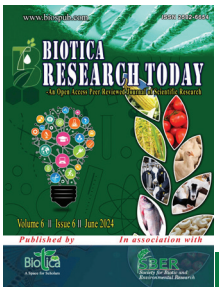
DOI: 10.54083/BioResToday/6.6.2024/327-335

Bio. Res. Today 2024, 6(6):327-335



Biotica Research Today

e-ISSN: 2582-6654

June, 2024

Article ID: RT1607 **Research Article**

Optimization of PCR Conditions for Improved Amplification Efficiency and Specificity on *PfHRP2/3* Genes Deletion in *Plasmodium falciparum*

Yusuf A.M.[1*], Umar A.M.[2], Eberemu N.C.[2], Auta T.[2], Wagini N.H.[1], Gidado S.M.[1], Zainab A. Yar'adua[1], Matazu N.U.[3] and Suleiman M.[1]

[1]Dept. of Biology, Faculty of Natural and Applied Sciences, Umaru Musa Yar'adua University Katsina, Katsina State (PMB 2218), Nigeria

[2]Dept. of Biological Sciences, Faculty of Life Sciences, Federal University Dutsinma, Katsina State (PMB 5001), Nigeria

[3]Dept. of Biochemistry, Faculty of Natural and Applied Sciences, Umaru Musa Yar'adua University Katsina, Katsina State (PMB 2218), Nigeria

Open Access

Corresponding Author

Yusuf A.M.

musa.aliyu@umyu.edu.ng

Conflict of interests: The author has declared that no conflict of interest exists.

How to cite this article?

Yusuf, A.M., Umar, A.M., Eberemu, N.C., *et al*., 2024. Optimization of PCR Conditions for Improved Amplification Efficiency and Specificity on PfHRP2/3 Genes Deletion in Plasmodium falciparum. *Biotica Research Today* 6(6), 327-335. DOI: 10.54083/BioResToday/6.6.2024/327-335.

Abstract

This study aimed to optimize PCR conditions for improved specificity and sensitivity by investigating the effects of DNA template dilutions, magnesium chloride ($MgCl_2$) titration concentrations, annealing temperatures and primer dimer formation. Real-Time PCR (RT-PCR) assays were conducted and agarose gel electrophoresis and polyacrylamide gel electrophoresis were employed for visualization and analysis of PCR products. For DNA template dilutions, RT-PCR revealed a concentration-dependent decrease in cycle threshold (CT) values, indicating higher initial copy numbers in undiluted DNA samples. Melting curve analysis confirmed single-species template DNA, while agarose gel electrophoresis demonstrated decreasing band intensities with dilution, alongside nonspecific amplification products in all samples. $MgCl_2$ titration concentrations showed optimal amplification at 1.5 mMol and 2 mMol, with weaker amplification at lower concentrations and nonspecific products at higher concentrations. Annealing temperature optimization revealed optimal efficiency at 58 °C and 55 °C, with reduced amplification at extremes and nonspecific products at higher temperatures. Primer dimer formation was observed, affecting amplification specificity, with the lower peak denaturing at a higher temperature indicative of primer dimerisation. Optimization strategies such as HotStart PCR and the use of monoclonal antibodies for Taq polymerase inhibition were discussed for improved specificity. Overall, systematic optimization of PCR conditions is crucial for achieving reliable and reproducible results, with considerations for DNA template concentration, $MgCl_2$ titration, annealing temperature and primer dimer formation essential for enhancing PCR performance.

Keywords: Annealing temperature, DNA template dilution, HotStart PCR, $MgCl_2$ titration, PCR optimization, Primer dimer

Introduction

The Polymerase chain reaction (PCR) refers to a technique employed to duplicate and increase the quantity of nucleic acids through cycles of heating and cooling at specific temperatures (95 °C, 60 °C and 72 °C) for set periods, conducted in PCR Thermocycler (programmed) (Rychlik *et al.*, 1990; Cha and Thilly, 1993). Basic single-step PCR (BPCR), PCR-restriction fragment length polymorphism (PCR-RFLP), nPCR; nested PCR and qPCR; real-time/ quantitative PCR are generally applied to identify diverse genetic sequences

Article History

RECEIVED on 08th April 2024 RECEIVED in revised form 07th June 2024 ACCEPTED in final form 14th June 2024

(Deepachandi *et al.*, 2019). Cobb and Clarkson (1994) described classical PCR as comprising three key steps: denaturation (where DNA melts and hydrogen bonds are disrupted, yielding two single-stranded DNAs), annealing (where primers bind to the DNA template) and elongation (where new double-stranded DNA is synthesized). According to Kwok *et al.* (1990), PCR mastermixes typically contain two short single-stranded primers specific to the target region, a heat-resistant DNA polymerase like Taq polymerase with a reaction buffer with magnesium and dNTPs. The amplified product or amplicon, can originate from various template sources, including synthetic, genomic, mitochondrial or plasmid DNA, as well as RNA sources like mRNA, miRNA or total RNA (Rychlik *et al.*, 1990). In Reverse Transcription-PCR (RT-PCR), RNA is copied using reverse transcriptase to produce cDNA; complimentary DNA, which subsequently acts as the template for the amplification process in PCR. This technique is essential for detecting RNA and for studies examining gene expression and the effects of mutations on mRNA stability. Standard PCR typically need post PCR amplification investigation, likes of agarose/ polyacrylamide gel-electrophoresis to evaluate the results (Stift *et al.*, 2003).

qPCR stand for the method that allows for the monitoring of PCR progression in real time, recording the accumulation of the product throughout the entire process within the PCR tube, eliminating the need for post-PCR analysis. This approach fundamentally changes how some may conduct qPCR for RNA and DNA (Maeda *et al.*, 2003; Rajalakshmi, 2017). During qPCR, the accumulation of the product is tracked by a rising fluorescent signal and two main chemistries are employed for detecting the PCR product: TaqMan, which uses the fluorogenic 5' nuclease activity and SYBR Green dye, a fluorescence molecule that binds to DNA (Paul *et al.*, 2010). Fluorescence is recorded at each cycle and the cycle at which the initial detectable signal emerges is termed the 'cycle threshold' (CT-value). The CT-value is the key for data analysis; a lower CT-value signifies a higher initial concentration of the template, whereas a higher CT-value indicates a lower starting concentration. The quantitative assessment of the PCR product is obtained from the CT-value, which is established using a standard curve (Toz *et al.*, 2013; Chen *et al.*, 2022).

SYBR Green dye is employed as a double-stranded DNA binding dye for detecting the accumulation of PCR products during cycles. It has an affinity for detecting dsDNA as well as nonspecific reactions within PCR products, yielding accurate results with properly optimized reactions. SYBR Green dye binds to both specific and nonspecific DNA products, producing amplification signals for all bound products (Salam *et al.*, 2010; Yehia *et al.*, 2012; Toz *et al.*, 2013; Mohammadiha *et al.*, 2017; Teimouri *et al.*, 2018).

After polymerization, PCR reactions are manually transferred onto polyacrylamide or agarose electrophoresis gels blotted with either SYBR green or an ethidium bromide dyes. This process allows the identification of the target products based on their sizes, which are then viewed under UV light, a method that has seen significant advancements (Erlich *et al.*, 1991). The DNA amplification as well as examination can be performed concurrently using SYBR Green as DNA binding dye, which specifically binds to double stranded DNA (dsDNA). These dyes are incorporated into mastermix to quantify the template during amplification. Fluorescence accumulates and is recorded in each cycle during the elongation phase, crossing the threshold line depending on the initial template concentrations (Gibson *et al.*, 1996). As SYBR Green fluorescence is tracked from start to finish during the temperature cycles, the rapid loss of fluorescence near the DNA's dissociation temperature indicates denaturation (Wittwer *et al.*, 1997). This occurs because the melting curves of DNA products are influenced by their AT/GC content, sequences, lengths and can be differentiated by these unique melting curves observed during PCR cycles as the temperature progresses through the denaturation step, affecting dsDNA fluorescence (Roux, 1995).

Due to SYBR Green's ability to attach to every dsDNA product inside reactions, issues such as the detection of weak signals, multiple bands or missing bands on the gel frequently arise. Several factors contribute to these problems, including DNA template dilutions, the materials or sources of DNA extraction, annealing-temperature, hydrogen bonding, dNTPs, The quantity and nature of primers utilized, enhancers and the concentrations of $MgCl_2$ (Roux, 1995; Wittwer *et al.*, 1997). These disparities necessitate PCR optimizations tailored to entity variables (Hecker and Roux, 1996).

DNA template dilutions play a crucial role in PCR as they form the starting amplification templates. Using higher amounts or dilutions of DNA can result in nonspecific products, reducing reaction specificity (Cha and Thilly, 1993). Excessive DNA dilutions also affect primer annealing by binding to magnesium, thereby inhibiting polymerase activity. Thus, optimizing DNA dilutions is essential, as DNA concentrations depend on the extraction materials and the source of the DNA (Harris and Jones, 1997).

Low annealing temperatures can lead to the detection of non-specific bands, while high temperatures may result in weak or absent primer annealing to single-stranded DNA (ssDNA) templates, causing reduced performance. The optimal temperature for primer annealing is typically 3-5 °C less than melting temperature of primers that are less stable (Rychlik *et al.*, 1990). Although various formulas exist to calculate T_m, none provide a universally reliable temperature for PCR, necessitating experimentation at different temperatures to determine the best annealing temperature (Hecker and Roux, 1996). Thus, optimizing the annealing temperature is crucial.

Magnesium acts as a coenzyme for Taq DNA polymerase and is required in substantial amounts. the DNA template, primers, dNTP and taq polymerase all interact with magnesium, diminishing the pool of free magnesium accessible for the enzyme (Innis and Gelfand, 1990). Chelating agents present in the DNA template and primers, such as EDTA, can also combine with magnesium (Lam *et al.*, 2004). Excess magnesium stabilizes dsDNA, hindering the denaturation step and decreasing performance, while high magnesium concentrations can cause primers to

anneal to incorrect ssDNA templates. Conversely, low magnesium concentrations reduce Taq polymerase activity (Markoulatos *et al.*, 2002). Therefore, optimizing magnesium concentration is essential to ensure enough free magnesium is available for Taq polymerase function despite the binding of other reaction components.

Hot start techniques are used to eliminate non-specific PCR products synthesized at lower environmental temperatures before amplification begins (Salam *et al.*, 2010; Yehia *et al.*, 2012; Toz *et al.*, 2013; Mohammadiha *et al.*, 2017; Teimouri *et al.*, 2018). These techniques prevent Taq polymerase extension at low temperatures by targeting key reaction components such as primers, dNTPs, Taq polymerase and magnesium (D'Aquila *et al.*, 1991; Paul *et al.*, 2010). Current hot start methods include using wax barriers to separate reaction components before activation, accessory proteins, thermolabile modified chemicals, antagonistic DNA polymerase antibodies to obstruct the enzymatic activity and dNTPs (Paul *et al.*, 2010; Mohammadiha *et al.*, 2017).

The purpose of this research was to investigate how different dilutions of DNA templates impact PCR amplification through qPCR and agarose gel electrophoresis analysis. Furthermore, it aimed to assess the effectiveness of various magnesium concentrations and annealing temperatures in optimizing PCR through polyacrylamide gel electrophoresis, with the goal of gathering data to determine the most suitable dilution and concentration conditions for amplifying *P. falciparum* genes.

Materials and Methods

Optimized PCR Master Mixes for Amplification of DNA Template Dilutions

The PCR amplification strategy was used for amplification of *P. falciparum* DNA, in which specific primers pairs (rFAL1-rFAL2) was used of the reactions. The PCR assay was carried out utilising a PCR Systems 97000 Applied Biosystems (RT-PCR and classical Thermocycler. The method used by Carbonara *et al.* (2012) was adapted. In the amplification process, 1 µL volume of the extracted DNA obtained from blood containing *P. falciparum* parasite was used. An optimized PCR reaction was prepared for five reactions. Each reaction tube, labeled 1 through 5, contained 9.5 µL of water, 12.5 µL of 2x SYBR PCR master mix and 1 µL each of forward and reverse primers, resulting in a total volume of 24 µL of master mix per tube. Tubes 1, 2, 3 and 4 were then supplemented with 1 µL each of undiluted DNA, DNA diluted 1/10, DNA diluted 1/100 and water, respectively. The amplification of rPLU for genus-specific and rFAL for species-specific sequences was carried out using an Applied Biosystems thermocycler (PE 97000) in a one-step qPCR block. The PCR tubes were subjected to an initial denaturation at 94 °C for 2 minutes, followed by an amplification process consisting of 40 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute. A final elongation step at 72 °C for 3 minutes was performed at the end of the reaction.

The qPCR protocol was carried out as previously outlined. After completing 40 cycles, 15 µL from each PCR reaction - containing undiluted DNA, 1/10 and 1/100 DNA dilutions and a water control - were taken for analysis using 2% agarose gel electrophoresis. The gel was stained with ethidium bromide and run for 45 minutes at 80 volts in a 1X TAE buffer. Visualization and image capture were conducted using a gel documentation system (Samal *et al.*, 2022).

PCR Annealing Temperatures Optimisation

In a 1.5 ml Eppendorf tube, a mixture was prepared for six reactions, comprising 106.5 µL of water, 15 µL of 10x reaction buffer, 4.5 µL of 50 mM $MgCl_2$, 3 µL of dNTPs, 6 µL each of forward and reverse primers, 3 µL of Taq polymerase and 6 µL of DNA template. A final volume of 25 µL was then pipetted into five PCR reaction tubes. These tubes were placed on a PCR gradient block and subjected to annealing temperatures ranging from 46 °C to 65 °C. The protocol included an initial denaturation at 94 °C for 2 minutes, followed by an amplification cycle consisting of 40 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute. A final elongation step at 72 °C for 3 minutes was included at the end of the reaction. Following this, polyacrylamide gel electrophoresis analysis was carried out as previously described.

PCR Optimization for Determining the Optimal Magnesium Concentration

In a 1.5 ml Eppendorf tube, a mixture was prepared for six reactions, consisting of 95 µL of water, 15 µL of 10x reaction buffer, 3 µL of dNTPs, 6 µL each of forward and reverse primers, 3 µL of Taq polymerase and 6 µL of DNA template. The prepared master mix, 22.5 µL per reaction, was then dispensed into five separate reaction tubes labeled 1 through 5. Tubes 1, 2, 3, 4 and 5 received 2.5 µL of $MgCl_2$ at concentrations ranging from 0.5 mM to 5 mM, respectively. The tubes were placed on a gradient PCR block and subjected to an initial denaturation step at 94 °C for 2 minutes. This was followed by an amplification cycle of 40 iterations, including steps at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute. The reaction concluded with a final elongation step at 72 °C for 3 minutes. Following this, polyacrylamide gel electrophoresis analysis was performed as previously described.

Results and Discussion

Impact of Different DNA Template Concentrations

Figure 1(a) shows the CT-values derived from real-time PCR analysis using SYBR Green dye for various DNA template dilutions. These values corresponded to 17.82 for undiluted DNA, 19.54 for a 1/10 dilution, 22.56 for a 1/100 dilution and 34.18 for water. The plateau stage, indicating no further difference in CT-values after 40 cycles when all primers had been utilized, was also depicted.

Figure 1(b) indicates a single peak, this suggests that the template DNA is derived from a singular species. The temperature at which all DNA template dilutions underwent denaturation (*i.e.*, undiluted DNA, 1/10, 1/100 dilutions and water dilutions) is 74.83 °C, as shown by the blue vertical line.

The impacts of different dilutions of DNA template observed on agarose gel and qPCR fluorescence results provide valuable insights into the PCR assay specificity and

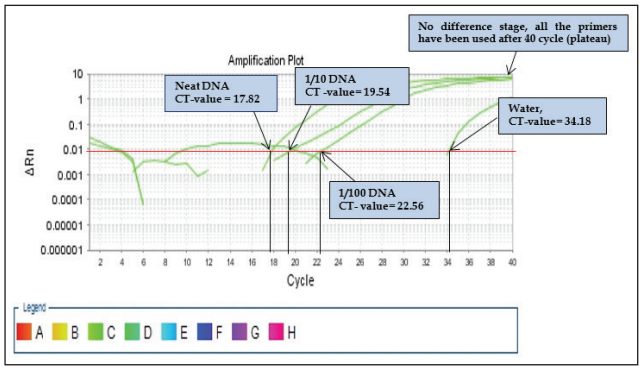


Figure 1(a): Real-time fluorescence detection by SYBR green showing CT-values of various dilutions of the DNA template (undiluted DNA, 1/10 dilution, 1/100 dilution and PCR-grade water as negative control)

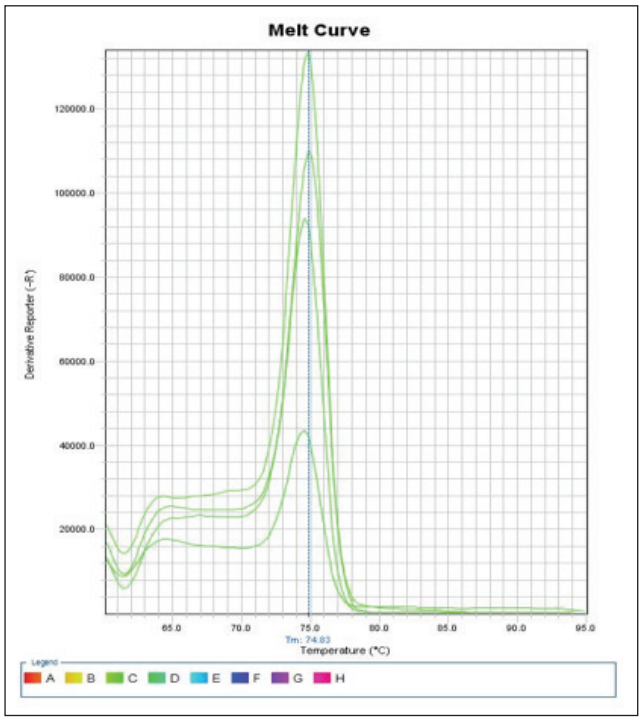


Figure 1(b): Melt curve of amplified DNA Templates dilutions and water as negative control

sensitivity. The CT-values obtained from RT-PCR, along with the melting curve analysis, indicate the efficiency of amplification and the homogeneity of the DNA template. Additionally, agarose gel electrophoresis results provide visual confirmation of PCR product specificity and the presence of non-specific amplification products.

The decrease in CT-values with increasing DNA template concentration (undiluted DNA, 1/10, 1/100 dilutions) in RT-PCR suggests a higher initial number of copy of the targeted DNA in the undiluted DNA sample, leading to earlier detection of fluorescence. This trend is consistent with the expected relationship between DNA template concentration and CT-value in PCR assays (Bustin *et al.*, 2009; Liu *et al.*, 2021). The plateau observed after 40 cycles indicates that the PCR reaction has reached its maximum efficiency, where further cycles do not result in a significant increase in fluorescence.

The single peak observed in the melting curve analysis at 74.83 °C confirms the specificity of the PCR amplification, indicating that the DNA template is derived from a single species. This finding is essential for ensuring the accuracy and reliability of the PCR assay, as it confirms that the amplified product corresponds to the target sequence of interest (Deepak *et al.*, 2007; Zang *et al.*, 2024).

Agarose gel electrophoresis results further validate the specificity of PCR amplification, with stronger bands observed in undiluted DNA and decreasing band intensity in 1/10 and 1/100 dilutions, consistent with the trends observed in RT-PCR CT-values. The presence of lower resolution nonspecific products in all samples indicates the potential for nonspecific amplification, which may be exacerbated at lower DNA template concentrations which substantiate with Fraga *et al.* (2014). The absence of signal from the water sample serves as a negative control, confirming that the observed bands are specific to DNA amplification and not due to contamination or artifacts.

Overall, the integration of RT-PCR, melting curve analysis and agarose gel electrophoresis provides a comprehensive assessment of PCR assay performance, including sensitivity, specificity and the potential for nonspecific amplification.

The RT-PCR protocol conducted is in accordance to an established described method. After completing 40 cycles, 10 µl of PCR reactions containing undiluted DNA, DNA diluted 1/10, DNA diluted 1/100 and a water control were subjected to analysis by means of 2% agarose gel electrophoresis. The gel was then stained with ethidium bromide. The figure 1(c) illustrates the outcomes of the gel electrophoresis, wherein undiluted DNA displayed a distinct and robust band, while 1/10 and 1/100 dilutions exhibited progressively weaker bands with decreasing DNA concentrations. Additionally, lower resolution nonspecific products were observed across all samples. The analyzed products were observed to fragment at 223 bp on the agarose gel, with no signal detected from the water sample.

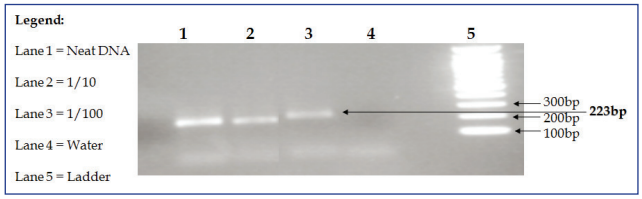


Figure 1(c): Various DNA templates (neat, 1/10, 10/100 and water as negative control) amplified using agarose electrophoresis gel

Effects of Magnesium Chloride Titration Concentrations

Various Magnesium chloride concentrations were tested using polyacrylamide gel electrophoresis (40%) with ethidium bromide added to stain the gel in order to ascertain their optimal efficacy in PCR. Figure 2 illustrates that 1.5 mMol and 2 mMol exhibited the most robust amplifications, albeit with the presence of additional nonspecific products across all concentrations. Conversely, 0.5 mMol and 3 mMol displayed slightly stronger amplifications, while 5 mMol demonstrated weaker amplifications.

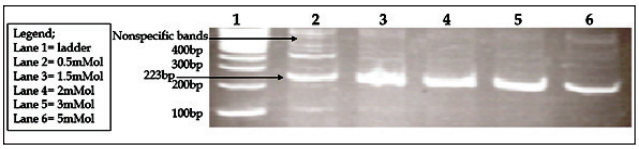


Figure 2: The Polyacrylamide gel bands fluorescence for varying Magnesium chloride concentration

The observed effects of different magnesium chloride ($MgCl_2$) titration concentrations on PCR efficiency, as determined by 40% polyacrylamide gel-electrophoresis and stained with ethidium bromide; figure 2 presents a crucial aspect of PCR optimization. The presence of nonspecific products in all concentrations except 2 mMol and 3 mMol (lanes 4 and 5) suggests an optimal range for $MgCl_2$ concentration in PCR reactions, with deviations leading to suboptimal or undesirable outcomes.

The amplification strength observed in the gel electrophoresis results can be attributed to the role of magnesium ions in PCR. Magnesium ions are essential cofactors for DNA polymerase activity, facilitating primer annealing, template binding and nucleotide incorporation during PCR amplification (Ordóñez and Redrejo-Rodríguez, 2023). However, the concentration of magnesium ions must be carefully titrated to balance their positive effects on PCR efficiency with the risk of nonspecific amplification.

The strongest amplifications observed at 2 mMol and 3 mMol $MgCl_2$ concentrations indicate an optimal range, where DNA polymerase activity is maximized without excessive nonspecific amplification. Here it corroborates with the findings of earlier studies demonstrating the $MgCl_2$ concentration importance in PCR optimization (Lundberg *et al.*, 1991; Zarlenga and Higgins, 2001; Kipf *et al.*, 2022).

Conversely, the weaker amplifications observed at 5 mMol $MgCl_2$ suggest an excess of magnesium ions, which can inhibit PCR efficiency by interfering with primer annealing and template binding. This phenomenon is well-documented in the literature, highlighting the need for careful $MgCl_2$ titration to avoid suboptimal PCR conditions (Hsiang *et al.*, 2010; Haanshuus *et al.*, 2019).

The slight amplifications observed at 0.5 mMol $MgCl_2$ concentration may indicate suboptimal conditions where the magnesium ion concentration is insufficient to support robust PCR amplification but not low enough to inhibit the reaction entirely. Fraga *et al.* (2014) and Kipf *et al.* (2022) suggested that further optimization experiments could explore narrower concentration ranges around these values to pinpoint the optimal $MgCl_2$ concentration for the specific PCR assay.

Generally, the observed effects of $MgCl_2$ titration concentrations on PCR efficiency underscore the importance of systematic optimization in molecular biology protocols to achieve reliable and reproducible results.

Effects of Annealing Temperatures

The PCR optimal annealing-temperature was explored in assessing the impact of various PCR annealing-temperatures efficiency. The figure 3 illustrates that nothing was observed at 65 °C, while weaker amplification was detected at 46 °C and 62 °C. The strongest amplifications were observed at 58 °C and 55 °C; however, additional nonspecific products were evident across all temperature bands.

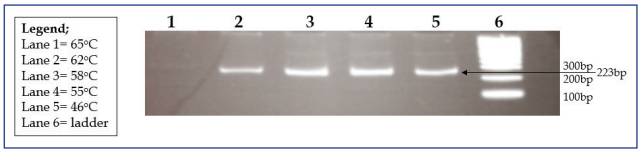


Figure 3: The results of five (5) different annealing temperatures amplified on Polyacrylamide gel in PCR optimisation

The observed effects of different annealing temperatures on PCR efficiency highlight the critical role of temperature optimization in achieving robust and specific amplification. Annealing temperature directly influences primer annealing to the template DNA during PCR, affecting both specificity and efficiency of amplification.

The lack of amplification at 65 °C suggests that this temperature is too high for efficient primer binding to the template DNA, leading to inadequate amplification. Conversely, the less strong amplification observed at 46 °C indicates that this temperature may be too low for optimal primer annealing, resulting in reduced PCR efficiency. These findings are consistent with the known dependence of PCR efficiency on annealing temperature, where temperatures too high or too low can compromise primer-template binding and subsequent amplification (Mamedov *et al.*, 2008; Karunanathie *et al.*, 2022).

The strongest amplifications observed at 58 °C and 55 °C suggest an optimal annealing temperature range where primer annealing is maximized, leading to efficient and specific amplification of the target sequence. However, the presence of additional nonspecific products at these temperatures indicates the importance of balancing PCR efficiency with specificity. These nonspecific products may arise from primer dimer formation or nonspecific primer binding, highlighting the need for careful optimization of PCR conditions to minimize nonspecific amplification (Taylor *et al.*, 2015; Sathyanarayana and Wainman, 2024).

Overall, the observed effects of annealing temperature on PCR efficiency underscore the importance of systematic temperature optimization to achieve optimal amplification conditions and minimize nonspecific products, ultimately ensuring the accuracy and reliability of PCR assays.

The Impact of Primer Dimerisation

At Figure 4, two peaks were observed, where the lesser peak corresponds to denature DNA templates at 92.73 °C (very high temperature). This goes along with the findings of Zheng *et al.* (2024) which outline that the effect of primer dimers on PCR efficiency and specificity underscores the importance of annealing temperature optimization, including ensuring appropriate melting temperatures and avoiding complementarily between primers, can help minimize the formation of primer dimmers.

The investigation into the optimal annealing temperature for PCR revealed notable trends. Figure 3 demonstrates

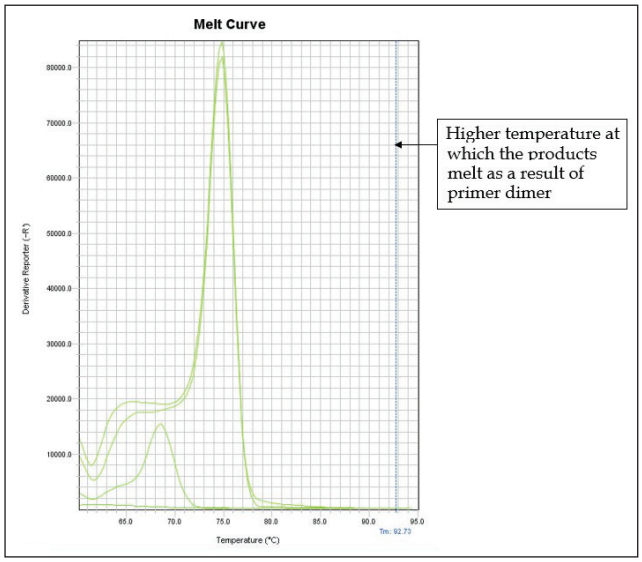


Figure 4: Showed the real-time PCR primer dimer curve

that stronger and cleaner amplification occurred at higher temperatures, particularly at 58 °C, with 55 °C showing slightly weaker results. Conversely, no amplification signal was detected at the highest temperature of 65 °C; while at 62 °C, strong amplification alongside additional nonspecific products was observed. At the lowest temperature of 46 °C, weak amplification relative to 58 °C was evident. This finding suggests that lower temperatures are directly correlated with reduced amplification and the presence of nonspecific bands. Conversely, temperatures higher than optimal may lead to decreased yield rates, likely influenced by primers annealing to nonspecific sites and the GC content of the primers (Rychlik *et al.*, 1990; Fraga *et al.*, 2014).

Furthermore, the absence of amplification at the highest annealing temperature of 65 °C aligns with earlier findings of Fraga *et al.* (2014) and Maruf *et al.* (2023), which suggest that amplification may be compromised at excessively high temperatures, resulting in reduced product yield (Fraga *et al.*, 2014; Zheng *et al.*, 2024).

In figure 4, double peaks indicate primer dimer formation, having the smaller peak indicating denaturation of DNA template at 92.73 °C; a very high temperature. This phenomenon is consistent with findings by Fraga *et al.* (2014), results from primer dimerization altering the sequence, leading to the presence of double-stranded DNA and multiple peaks.

Regarding $MgCl_2$ titration concentrations, figure 2 illustrates that 1.5 mMol and 2 mMol exhibited the strongest amplifications, while 0.5 mMol and 3-5 mMol resulted in reduced product yield. Although 1.5 mMol and 2 mMol displayed similar bands, 1.5 mMol produced the best results. The reduction in $MgCl_2$ concentrations could affect the kinetics of primer-template interactions at amplification temperatures or diminish the activity of the polymerase enzyme, aligning with the observations of Markoulatos *et al.* (2002), who noted a decrease in Taq polymerase activity with lower concentrations. Moreover, the occurrence of nonspecific products across all concentrations is attributed to the stabilization of double-stranded DNA in the presence of surplus magnesium, resulting in incorrect primer annealing, in agreement with the observations of Cobb and Clarkson (1994).

Furthermore, the inclusion of organic solvents such as dimethylsulfoxide and glycerol to enhance specificity and product yield, as well as the optimization of dNTPs, Taq polymerase and primer concentrations, should be considered (Pomp and Medrano, 1991; Roux, 1995).

The HotStart technique is recommended for preventing Taq polymerase activity at lower temperatures, thereby minimizing nonspecific product formation and primer dimerization (Salam *et al.*, 2010; Yehia *et al.*, 2012; Toz *et al.*, 2013; Mohammadiha *et al.*, 2017; Teimouri *et al.*, 2018; Asif *et al.*, 2021). This approach involves incubating essential components, such as Taq polymerase, at room temperature until the initial cycle temperature surpasses the reactant melting temperature, effectively preventing nonspecific and primer dimer formation (Akbari *et al.*, 2005; Karunanathie *et al.*, 2022). Currently, the utilization of monoclonal antibodies (MAbs) to purify Taq DNA polymerase is gaining traction due to its ability to temporarily block enzyme activity (Sathyanarayana and Wainman, 2024). According to Zheng *et al.* (2024), when MAbs and Taq polymerase are combined and added to PCR tubes at room temperature, they produce specific DNA products during higher temperature cycles, even when dealing with complex DNA containing lower copy numbers. This method allows for master mixes to be prepared at room temperature without the need for tube opening or the use of mineral oil or waxes (Paul *et al.*, 2010; Zheng *et al.*, 2024).

Recommendations

Thorough extraction of DNA templates, precise adjustment of $MgCl_2$ concentrations and meticulous control of annealing temperature is crucial for optimizing PCR efficiency and specificity. Consideration of strategies such as HotStart PCR and the use of monoclonal antibodies can enhance specificity and sensitivity, particularly in the presence of primer dimerisation. Further optimization experiments should explore narrower concentration ranges and alternative PCR techniques, such as nested PCR, to further refine assay performance.

Conclusion

Optimization of PCR conditions is essential for achieving reliable and reproducible results in molecular biology experiments. This study highlights the importance of systematic experimentation to identify optimal conditions for PCR assays. By carefully titrating DNA template concentration, $MgCl_2$ concentration and annealing temperature, researchers can enhance amplification efficiency and specificity while minimizing nonspecific products. Additionally, strategies such as HotStart PCR and the use of monoclonal antibodies offer promising approaches to improve assay performance. Overall, systematic optimization of PCR conditions is critical for advancing molecular biology research and ensuring the accuracy of experimental findings.

Acknowledgement

We would like to express our gratitude to the Tertiary Education Trust Fund (TET Fund), Nigeria for financial support. We also acknowledge Management of Umaru Musa Yar'adua University Katsina, Nigeria and Sheffield Hallam University, UK for unconditional valuable contributions to this research.

References

Akbari, M., Hansen, M.D., Halgunset, J., Skorpen, F., Krokan, H.E., 2005. Low copy number DNA template can render polymerase chain reaction error prone in a sequence-dependent manner. *The Journal of Molecular Diagnostics* 7(1), 36-39. DOI: https://doi.org/10.1016/S1525-1578(10)60006-2.

Asif, A.A., Hussain, H., Kumaran, S.S., Syed, S.B., Vanka, V., Tharoor, M., Rangwala, U.S., Rathore, U., Singhal, M., Chatterjee, T., 2021. Efficacy of subcutaneous interferon-beta in COVID-19: A meta-analysis and systematic review. *Journal of Community Hospital Internal Medicine Perspectives* 11(6), 760-768. DOI: https://doi.org/10.1080/20009666.2021.1984644.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* 55(4), 611-622. DOI: https://doi.org/10.1373/clinchem.2008.112797.

Carbonara, T., Pascale, R., Argentieri, M.P., Papadia, P., Fanizzi, F.P., Villanova, L., Avato, P., 2012. Phytochemical analysis of a herbal tea from *Artemisia annua* L. *Journal of Pharmaceutical and Biomedical Analytics* 62, 79-86. DOI: https://doi.org/10.1016/j.jpba.2012.01.015.

Cha, R.S., Thilly, W.G., 1993. Specificity, efficiency and fidelity of PCR. In: PCR Methods and Applications. *Genome Research* 3(3), S18-S29. DOI: https://doi.org/10.1101/gr.3.3.s18.

Chen, S., Sun, Y., Fan, F., Chen, S., Zhang, Y., Zhang, Y., Meng, X., Lin, J.M., 2022. Present status of microfluidic PCR chip in nucleic acid detection and future perspective. *TrAC Trends in Analytical Chemistry* 157, 116737. DOI: https://doi.org/10.1016/j.trac.2022.116737.

Cobb, B.D., Clarkson, J.M., 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Research* 22(18), 3801-3805. DOI: https://doi.org/10.1093/nar/22.18.3801.

D'Aquila, R.T., Bechtel, L.J., Videler, J.A., Eron, J.J., Gorczyca, P., Kaplan, J.C., 1991. Maximizing sensitivity and specificity of PCR by pre-amplification heating. *Nucleic Acids Research* 19(13), 3749. DOI: https://doi.org/10.1093/nar/19.13.3749.

Deepachandi, B., Weerasinghe, S., Soysa, P., Karunaweera, N., Siriwardana, Y., 2019. A highly sensitive modified nested PCR to enhance case detection in leishmaniasis. *BMC Infectious Diseases* 19, 623. DOI: https://doi.org/10.1186/s12879-019-4180-3.

Deepak, S.A., Kottapalli, K.R., Rakwal, R. oros, G., Rangappa, K.S., Iwahashi, H., Masuo, Y., Agrawal, G.K., 2007. Real-time PCR: Revolutionizing detection and expression analysis of genes. *Current Genomics* 8(4), 234-251. DOI: https://doi.org/10.2174/138920207781386960.

Erlich, H.A., Griffith, R.L., Bugawan, T.L., Ziegler, R., Alper, C., Eisenbarth, G., 1991. Implication of specific DQB1 alleles in genetic susceptibility and resistance by identification of IDDM siblings with novel HLA-DQB1 allele and unusual DR2 and DR1 haplotypes. *Diabetes* 40(4), 478-481. DOI: https://doi.org/10.2337/diab.40.4.478.

Fraga, D., Meulia, T., Fenster, S., 2014. Real-time PCR. *Current Protocols Essential Laboratory Techniques* 8, 10-40. DOI: https://doi.org/10.1002/9780470089941.et1003s08.

Gibson, U.E., Heid, C.A., Williams, P.M., 1996. A novel method for real time quantitative RT-PCR. *Genome Research* 6, 995-1001. DOI: https://doi.org/10.1101/gr.6.10.995.

Haanshuus, C.G., Mørch, K., Blomberg, B., Strøm, G.E.A., Langeland, N., Hanevik, K., Mohn, S.C., 2019. Assessment of malaria real-time PCR methods and application with focus on low-level parasitaemia. *PloS ONE* 14(7), e0218982. DOI: https://doi.org/10.1371/journal.pone.0218982.

Harris, S., Jones, D.B., 1997. Optimisation of the polymerase chain reaction. *British Journal of Biomedical Science* 54(3), 166-173.

Hecker, K.H., Roux, K.H., 1996. High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. *Biotechniques* 20(3), 478-485. DOI: https://doi.org/10.2144/19962003478.

Hsiang, M.S., Lin, M., Dokomajilar, C., Kemere, J., Pilcher, C.D., Dorsey, G., Greenhouse, B., 2010. PCR-based pooling of dried blood spots for detection of malaria parasites: Optimization and application to a cohort of Ugandan children. *Journal of Clinical Microbiology* 48(10), 3539-3543. DOI: https://doi.org/10.1128/JCM.00522-10.

Innis, M.A., Gelfand, D.H., 1990. Optimization of PCRs. In: *PCR Protocols: A Guide to Methods and Applications*. (Eds.) Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. Academic Press, Elsevier Inc. pp. 3-12. DOI: https://doi.org/10.1016/B978-0-12-372180-8.50005-6.

Karunanathie, H., Kee, P.S., Ng, S.F., Kennedy, M.A., Chua, E.W., 2022. PCR enhancers: Types, mechanisms and applications in long-range PCR. *Biochimie* 197, 130-143. DOI: https://doi.org/10.1016/j.biochi.2022.02.009.

Kipf, E., Schlenker, F., Borst, N., Fillies, M., Kirschner-Schwabe, R., Zengerle, R., Eckert, C., von Stetten, F., Lehnert, M., 2022. Advanced minimal residual disease monitoring for acute lymphoblastic leukemia with multiplex mediator probe PCR. *The Journal of Molecular Diagnostics* 24(1), 57-68. DOI: https://doi.org/10.1016/j.jmoldx.2021.10.001.

Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C., Sninsky, J.J., 1990. Effects of primer-template mismatches on the polymerase chain

reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Research* 18(4), 999-1005. DOI: https://doi.org/10.1093/nar/18.4.999.

Lam, N.Y.L., Rainer, T.H., Chiu, R.W.K., Lo, Y.M.D., 2004. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. *Clinical Chemistry* 50(1), 256-257. DOI: https://doi.org/10.1373/clinchem.2003.026013.

Liu, Z., Sun, J., Zhao, G., Xiong, S., Ma, Y., Zheng, M., 2021. Transient stem-loop structure of nucleic acid template may interfere with polymerase chain reaction through endonuclease activity of *Taq* DNA polymerase. *Gene* 764, 145095. DOI: https://doi.org/10.1016/j.gene.2020.145095.

Lundberg, K.S., Shoemaker, D.D., Adams, M.W.W., Short, J.M., Sorge, J.A., Mathur, E.J., 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene* 108(1), 1-6. DOI: https://doi.org/10.1016/0378-1119(91)90480-Y.

Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Kokeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F., Takashiba, S., 2003. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunology & Medical Microbiology* 39(1), 81-86. DOI: https://doi.org/10.1016/S0928-8244(03)00224-4.

Mamedov, T.G., Pienaar, E., Whitney, S.E., TerMaat, J.R., Carvill, G., Goliath, R., Subramanian, A., Viljoen, H.J., 2008. A fundamental study of the PCR amplification of GC-rich DNA templates. *Computational Biology and Chemistry* 32(6), 452-457. DOI: https://doi.org/10.1016/j.compbiolchem.2008.07.021.

Markoulatos, P., Siafakas, N., Moncany, M., 2002. Multiplex polymerase chain reaction: A practical approach. *Journal of Clinical Laboratory Analysis* 16(1), 47-51. DOI: https://doi.org/10.1002/jcla.2058.

Maruf, S., Sagar, S.K., Rashid, M.M.U., Nath, P., Islam, M.S., Ghosh, P., Rashid, M.U., Mondal, D., El Wahed, A.A., Basher, A., 2023. Revisiting the diagnosis and treatment of Para Kala-azar Dermal Leishmaniasis in the endemic foci of Bangladesh. *PLoS ONE* 18(1), e0280747. DOI: https://doi.org/10.1371/journal.pone.0280747.

Mohammadiha, A., Dalimi, A., Mahmoodi, M.R., Parian, M., Pirestani, M., Mohebali, M., 2017. The PCR-RFLP-based detection and identification of the *Leishmania* species causing human *Cutaneous leishmaniasis* in the Khorasan-Razavi Province, Northeast of Iran. *Journal of Arthropod-Borne Diseases* 11(3), 383-392.

Ordóñez, C.D., Redrejo-Rodríguez, M., 2023. DNA polymerases for whole genome amplification: Considerations and future directions. *International Journal of Molecular Sciences* 24(11), 9331. DOI: https://doi.org/10.3390/ijms24119331.

Paul, N., Shum, J., Le, T., 2010. Hot start PCR. In: *RT-PCR Protocols: Second Edition*, *Methods in Molecular Biology*, Volume 630. (Eds.) King, N. Humana Press, Totowa, NJ. pp. 301-318. DOI: https://doi.org/10.1007/978-1-60761-629-0_19.

Pomp, D., Medrano, J.F., 1991. Organic solvents as facilitators of polymerase chain reaction. *Biotechniques* 10(1), 58-59.

Rajalakshmi, S., 2017. Different types of PCR techniques and its applications. *International Journal of Pharmaceutical*, *Chemical and Biological Sciences* 7(3), 285-292.

Roux, K.H., 1995. Optimization and troubleshooting in PCR. In: *PCR Methods and Applications*. *Genome Research* 4(5), S185-S194. DOI: https://doi.org/10.1101/gr.4.5.S185.

Rychlik, W., Spencer, W.J., Rhoads, R.E., 1990. Optimization of the annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Research* 18(21), 6409-6412. DOI: https://doi.org/10.1093/nar/18.21.6409.

Salam, M.A., Mondal, D., Kabir, M., Ekram, A.R.M.S., Haque, R., 2010. PCR for diagnosis and assessment of cure in kala-azar patients in Bangladesh. *Acta Tropica* 113(1), 52-55. DOI: https://doi.org/10.1016/j.actatropica.2009.09.005.

Samal, K.C., Ramakrishna, B., Sahoo, J.P., Behera, L., 2022. Molecular characterization and validation of micro-satellite markers linked to powdery mildew disease resistance in mini core germplasm of Urdbean [*Vigna mungo* (L.) Hepper]. *Research Biotica* 4(2), 62-68. DOI: https://doi.org/10.54083/ResBio/4.2.2022/62-68.

Sathyanarayana, S.H., Wainman, L.M., 2024. Laboratory approaches in molecular pathology: The polymerase chain reaction. Chapter 2. In: *Diagnostic Molecular Pathology: A Guide to Applied Molecular Testing*, Second Edition. (Eds.) Coleman, W.B. and Tsongalis, G.J. Academic Press, Elsevier Inc. pp. 13-25. DOI: https://doi.org/10.1016/B978-0-12-822824-1.00041-9.

Stift, G., Pachner, M., Lelley, T., 2003. Comparison of RAPD fragments separation in agarose and polyacrylamide gel by studying *Cucurbita* species. *Cucurbit Genetics Cooperative Report* 26, 62-65.

Taylor, S.C., Carbonneau, J., Shelton, D.N., Boivin, G., 2015. Optimization of droplet digital PCR from RNA and DNA extracts with direct comparison to RT-qPCR: Clinical implications for quantification of Oseltamivir-resistant subpopulations. *Journal of Virological Methods* 224, 58-66. DOI: https://doi.org/10.1016/j.jviromet.2015.08.014.

Teimouri, A., Mohebali, M., Kazemirad, E., Hajjaran, H., 2018. Molecular identification of agents of human *Cutaneous leishmaniasis* and canine visceral leishmaniasis in different areas of Iran using internal transcribed spacer 1 PCR-RFLP. *Journal of Arthropod-Borne Diseases* 12(2), 162-171. DOI: https://doi.org/10.18502/jad.v12i2.42.

Toz, S.O., Culha, G., Zeyrek, F.Y., Ertabaklar, H., Alkan, M.Z., Vardarli, A.T., Gunduz, C., Ozbel, Y., 2013. A real-time ITS1-PCR based method in the diagnosis and species identification of *Leishmania* parasite from human and dog clinical samples in Turkey. *PLoS Neglected Tropical Diseases* 7(5), e2205. DOI: https://doi.org/10.1371/

journal.pntd.0002205.

Wittwer, C.T., Herrmann, M.G., Moss, A.A., Rasmussen, R.P., 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques* 22(1), 130-138. DOI: https://doi.org/10.2144/97221bi01.

Yehia, L., Adib-Houreih, M., Raslan, W.F., Kibbi, A.G., Loya, A., Firooz, A., Satti, M., El-Sabban, M., Khalifeh, I., 2012. Molecular diagnosis of *Cutaneous leishmaniasis* and species identification: Analysis of 122 biopsies with varied parasite index. *Journal of Cutaneous Pathology* 39(3), 347-355. DOI: https://doi.org/10.1111/j.1600-0560.2011.01861.x.

Zang, P., Xu, Q., Li, C., Tao, M., Zhang, Z., Li, J., Zhang, W., Li, S., Li, C., Yang, Q., Guo, Z., Yao, J., Zhou, L., 2024. Self-correction of cycle threshold values by a normal distribution-based process to improve accuracy of quantification in real-time digital PCR. *Analytical and Bioanalytical Chemistry* 416, 2453-2464. DOI: https://doi.org/10.1007/s00216-024-05208-w.

Zarlenga, D.S., Higgins, J., 2001. PCR as a diagnostic and quantitative technique in veterinary parasitology. *Veterinary Parasitology* 101(3-4), 215-230. DOI: https://doi.org/10.1016/S0304-4017(01)00568-4.

Zheng, J., Guo, N., Huang, Y., Guo, X., Wagner, A., 2024. High temperature delays and low temperature accelerates evolution of a new protein phenotype. *Nature Communications* 15, 2495. DOI: https://doi.org/10.1038/s41467-024-46332-6.