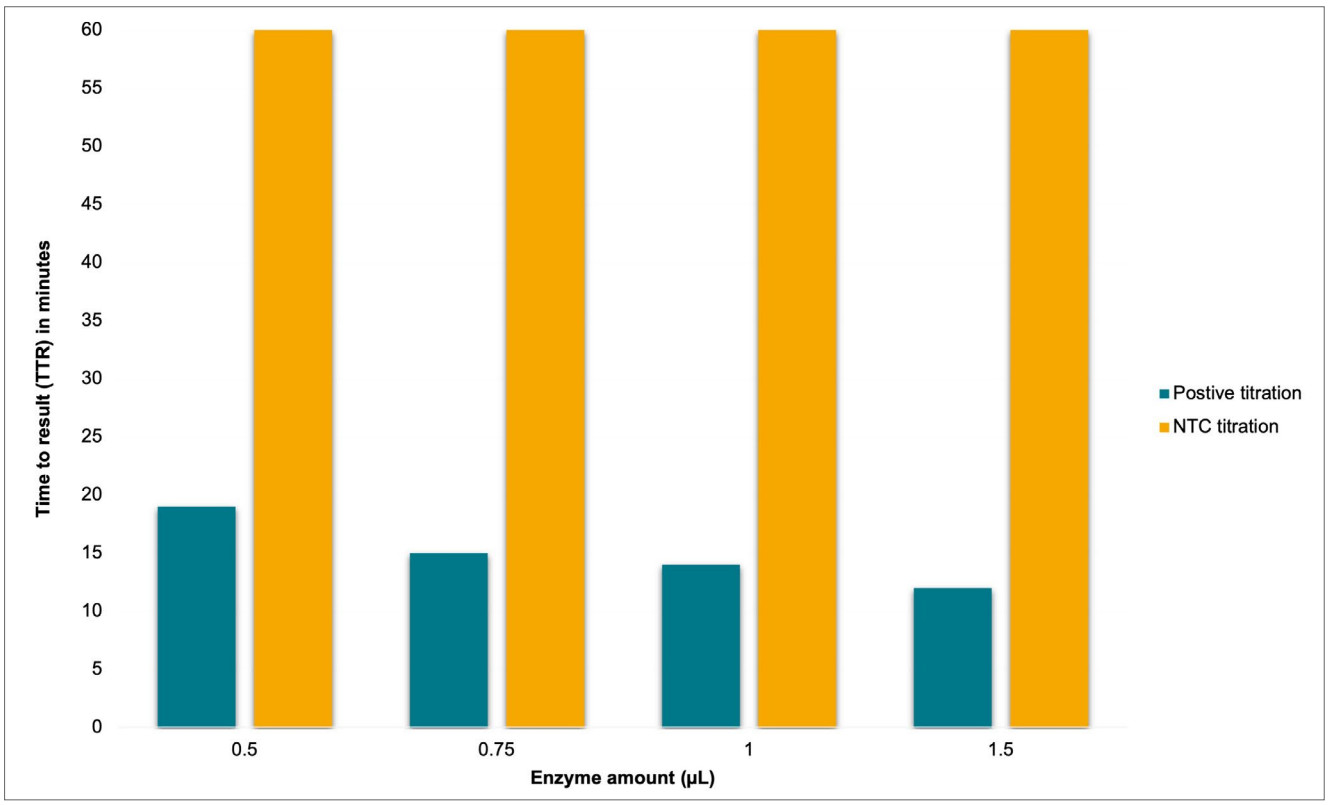

Figure 4. Enzyme titration using real-time fluorescent detection of amplified products. Four enzyme concentrations were titrated. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) in conjunction with Green Fluorescent dye. In this example 1.5 µL LavaLAMP DNA Enzyme improved the positive time to result without increasing background amplification.

7.4 Primer concentration titration (optional)

Depending on the primer-template combination, it may be necessary to optimise 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 optimisations, the primer concentration titration should be titrated from 0.25-1x. 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. Biosearch Technologies does not recommend increasing primer concentration above the recommended levels.

Recommendations

- Set target input at a moderate level
- 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.

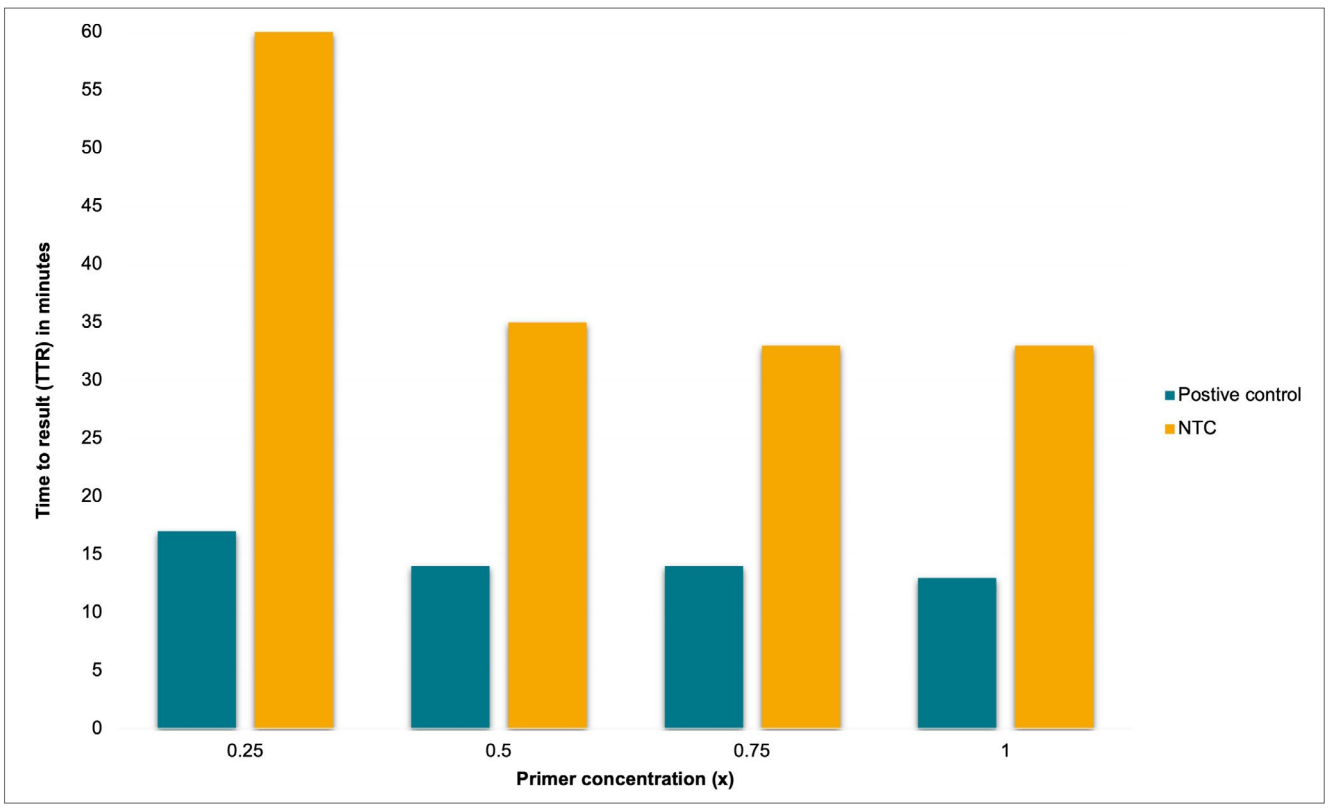

Figure 5. Primer concentration effects using real-time fluorescence detection of amplified products. Four primer concentrations were titrated from 0.25x to1.0x. Real-time detection was performed on a CFX96 Thermal Cycler (Bio-Rad) in conjunction with Green Fluorescent Dye. In this example, lower primer concentration significantly decreased background amplification without negatively affecting the positive sample time to results values.

8. Typical LAMP results

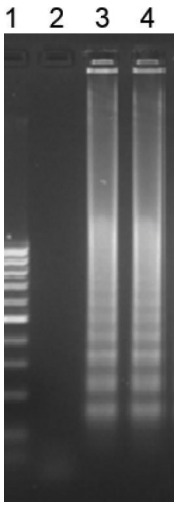

Figure 6: Reaction products from a Positive Control Target.
Lane 1: 100 bp marker,
Lane 2: No Template Control reaction.
Lanes 3 and 4: A distinct banding pattern is seen among the smear, which is indicative of a positive LAMP reaction.

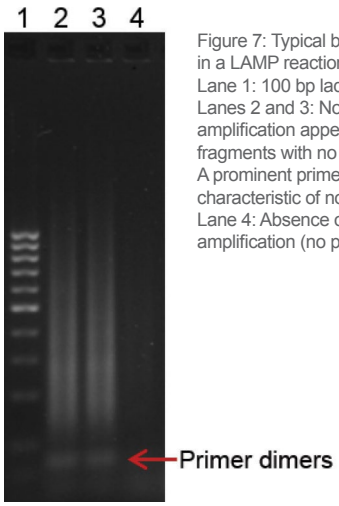

Figure 7: Typical background amplification in a LAMP reaction.
Lane 1: 100 bp ladder,
Lanes 2 and 3: Non-specific or background amplification appears as a smear of DNA fragments with no visible or distinct bands. A prominent primer dimer band is also characteristic of non-specific amplification.
Lane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

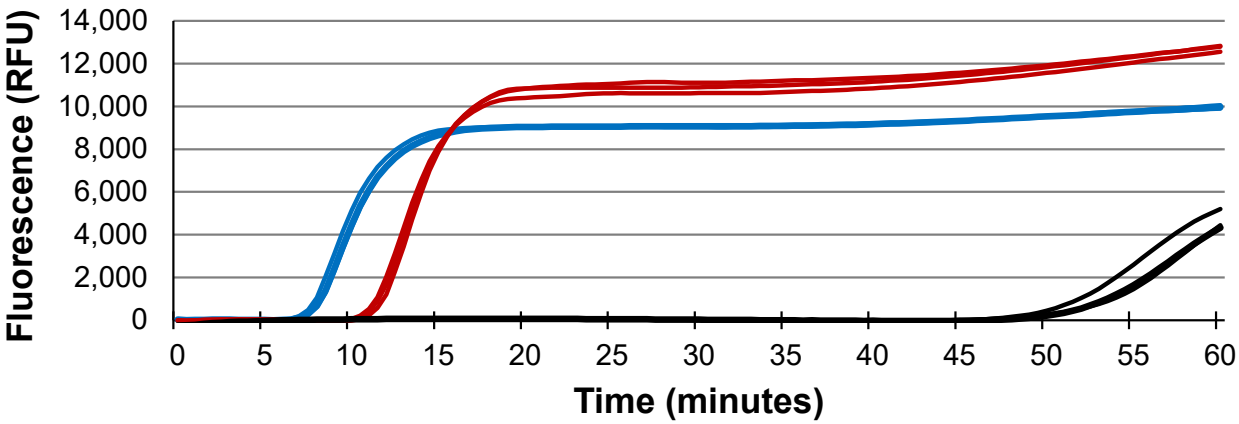

Figure 6. Early fluorescent signals from positive LAMP reactions and late background signals from NTC Controls after extended incubation. LAMP reactions were run in a real-time thermal cycler and the fluorescent signal from each reaction was captured over a 60-minute reaction time. The red and blue lines represent the fluorescent signals from Positive Control reactions with varying amounts of target. The black lines are the non- specific background amplification signals that can arise later in a No Target Control (primers) reaction.

9. Further support

If you require any further support, please contact our technical support team at techsupport@lgcgroup.com.

@LGCBiosearch | biosearchtech.com

GEN/1135/SW/0723

For Research Use Only. Not for use in diagnostic procedures.

bsi. ISO 13485 Medical Devices Quality Management
FM 598408

BIOSEARCH™ TECHNOLOGIES
GENOMIC ANALYSIS BY LGC