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Loop-mediated Isothermal Amplification (LAMP) with Eppendorf ThermoMixer® C and ThermoTop®

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Abstract

The Covid-19 pandemic showed a silver lightning in the molecular biology world by spurring on improvements of many methods especially for the detection of nucleic acids. Loop-mediated isothermal amplification (LAMP) has become a popular detection method due to its operational simplicity and speed.

Using the WarmStart® Colorimetric LAMP 2X Master Mix (NEB), the human apolipoprotein L1 gene (apoL1) was detected in just 30 min using an Eppendorf ThermoMixer® C equiped with an Eppendorf ThermoTop®. The sensitivity of this test is as high as 0.1 ng with 45 min incubation.

Introduction

The amplification of nucleic acids is a widespread tool for the detection of various microorganisms and pathogens. Besides commonly used PCR-based methods, isothermal amplification offers an alternative for the detection of nucleic acids. Recently, the interest in isothermal amplification grew rapidly because of the Covid-19 pandemic. Some isothermal amplification methods are now already in use as PoC-NAT-tests (Point of care nucleic acid amplification tests) for the Coronavirus.

One of these methods is loop-mediated isothermal amplification (LAMP). LAMP was developed in 2000 by Notomi et al. [1]. During the reaction, a dumbbell-shaped DNA loop is created through primer annealing and the amplification is performed by polymerases with strand-displacement activity. This eliminates the need for an additional denaturation step and leads to continuous amplification of DNA under isothermal conditions. By adding reverse transcriptase to the assay, LAMP can also be used for the detection of RNA.

Successful LAMP reactions can be instantly observed through turbidimetry or colorimetric dyes without the necessity of an additional time-consuming step like gel electrophoresis. The simplicity of the LAMP method even allowed the development of a SARS-CoV-2-Test that can be performed at home using only a smartphone and no laboratory equipment [2].

In this article we use the Eppendorf Thermomixer C to optimize a LAMP protocol for the detection of the human apoL1 gene with two different polymerases. We further assess the detection sensitivity of the LAMP

Material and Methods

The human apolipoprotein L1 gene (apoL1) was amplified in 0.1 ml Eppendorf PCR tube strips from human genomic DNA (Promega) using a 10X primer mix prepared with 20 µM of FIP and BIP primers and 8 µM of F3 and B3 primers. The primer sequences used are listed in Table 1 [3].

Primer	Sequence (5′ – 3′)
F3	TCAGAGGTCATCTCACCCAC
B3	TTCTTCTCCATCACCCCAGA
FIP	GACCCTGGGCAAGTCATTCCCCAACTTGTGCCAGGCCCTG
BIP	GAGCAGGTAGATCATAGGACAACAGAGGGACAGAGGTGAG

Table 1: Sequences of LAMP primers for amplification of human ApoL1 [3].

The reaction was performed using the WarmStart® Colorimetric LAMP 2X Master Mix (NEB, Cat. No.: M1804S) consisted of 10 µl WarmStart Colorimetric LAMP 2X Master Mix, 2 µl primer mix, 1 µl of template DNA and 7 µl nuclease-free H_2O.
Reactions were performed using the Isothermal Master Mix (OptiGene, Cat. No.: ISO-004nd) containing 12 µl of the Isothermal Master Mix, 2 µl primer mix, 1 µl of template DNA and 5 µl nuclease-free H_2O.

Gradient optimization

In the first experiment, all samples were prepared with the WarmStart Master Mix and containing 50 ng of template DNA each. The samples were incubated on a Mastercycler X50a using a temperature gradient to find the optimal reaction temperature. The gradient was programmed vertically ranging from 59.8 °C to 68 °C, and samples were placed at 61.3 °C, 62.9 °C, 64.9 °C and 66.5 °C. After incubation for 45 min, successful reactions were observed through the colorimetric dye included in the master mix and additionally verified using 1.5 % agarose gel electrophoresis.

Assessment of test sensitivity

To observe how quickly lower template concentrations can be detected, the template DNA was diluted and the color of the colorimetric dye was examined at different timepoints. The final samples contained the WarmStart Colorimetric LAMP 2X Master Mix with 0.001 ng, 0.01 ng, 0.1 ng, 1 ng, 10 ng, 50 ng or 100 ng of template DNA and were incubated at 65 °C on the Eppendorf ThermoMixer® C for up to 60 min. The ThermoMixer® C was equipped with ThermoTop® to prevent the formation of condensate in small vessels, thus keeping the reaction volume constant and temperature homogenous [4]. During the incubation, samples were taken out after 15 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min and 55 min and photographed for later comparison of their color. The samples were put back on the heat as quickly as possible, so that they would be incubated for the full 60 min in total and the effect on the reaction would be as small as possible.

Assessment of LAMP technique

To assess the robustness of the loop-mediated isothermal amplification technique, a second LAMP experiment was performed using a different reaction kit (Isothermal Master Mix). The LAMP reaction containing the master mix and 50 ng of template DNA were incubated at 65 °C on the Eppendorf ThermoMixer C with the ThermoTop for 45 min. Additionally, primer-specific PCR was performed using the F3/B3 primers for comparison. PCR samples consisted of 10 µl GoTaq® Green Master Mix (Promega, Cat. No.: M7122), 2 µl F3/B3 primers (final concentration: 1 µM), 1 µl template DNA (50 ng) and 7 µl nuclease-free H_2O. The PCR protocol described in Table 2 was run on the Mastercycler X50a. All amplified DNA was subsequently visualized on a 1.5 % agarose gel.

Header	Lid	105°C	
	Temperature mode	Standard	
	Block settings	Aluminum 96	
	Energy saving mode	ON	
PCR	**Temp [°C]**	**Time [mm:ss]**	**Cycles**
Pre-heating	95	pause	1
Initial denaturation	95	2:00	
Denaturation	95	0:30	30
Annealing	Gradient 60 – 68 °C	1:00	
Elongation	72	1:00	
Final elongation	72	5:00	1
	4	pause	

Table 2: PCR program for amplification of ApoL1 with F3/B3 primers.

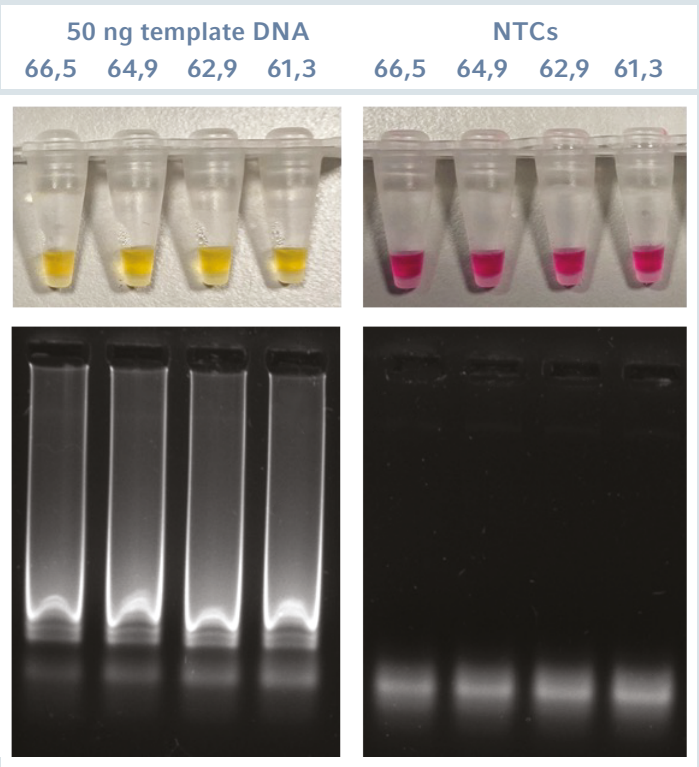

Figure 1: Results of the LAMP reaction using the WarmStart Colorimetric LAMP 2X Master Mix with different incubation temperatures. Shown is the color of the reaction samples with 50 ng of template DNA and the NTCs (No template control samples), as well as the reactions loaded on a 1.5 % agarose gel.

Results and Discussion

Gradient optimization

The WarmStart LAMP reaction was performed using a temperature gradient around 65 °C to determine the actual optimal reaction temperature based on the manufacturers' recommendation of the optimal temperature given for the polymerase, as well as this apoL1 LAMP system [3].

The colorimetric dye contained in the WarmStart kit is originally pink and will turn yellow upon successful amplification of DNA. The results showed that all LAMP reactions containing template DNA were successful regardless of the incubation temperature. This result was further verified by agarose gel electrophoresis, as shown by the characteristic smear pattern indicating positive reactions. Although not quantitative, all four reactions showed similar band intensity, indicating that the isothermal system being tested is not temperature sensitive within the defined range (Figure 1). As such, 65 °C was selected as the incubation temperature for subsequent experiments for the reasons: 1) WarmStart kit recommendation, 2) ApoL1 LAMP reaction protocol [3] and 3) Isothermal Master Mix recommendation.

Figure 2 LAMP reactions with template dilutions after different incubation times. Color change from pink to yellow indicates positive amplification. Discoloration of pink colorimetric dye indicates over incubation, whereupon observation of further incubation is not longer reliable.

Assessment of test sensitivity

The colorimetric dye contained in the WarmStart Colorimetric LAMP 2X Master Mix allowed instant identification of amplification of DNA during the incubation of the LAMP reaction. This in turn, allowed qualitative assessment of the sensitivity of the LAMP assay. As expected, the reaction took longer to change color with less template DNA (Figure 2).

The samples with 100 ng, 50 ng and 10 ng of template DNA were observed to begin to change color after 25 min. After 30 min, they had turned completely yellow, indicating positive detection of the apoL1 gene.

Under tested conditions, this protocol showed a sensitivity limit of 0.1 ng template DNA, which started to turn positive yellow after 45 min. After incubation for 60 min, the color of the NTC was observed to have started to change, indicating that the assay was no longer reliable with further heating. Hence, the assay was terminated at this time point and no further incubation was tested.

Assessment of LAMP technique

To assess the viability of the loop-mediated isothermal amplification (LAMP) technique for DNA detection, the apoL1 gene experiment was repeated using another reaction kit without a colorimetric dye.

The results, processing times and costs were compared to primer-specific PCR technique to analyze respective pros and cons.

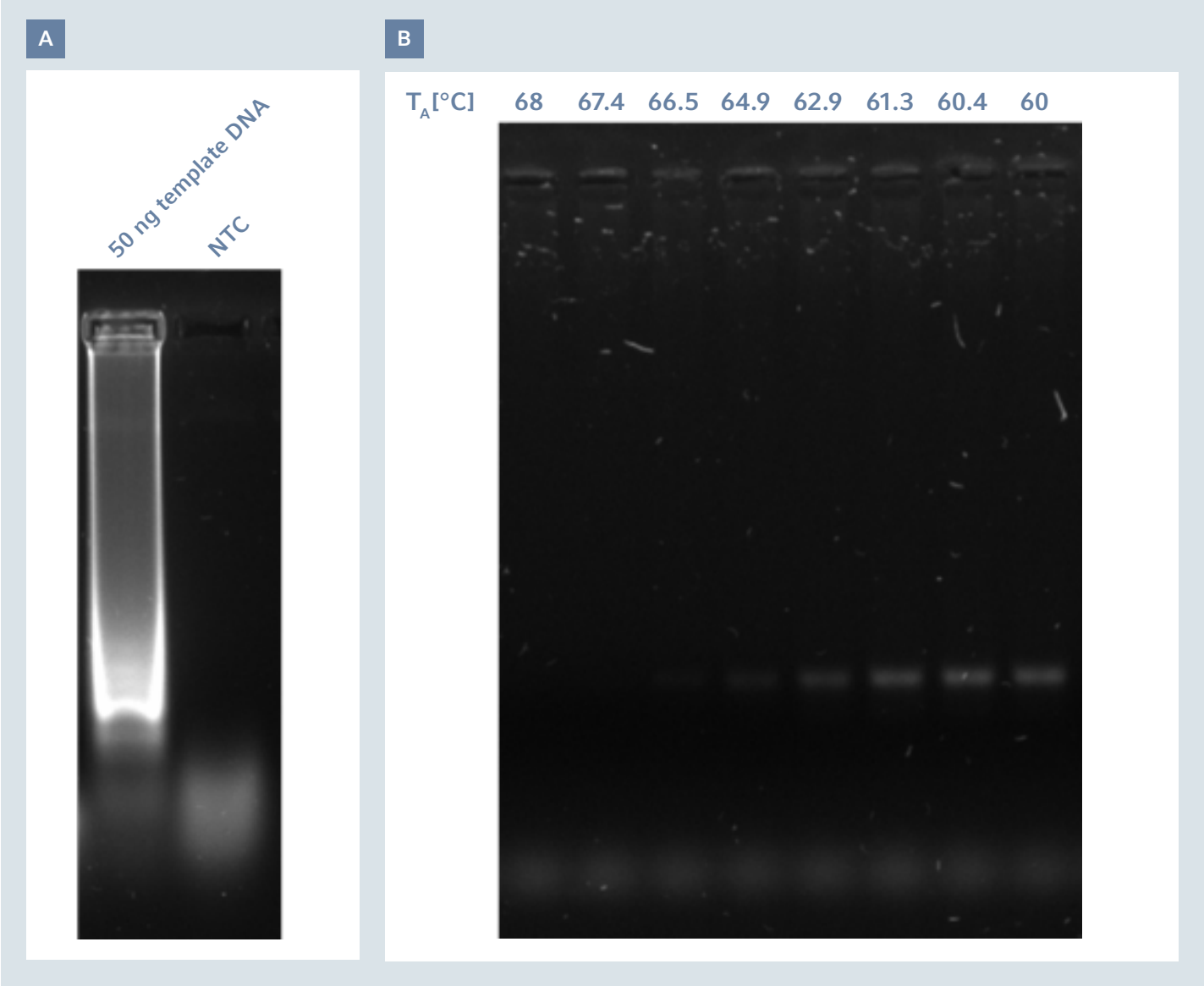

Figure 3: Comparison of amplification of apoL1 gene via LAMP vs. conventional primer-specific PCR techniques. **(A)** Results of the LAMP reaction performed at 65 °C using the Isothermal Master Mix. **(B)** Primer-specific PCR using F3/B3 primers with 50 ng of template DNA showed successful amplification with ca. 60-65 °C annealing temperature (T_A).

Amplification of the apoL1 gene was successfully achieved using the Isothermal Master Mix, as was shown by the characteristic smear on the gel (Figure 3A). The reaction took 45 min. In comparison, primer-specific PCR technique used to achieve positive DNA amplification of the ~200 bp amplicon (Figure 3B) took 1:45 h.
For LAMP kits that do not contain a colorimetric dye like Isothermal Master Mix, visualization of DNA amplification can be done via gel electrophoresis, which is the same as PCR. Hence, while cost and detection results are comparable, LAMP detection of this gene system has a slight total run time advantage over traditional primer-specific PCR. However, existing fast PCR reagents in the market can speed up PCR reactions. Additionally, the PCR protocol can still be optimized to reduce its total run time. Hence, both LAMP and PCR techniques are considered to be comparable to each other as a viable DNA detection technique.

However, with the introduction of a LAMP kit pre-mixed with colorimetric dye for direct end-point detection, the scale tilts over to the side of the LAMP technique, as it does not require additional equipment and saves the step of running a gel. Although similar results might be achievable using qPCR on the side of primer-specific detection technique, a real-time cycler is required. The cost of qPCR kits is also generally higher than end-point PCR kits, albeit comparable to commercial colorimetric LAMP kits.
However, inexpensive colorimetric dyes (e.g. hydroxynaphthol blue) could be used for visualization to circumvent the issue of higher cost. This would lower the cost of LAMP assays compared to using commercial colorimetric LAMP kit, but it would require the user to put effort in optimizing the required concentrations and parameters for the protocol.

Conclusion

The LAMP technique proves to be a viable method for DNA detection, as shown herein whereby the apoL1 gene was positively identified after 30 min. Moreover, this technique has high sensitivity of up to 0.1 ng template DNA, fast reaction completion time can be directly visualized via simple colorimetric detection.

LAMP assays can be carried out successfully using "contamination-free" dry block incubation with Eppendorf Thermomixer® C, augmented by Eppendorf ThermoTop® heated lid to keep stable incubation reaction environment by preventing the formation of condensate in the vessels. The use of Mastercycler® X50 allows fast and simple optimization of reaction temperature when setting up a new LAMP assay.

Literature

[1] Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic acid research, 28 (12).
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[3] Kanchanaphum, P. (2018). Identification of human DNA by loop-mediated isothermal amplification (LAMP) technique combined with white ring precipitation of $Cu(OH)_2$. Songklanakarin Journal of Science and Technology, 40 (4), 738 – 742.
[4] Käppler-Hanno, K. Eppendorf ThermoTop® - condens.protect®. Effective protection against sample condensation. Eppendorf White Paper (9): December 2012.

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