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OPTIMIZATION AND EFFICIENCY ANALYSIS OF REAL-TIME PCR FOR LEPTOSPIRA SPP. DIAGNOSIS BASED ON THE LIPL32 GENE

Optimasi dan Analisis Efisiensi Real-Time PCR untuk Diagnostik *Leptospira spp.* Berbasis Gen LipL32

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ABSTRACT
Leptospirosis is a widespread zoonotic infection that endangers the health of both humans and animals, particularly in tropical and subtropical regions. Therefore, timely, sensitive and specific laboratory confirmation is essential for early clinical management. The *LipL32* gene is a highly conserved virulence factor in pathogenic *Leptospira*. Real-time PCR provides rapid detection with high sensitivity and specificity. This study optimized real-time PCR conditions by evaluating annealing temperatures (60°C, 61°C, 62°C), primer concentrations (500 nM, 700 nM, 900 nM), and probe concentrations (250 nM, 300 nM). PCR efficiency was analyzed using absolute quantification with serial DNA dilutions (10^0 to 10^{-4}). The optimal conditions were 60°C annealing temperature, 500 nM primers, and 300 nM probes. Near-ideal efficiency (97%) was achieved at 60°C with 500 nM primers and 250 nM or 300 nM probes, while 103% efficiency was observed at 61°C with 500 nM primers and 250 nM probes. This optimization enhances *Leptospira* detection accuracy using real-time PCR.

Keywords: *Leptospirosis, LipL32, Molecular Diagnosis, PCR efficiency, Real-time PCR*

ABSTRAK
Leptospirosis adalah infeksi zoonosis yang tersebar luas yang membahayakan kesehatan manusia dan hewan, terutama di daerah tropis dan subtropis. Oleh karena itu, konfirmasi laboratorium yang tepat waktu, sensitif dan spesifik sangat penting untuk penanganan klinis dini. Gen *LipL32* telah diidentifikasi sebagai faktor virulensi yang sangat terkonservasi dalam semua *Leptospira* patogen. Real-time PCR menawarkan deteksi cepat dengan sensitivitas dan spesifisitas tinggi. Penelitian ini mengoptimasi kondisi real-time PCR dengan mengevaluasi suhu annealing (60°C, 61°C, 62°C), konsentrasi primer (500 nM, 700 nM, 900 nM), dan konsentrasi probe (250 nM, 300 nM). Efisiensi PCR dianalisis menggunakan absolute quantification dengan pengenceran serial DNA (10^0 hingga 10^{-4}). Kondisi optimal yang diperoleh adalah suhu annealing 60°C, primer 500 nM, dan probe 300 nM. Efisiensi reaksi mendekati ideal (97%) diperoleh pada suhu annealing 60°C dengan primer 500 nM dan probe 250 nM atau 300 nM, sedangkan efisiensi 103% diperoleh pada suhu 61°C dengan primer 500 nM dan probe 250 nM. Optimasi ini penting untuk meningkatkan akurasi deteksi *Leptospira spp.* menggunakan real-time PCR.

Kata kunci: *Diagnostik Molekuler, Efisiensi PCR, Leptospirosis, LipL32, Real-time PCR*

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INTRODUCTION

Leptospirosis is a zoonotic disease caused by infection with *Leptospira* spp., a bacterium widely distributed across various regions, particularly in tropical and subtropical areas. This disease has become a serious public health challenge, with the number of cases increasing each year. According to the latest data, more than one million cases of leptospirosis occur worldwide annually, resulting in approximately 60,000 deaths (Wang and Dunn 2024). Transmission occurs through contact with the urine of infected animals, either directly or through contaminated environments (Limothai et al. 2021).

Early diagnosis of leptospirosis is crucial to reducing disease severity and preventing further complications. Various diagnostic methods have been used, including serological tests such as the Microscopic Agglutination Test (MAT) and molecular-based techniques like Polymerase Chain Reaction (PCR). Although MAT remains the gold standard, it has limitations in terms of time and the need for specialized laboratory facilities. In contrast, real-time PCR offers advantages such as rapid detection, high sensitivity and specificity, and quantification capability (Waggoner et al. 2015; Gayathri et al. 2022; Valente et al. 2024).

The outer membrane of Leptospira consists of antigenic and virulence-determining components, including lipoproteins, lipopolysaccharides and peptidoglycans. The outer membrane of spirochetes and gram-negative bacteria acts as a permeability barrier (Villarreal-Julio et al. 2022). Among the outer membrane components of *Leptospira*, Leptospira lipoprotein 32 (LipL32) is the most abundant outer membrane component found in pathogenic *Leptospira* but is absent in non-pathogenic strains (González et al. 2013; Hsu and Yang 2022). Therefore, real-time PCR methods targeting this gene have great potential to improve the accuracy of leptospirosis diagnosis. However, amplification efficiency in real-time PCR is influenced by several key factors, including annealing temperature, primer concentration, and probe concentration. Optimizing these parameters aims to enhance specificity, sensitivity, and amplification efficiency to ensure more accurate and reliable detection results (Karuniawati et al. 2012).

Leptospira thrives and persists in the kidneys of reservoir animals. Rodents, particularly rats, serve as the primary vectors or reservoirs for leptospirosis. Efforts to confirm the presence of Leptospira and the predominant serovar varieties in the environment can be conducted through rodent surveillance and bacterial identification in these animals (Otto et al. 2015). Kidney samples from rats can be used for culture examination and PCR analysis (Handayani, 2019). The relative kidney weight of a rat weighting approximately 200 grams is around 0.8 grams (Annisa et al., 2021).

Although real-time PCR targeting the LipL32 gene is widely used for detecting Leptospira, the efficiency of the assay still varies markedly depending on the oligonucleotide and thermocycling parameters used. Comprehensive optimisation studies are lacking, particularly for samples derived from reservoir animals in the field (Galloway and Hoffmaster 2015; Green and Sambrook 2018). This study therefore addresses this gap by systematically evaluating the effects of varying the annealing temperature, primer and probe concentrations on the efficiency of a LipL32-based real-time PCR assay for the accurate detection of Leptospira spp.

METHODS

Study site and Materials

The research was conducted at the Molecular Biology Laboratory of Loka Public Health Laboratory Pangandaran, located at JL. Raya Pangandaran KM 3, Babakan Village, Pangandaran District, Pangandaran Regency, from September 2024 to December 2024.

The materials used in this study included the Geneaid DNA Mini Kit (Tissue), GoTaq Probe qPCR Master Mix Kit (Promega), LipL32 Target Gene Primers and Probe Set, Nuclease-Free Water, and kidney tissue samples from rats that were confirmed positive for *Leptospira spp.*

Procedur

DNA Isolation Procedur

DNA isolation was performed from rat kidney tissue using the Geneaid DNA Mini Kit (Tissue). A 30 mg tissue sample was transferred into a 1.5 mL microtube containing 200 µL lysis buffer and 20 µL Proteinase K, then incubated at 60°C for 30 minutes until the tissue was completely degraded. After incubation, the lysate was then transferred to a spin column and centrifuged at 14.000 ×g for 2 minutes at room temperature. Subsequent wash steps were performed in accordance with the manufacturer's protocol, with centrifugation at 14.000 ×g for 1 minute after each step. The purified DNA was eluted in 100 µL of elution buffer.

Serial DNA Dilution

The isolated DNA was serially diluted 10-fold across 5 variations, each with a final volume of 100 µL.

Real-Time PCR Reaction Setup

The PCR reaction was carried out using the real-time PCR method with the GoTaq Probe qPCR Master Mix (Promega). The reaction mix had a total volume of 20 µL, which included the master mix, primers and probe from Stoddard (2013). A 5 µL volume of DNA template was added to each reaction.

To determine the optimal conditions, variations were tested for annealing temperatures (60°C, 61°C, and 62°C), primer concentrations (500 nM, 700 nM, and 900 nM), and probe concentrations (250 nM and 300 nM). All PCR reactions were run on a Qiagen Rotor-Gene Q Thermal Cycler using an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds (denaturation), the varied annealing temperature, and a 1-minute extension.

PCR Efficiency Analysis

PCR amplification efficiency was evaluated using the absolute quantification method by constructing a linear regression curve from the amplification results of serially diluted DNA. The slope of the standard curve was used to calculate the reaction efficiency, with an optimal range between 90-110%. Q-Rex software was utilized to analyze both the slope and the coefficient of determination (R^2). The results of this analysis were used to determine the optimal PCR conditions to ensure maximum amplification efficiency.

RESULT AND DISCUSSION

Amplification Curve

The real-time PCR amplification results showed variations in cycle quantification (Cq) values based on changes in annealing temperature, primer concentration, and probe concentration. The results indicated that the Cq values increased with higher dilution levels, reflecting a decrease in the amount of target DNA. Positive controls consistently produced results across all temperatures, while the no-template control (NTC) showed no amplification (Cq = 0), confirming that the reaction was free from contamination (Table 1).

Table 1 Cq value of the real-time PCR amplification result

Dilution	Cq value for Primer Concentration of 500 nM						Cq value for Primer Concentration of 700 nM						Cq value for Primer Concentration of 900 nM					
	Probe 250 nM			Probe 300 nM			Probe 250 nM			Probe 300 nM			Probe 250 nM			Probe 300 nM		
	60°C	61°C	62°C	60°C	61°C	62°C	60°C	61°C	62°C	60°C	61°C	62°C	60°C	61°C	62°C	60°C	61°C	62°C
10^0	21.70	21.83	21.59	21.86	21.50	21.96	21.68	2170	22.95	21.91	21.88	21.29	21.59	23.10	21.71	21.49	21.97	21.37
10^{-1}	23.25	25.32	25.37	25.06	25.41	24.85	25.41	25.16	25.38	25.31	25.03	24.24	25.12	25.04	24.85	24.88	25.04	25.30
10^{-2}	27.47	28.97	28.76	28.92	28.95	29.13	28.43	29.68	29.42	26.96	29.06	29.12	28.74	29.04	28.60	28.81	29.51	28.55
10^{-3}	30.73	31.31	31.57	34.06	32.02	32.64	31.24	31.41	31.79	30.81	31.64	32.23	31.54	32.15	32.37	32.32	31.66	30.06
10^{-4}	34.93	35.11	34.25	34.34	33.31	35.60	34.15	37.20	35.11	34.66	34.22	36.08	32.28	34.71	38.02	35.41	37.00	38.02
PC	22.89	21.95	22.45	21.96	21.95	22.07	21.76	22.34	22.07	21.74	22.34	22.07	21.69	22.34	22.07	21.60	22.34	22.07
NTC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

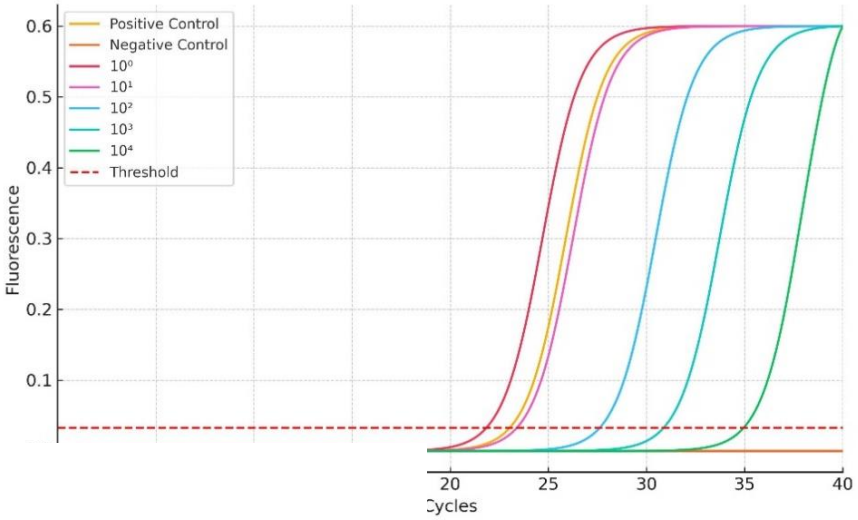


Figure 1 Amplification Curve at an Annealing Temperature of 60°C, Primer Concentration of 500 nM, and Probe Concentration of 250 nM

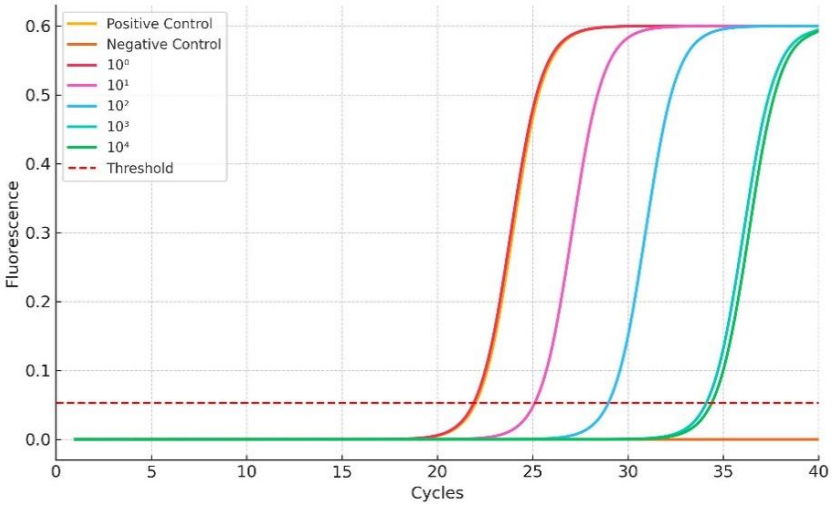


Figure 2 Amplification Curve at an Annealing Temperature of 60°C, a Primer Concentration of 500 nM, and a Probe Concentration of 300 nM

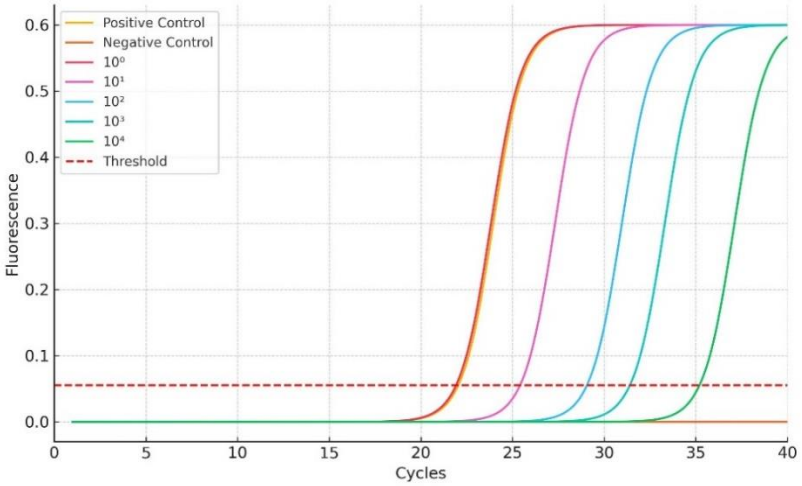


Figure 3 Amplification Curve at an Annealing Temperature of 61°C, a Primer Concentration of 500 nM, and a Probe Concentration of 250 Nm

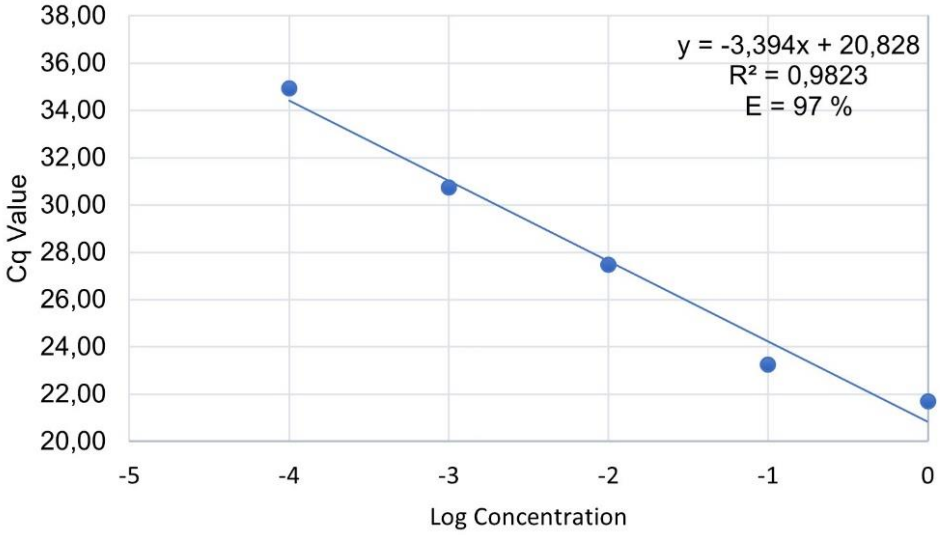


Figure 4 Linear Regression Plot at an Annealing Temperature of 60°C, Primer Concentration of 500 nM, and Probe Concentration of 250 nM

The results of the amplification curve at an annealing temperature of 60°C using a primer concentration of 500 nM and a probe concentration of 250 nM (Figure 1) gave sequential Cq values of 21.70; 23.25; 27.47; 30.73; 34.93. A coefficient of determination (R^2) value of 0.9823 was obtained with a linear regression equation y = -3.394x + 20.828 and a reaction efficiency (Eff.) value of 97%. At an annealing temperature of 60°C, using 500 nM primer concentration and 300 nM probe concentration (Figure 2), the Cq values were 21.86; 25.06; 28.92; 34.06; 34.34. The R^2 value was 0.9570, with a linear regression equation y = -3.396x + 22.056 and a reaction efficiency (Eff.) of 97%. At an annealing temperature of 61oC, using a primer concentration of 500 nM and a probe concentration of 250 nM (Figure 3), the Cq values were 21.83; 25.32; 28.97; 31.31; 35.11, respectively. The R^2 value obtained was 0.99568 with a linear regression equation y = -3.255x + 21.998 and a reaction efficiency (Eff.) of 103%.

The correct annealing temperature is crucial for ensuring optimal primer binding. If the annealing temperature is too high, primer binding becomes challenging, leading to less efficient PCR product formation; however, if it is too low, the reaction may become nonspecific (Thermo Fisher Scientific, 2023). Based on the optimization results, an annealing temperature of 60°C yielded reaction efficiency values most consistently within the 90-110% range. This finding is supported by the research of Ahmed et al., (2020), which demonstrated that optimizing the hybridization, annealing, and extension temperatures to 60°C is ideal for the DNA polymerase enzyme used in the LiPL32 amplification reaction.

Primer concentration can significantly affect amplification efficiency and the formation of primer dimers, potentially leading to inaccurate results. The primer concentrations used in this study were 500 nM, 700 nM, and 900 nM, in accordance with the recommended concentrations for the Promega master mix reagents. Among these, 500 nM yielded reaction efficiencies most consistently within the 90% to 110% range across all annealing temperature variations.

In molecular diagnostics, LipL32 is targeted in PCR assays, with an optimized protocol that enhances sensitivity by fine-tuning both primer and probe concentrations (Galloway and Hoffmaster 2015). If the primer concentration is too high, it can lead to saturation of the real-time PCR amplification curve, resulting in increased artifacts and potential misinterpretation of results. Conversely, if the concentration is too low, the target may not be detected, as fewer template molecules require more cycles to reach the threshold, thereby increasing the Cq value and potentially leading to false negatives (Ng, 2023).

A probe concentration of 300 nM yielded reaction efficiency values most frequently within the 90% to 110% range compared to other concentrations. Primer and probe concentrations must be adjusted—often through a series of PCR reactions with varying concentrations—to achieve optimal reaction efficiency and reproducibility (Green and Sambrook 2018). Lower primer and probe concentrations may cause the real-time PCR amplification curve to plateau at lower fluorescence intensities, underscoring the importance of finding the optimal balance to avoid these issues (Ng 2023).

Achieving a reaction efficiency of 100% is the ideal theoretical target but is rarely met in practice due to various factors that influence the PCR process. Reaction efficiency is critical for accurate real-time PCR analysis, as it affects the cycle quantification (Cq) values and, consequently, the estimation of the target nucleic acid concentration (Ruijter et al. 2021). Determining the reaction efficiency is one of the initial steps in setting up a real-time PCR assay.

Lower efficiency values are typically attributed to suboptimal primer design, reagent concentrations, or reaction conditions. Secondary structures—such as primer dimers and hairpins—or an incorrect melting temperature (Tm) can adversely affect primer annealing, leading to poor amplification. Additionally, since each successive dilution contains a lower amount of initial DNA, differences in Cq values occur among serially diluted samples (Biosistemika 2017)

Absolute Quantification Analysis

Absolute quantification in real-time PCR is a method used to determine the exact number of nucleic acid molecules in a sample, providing a more precise measurement compared to relative quantification. This technique involves using a standard curve to calculate the initial quantity of the target sequence, thereby enabling statistical confidence in the results (Farrell 2023). Absolute quantification is performed by serially diluting the DNA template to determine the number of target copies in the test sample. The Cq value from each dilution level is then plotted against the standard curve to determine PCR efficiency.

Table 2 Reaction Efficiency Results

[Primer Consentration]. [Probe concentration]	*Annealing Temp.*		
	60ºC	61ºC	62ºC
500 nM 250 nM	97%	103%	108%
500 nM 300 nM	97%	114%	93%
700 nM 250 nM	111%	86%	112%
700 nM 300 nM	110%	109%	85%
900 nM 250 nM	129%	114%	77%
900 nM 300 nM	92%	87%	83%

Table 2 presents the reaction efficiency values for each variation of primer concentration (500 nM, 700 nM, and 900 nM), probe concentration (250 nM and 300 nM), and annealing temperature (60°C, 61°C, and 62°C). A positive control (PC) using leptospira-positive kidney samples was performed to confirm the functionality of the nucleic acid template or the primer-probe set, while a negative template control (NTC) using nuclease-free water (NFW) was used to detect any contamination in the PCR reagents.

Reaction efficiencies approaching 100% were achieved at an annealing temperature of 60°C with a primer concentration of 500 nM and probe concentrations of both 250 nM and 300 nM, resulting in a reaction

efficiency of 97%. Additionally, at an annealing temperature of 61°C with a primer concentration of 500 nM and a probe concentration of 250 nM, the reaction efficiency reached 103%.

The reliability of the standard curve is influenced by factors such as reaction efficiency and correlation coefficient, which must be optimized across different PCR systems to ensure consistent results (Than et al. 2023). However, challenges arise at low concentrations, where the log-linear model of the standard curve may not accurately capture variability. This necessitates an enhanced modeling approach that incorporates probabilistic concepts to better account for random errors and improve data representation at these levels. This is particularly critical in applications where low concentrations are crucial, as inaccurate analysis can affect interpretation and decision-making(Whale et al. 2022; Schmidt et al. 2023). Therefore, the development and validation of robust standard curves is essential for accurate absolute quantification in real-time PCR, ensuring reliable results.

Although the optimisation produced promising results, there are several areas that could be improved in the future. Our study focused on the LipL32 gene and was conducted using a single real-time PCR platform and DNA extracted from rat kidney samples. Therefore, broader evaluations across additional genes, specimen types and instruments were beyond the scope of this study. Similarly, the absence of a melt-curve step means there is an extremely small risk of a non-specific signal being produced. Future studies should address these issues by validating these conditions on additional virulence genes, diverse clinical/environmental specimens and multiple real-time PCR instruments to confirm robustness.

CONCLUSION

Based on the research findings, the optimal annealing temperature was determined to be 60°C, with a primer concentration of 500 nM and a probe concentration of 300 nM. Reaction efficiencies close to 100% were achieved at 60°C using a primer concentration of 500 nM along with probe concentrations of 250 nM and 300 nM, resulting in an efficiency of 97%. Additionally, at an annealing temperature of 61°C with a primer concentration of 500 nM and a probe concentration of 250 nM, the reaction efficiency reached 103%.

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