Original Research Article

Effect of DNA Template Concentration on Standard Polymerase Chain Reaction

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ABSTRACT

The polymerase chain reaction (PCR) was an essential fundamental procedure widely used in molecular biology to amplify specific deoxyribonucleic acid (DNA) sequences. To investigate the efficiency and specificity of PCR using DNA extracted from human blood cells as sample, several concentrations of DNA templates were used to obtain accurate and reproducible results during this optimisation process. The principal objective was to identify which concentration was the best for amplifying the DNA target sequence and study the effects of the scale of concentrations to standard PCR. For this study, a series of PCR were conducted using various concentrations of DNA template ranging from 1 ng/µL to 100 ng/µL per reaction under optimized conditions of primer concentrations, annealing temperature and extension time. The results demonstrated that DNA template concentration significantly influenced the efficiency and specificity of PCR as the intensity of the target gene amplification band was increased on the agarose gel electrophoresis under ultraviolet light indicating the PCR product yield. Formation of primer-dimers became more prominent as the DNA template concentration reduced resulting in non-specific amplification. The concentrations ranging from 10 ng/µL to 70 ng/µL were the most optimal reactions profiting a remarkable feat of target gene amplification while concentrations less than 1 ng/µL produced none and non-specific outcomes retaining little amount of target gene amplification. In conclusion, our study highlights the pivotal role of DNA template concentration optimises PCR to increase reliability and reproducibility, extending our understanding of genetic analysis, diagnostic, and forensic sciences.

Keywords: Polymerase chain reaction (PCR), DNA template concentration, Amplification efficiency & specificity

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1.0 Introduction

Polymerase chain reaction (PCR) is a key technique that had been transforming many industries such as the diagnostic medicine and scientific research due to its powerful amplification technique that can generate an ample supply of a specific segment of deoxyribonucleic acid (DNA) (i.e., an amplicon) from only a small amount of starting material (i.e., DNA template or target sequence) (1). PCR was discovered by Dr. Kary Mullis in 1985, shortly after the double-stranded DNA structure was visualised that opened up variety ground breaking innovation as DNA were possible to be produced rapidly and precisely. This is because for the first time, it allowed for specific detection and production of large amounts of DNA (2). Thus, the 1993 Nobel Prize in chemistry was especially significant because it highlighted the new era in molecular biology with a critical achievement, that of the invention of the PCR method (3).

The sample that was utilised in this study was human blood cells which contained many types of cells: white blood cells (monocytes, lymphocytes, neutrophils, eosinophils, basophils, and macrophages), red blood cells (erythrocytes), and platelets (4). The extracted blood samples that related with the gene Insulin-induced gene 2 (INSIG2) taken from the research facility was used for this PCR reaction. Previous studies have shown that INSIG2 polymorphisms were associated with obesity, weight gain, and hypercholesterolemia (5).

To achieve an accurate and reproducible results, the PCR was optimised with suitable conditions including the concentration of DNA template which was paramount of this methodology. The amount of total DNA in a PCR has a marked effect on the outcome of a PCR procedure (1). It was hypothesised that altering the DNA template concentration would influence the PCR outcomes. Target gene amplification can be enhanced when the concentration of DNA template was optimal and allows for non-specific amplification generated due to having too high of DNA concentration or not enough amplification due to having too low of DNA concentration. Optimisation of DNA input is important because higher amounts increase the risk of non-specific amplification whereas lower amounts reduce yields (6). This research aimed to examine the effect of different DNA template concentration on standard PCR and assessed the impact on efficiency and specificity of PCR multiplication. Template DNA, DNA quality and purity will have a substantial effect on the likelihood of a successful PCR experiment (1).

These objectives were accomplished by a qualitative approached that employed available visual representation and comparative analysis between research papers to evaluate effects of various DNA template concentrations. This type of PCR offers qualitative answers on whether or not a particular target sequence is present, but no quantitative information (7).

2.0 Materials and Methods

2.1 Materials and Apparatus

The apparatus and materials used for this experiment were categorised into two parts, for the PCR process and for the DNA analysis. These following apparatus and materials were thermal cycler or the PCR machine (Bio-Rad, USA), PCR tubes or plates, micropipettes, and their tips, 0.5 ml tubes, 1.5 ml tubes, 2.0 ml Eppendorf tube, microcentrifuge, and agarose

gel with gel tray, chamber, electrodes, power supply, gel imaging system or UV transilluminator (Alpha Imager, Alpha Innotceh, USA). Reagents such as DNA template, forward and reverse primers, DreamTaq™ Green PCR Master Mix (Thermo Scientific, USA) (composed of DNA polymerase, nucleotides (dNTPs), $MgCl_2$ and buffer solution), distilled water (dH_20), DNA loading dye, 100 bp DNA size marker or DNA ladder (Vivantis Technologies, Malaysia), and other laboratory apparatus like racks, markers.

2.2 PCR Preparation

This study was done thoroughly starting from the pre-preparation, during the process, and analysis of the end. Firstly, the primer that was necessary for DNA amplification to amplify the INSIG2 gene was designed using online software Primer3Plus that was provided by National Center for Biotechnology Information (NCBI). The list of primers and their properties were as listed in Table 1. The designed primers were aligned with genomic sequence NC_000016.10 from the NCBI Reference Sequence (Homo sapiens chromosome 2, GRCh38.p14 Primary Assembly - Nucleotide - NCBI (nih.gov).

After that, the DNA samples were diluted to 12 concentrations from 100 ng/µL to 1 ng/µL by gradient dilution. About 9 µl of DNA sample taken from the stock solution and added with 1 µL of dH_20 produced a concentration of 90 ng/µL and so on with the lowest of 0.1 µL of DNA sample with 9.9 µL of dH_20 into 1.5 ml tubes for every dilution. Table 2 showed the calculation while Table 3 and 4 provided the dilution concentration of each sample.

Table 1: Primers Properties and Sequence of DNA Amplified

Properties	
Gene	*INSIG2*
Nucleotide change	Intronic Mutation
Position	Chr2:118078449 (GRCh38.p13)
CF INSIG2	AGC TGA AGT ACA AGG ACT TGA (Tm 57.7 °C)
CR INSIG2	ACC AAA GCC AGC CAT AAG C (Tm 57.7 °C)
5’ End Sequence	
TACATTTATT TCAACCGAGA GATGAAGGAA ACAAAGGTCC	
AGATTGGGTA CTAACTGGCT CAAGGTCACC TAGCTAGTTA	
GCTGAAGTAC AAGGACTTGA AGTTGATCTA ATGTTCTCTC	
TCCTACCTCC CTCCAATACC CCATCGGAAT TGAAATCATT	
GCAATAGCCA CTGCCAAGTA CTTAACAATG GATATTTGAT	
3’ End Sequence	
GTGGTCCTTT AGGTCTGTAC CAGGGTGGTT TTCAGTTTTT	
TACTTCATCA GCACAGGAAG GCAAGGACAT CGCACTGACT	
CTCATATTTC ATCCTTAGCT CTCGATTCCC TTGGTTTTAC	
ATTTCTGACT CTTCCTTCAG TTTCTTGTAG GGCTTTATGA	
TTTAATTTAA TTTCAGCTTA TGGCTGGCTT TGGTACCCAG	

Table 2: Calculation for Concentration of DNA with ID number C1

Template Conc. (ng/µL)	Calculation
100	$\frac{(100\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 10\ \mu L$
90	$\frac{(90\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 9\ \mu L$
80	$\frac{(80\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 8\ \mu L$
70	$\frac{(70\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 7\ \mu L$
60	$\frac{(60\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 6\ \mu L$
50	$\frac{(50\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 5\ \mu L$
40	$\frac{(40\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 4\ \mu L$
30	$\frac{(30\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 3\ \mu L$
20	$\frac{(20\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 2\ \mu L$
10	$\frac{(10\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 1\ \mu L$
0.5	$\frac{(5\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 0.5\ \mu L$
0.1	$\frac{(1\ ng/\mu L) \times (10\ \mu L)}{(100\ ng/\mu L) \times (X)}, X = 0.1\ \mu L$

Table 3: Dilution Concentration of DNA Sample (ID Number: 1)

Dilution	Contents (µL)		Conc. (ng/µL)
	DNA Template	Distilled Water	
1	10	0	100
2	9	1	90
3	8	2	80
4	7	3	70
5	6	4	60
6	5	5	50
7	4	6	40
8	3	7	30
9	2	8	20
10	1	9	10
11	0.5	9.5	5
12	0.1	9.9	1

Table 4: Dilution Concentration of DNA Sample (ID number: 2)

Dilution	Contents (µl)		Conc. (ng/µL)
	DNA Template	Distilled Water	
1	10	0	78
2	8.7	1.3	68
3	7.4	2.6	58
4	6.2	3.8	48
5	4.9	5.1	38
6	3.6	6.4	28
7	2.3	7.7	18
8	1.3	8.7	10
9	1.0	9.0	8
10	0.6	9.4	5
11	0.4	9.6	3
12	0.1	9.9	1

2.3 PCR Process

The required materials and apparatus for the procedure were set up to prepare a master mix. The master mix consisted of dNTPs, DNA polymerase, $MgCl_2$ and buffer solution that was pre-mixed in a 12.5 µL Green Taq solution combined with 9.5 µL dH_20 was mixed in a 2.0 ml tube. By using a micropipette and their sterile tips, 1.0 µL DNA template, 1.0 µL forward and reverse primers each were added into 0.5 ml PCR tubes with the remaining 22.0 µL master mix into the mixture formulating a total of 25.0 µL in a

tube. It was recommended to have an accurate pipetting skill in order to achieve quality effectiveness of PCR especially during transferring solution into the PCR tube and used of separate tips for every delivery to avoid any cross-contamination. The tubes were then inserted into a mini centrifuge to ensure homogeneity. After that, the PCR reaction was conducted using the PCR machine or Thermal Cycler. The PCR tubes with the mixture were placed into the thermal cycler with the appropriate primer melting temperature (T_m) and expected size of the amplicon. The PCR protocol for this reaction was according to the Table 5 below:

Table 5: PCR Protocol

Process	Temperature (°C)	Time Taken (min)
Pre-denaturation	95 °C	1
Thermal Cycling Steps		
Denaturation	95	0.5
Annealing	55	0.5
Extension	75	0.5
End of 30X Cycles		
Extra Elongation	75	5
Cooling down	12	3

2.4 Gel Electrophoresis

For the agarose gel electrophoresis, 1.5% of agarose gel was prepared. Mixture of 1.5 g agarose powder dissolved in 100 ml 1X Tris-borate-EDTA (TBE) buffer was heated until it was completely dissolved and was added Bio-Safe DNA Staining Solution (Bio-Rad, USA) loading 1µL. Then, a gel tray was put inside the gel box with combs inserted creating wells for sample loading. Warmed agarose solution was poured into the gel tray until they were fully submerged and was allowed to cooled down and harden after 20-30 minutes. Ten-µL of PCR mixture and 5 µL of DNA ladder were added to separate wells accordingly. To run the gel electrophoresis effectively, the wells were to be situated at the negative electrode and the chamber was filled with TBE buffer. This process took 40 minutes until acquired DNA bands had migrated to the appropriate distance. Finally, visualisation of the DNA bands were done by ultraviolet (UV) transilluminator called Alpha Imager (Alpha Innotech, USA) or a gel imaging system under the UV light. By comparing the band with the DNA ladder marker, the results were identified based on the presence, intensity or size of the amplified DNA fragments.

3.0 Results

The results that had been acquired were observed under UV transilluminator and analysed based on their band presence, intensity, and size. For the results, two human blood samples were used 100 ng/µL for sample 1 and 78 ng/µL for sample 2 with specific primers CF Insig and CR Insig for the annealing process. Several parameters were targeted to classify the results which were the amplification efficiency, specificity, non-specific amplification, no amplification, optimisation of concentration and reproducibility. According to the study's findings, the outcomes of regular PCR were significantly influenced by the concentration of DNA template. Low template concentrations resulted in less effective PCR reactions and lower-quality amplification products. High template concentrations improved the efficiency of the PCR reaction but increased the likelihood of non-specific amplification products. The amplification products frequently showed unique migration patterns on the gel and were bigger than the targeted product.

For this investigation, 10 ng/µL to 70 ng/µL were shown to be the ideal template concentration. At these concentrations, the PCR reaction was efficient and the amplifications were ideal. The band can be detected easily and consistent in their sizes and migration trends. The following figures show the results of this study. Figures 1 and 2 showed the UV visualization of the PCR amplification for both DNA samples. Table 6 and 7 show the DNA concentration for each dilution of the DNAs, whether the PCR is successful indicated by presences of band and the efficiency of the amplification as indicated by the intensity.

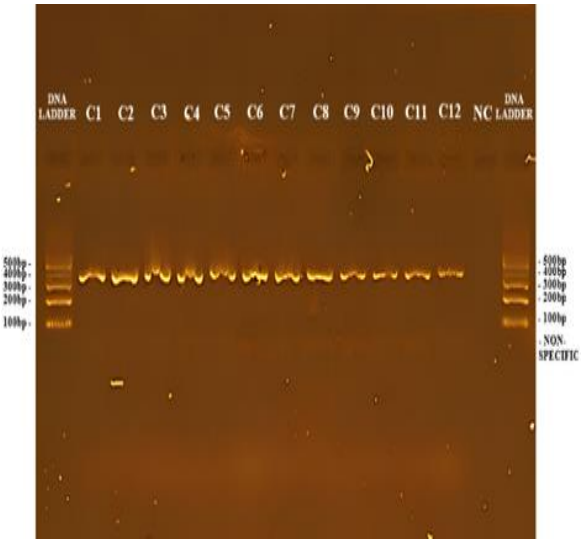

Figure 1: PCR result for DNA Sample 1
Note: C1-C12 are DNA, 100, 90, 80, 70, 60, 50, 40, 30 20, 10, 5 and 1 ng/µL respectively, NC being non-template control

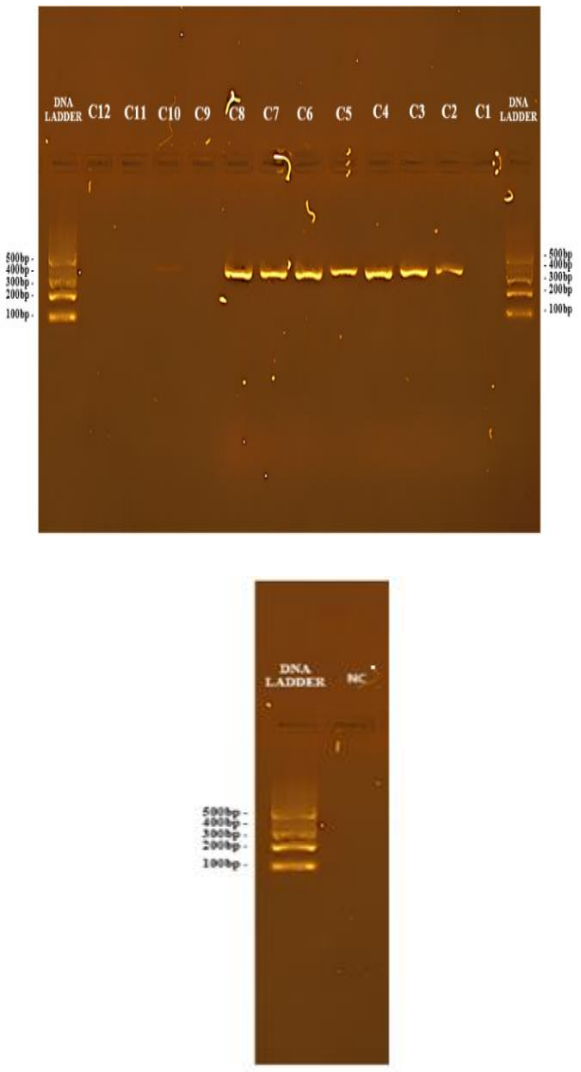

Figure 2: PCR result for DNA Sample 2
Note: C1-C12 are DNA, 78, 68, 58, 48, 38, 28, 18, 10, 8, 5, 3 and 1 ng/µL respectively, the non-template control, NC was run on a separate gel.

Intensity of the band determined the effectiveness of amplification of a PCR product. For DNA Sample 1, Table 6 showed the intensity of the band on the agarose gel under UV light. Studying with the 100 bp DNA marker, which has 100-3000 bp length of DNA fragment, and PCR amplified specific fragments showing at 400 bp. The amplification of DNA can be observed throughout concentration range of 100 ng/µL to 30 ng/µL with a high intensity and from 20 to 5 ng/µL with a moderate intensity. However, at 1 ng/µL, the band was only presence at low intensity. For DNA Sample 2, the Table 7 showed the concentration of 78 ng/µL had no presence of band although it was within the range of 100 ng/µL until 10 ng/µL of estimated amplicons. From 68 ng/µL to 10 ng/µL on the other hand had high intensity, while from 8 ng/µL to 1 ng/µL the band

Table 6: DNA Sample 1 Observed

Concentration	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**
ng/µL	100	90	80	70	60	50	40	30	20	10	5	1
Parameter												
Presence of Band	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Intensity	High	High	High	High	High	High	High	High	Mid	Mid	Mid	Low

Table 7: DNA Sample 2

Concentration	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**
ng/µL	78	68	58	48	38	28	18	10	8	5	3	1
Parameter												
Presence of Band	✗	✓	✓	✓	✓	✓	✓	✓	✗	✓	✗	✗
Intensity	None	High	High	High	High	High	High	High	None	Low	None	None

was faint to none existence.

According to the results, both samples 1 and 2 showed a good specificity that corresponded to the DNA ladder and positive control except for the concentration 78 ng/µL for sample 2 which had no presence of band. For the size of bands for sample 1, concentrations from 100 ng/µL to 10 ng/µL appeared larger compared to concentrations lower than 10 ng/µL. For the size of bands of sample 2, concentrations of 68 ng/µL to 10 ng/µL appeared larger than concentration of 78 ng/µL and concentration lower than 10 ng/µL. These low intensity, smaller sized bands below 100 bp when compared to the DNA ladder indicated production of primer dimers during PCR (8)

4.0 Discussion

4.1 Overview

The polymerase chain reaction is carried out in a reaction mixture which comprises the DNA extract (template DNA), Taq polymerase, the primers, and the four deoxyribonucleoside triphosphates (dNTPs) in excess in a buffer solution (9). DNA Template is the sample DNA that contains the target sequence (10). By diluting the DNA template concentration, various concentrations were acquired and assayed with gel electrophoresis. Through these gels, particles in smaller mass and size can move faster, whereas the larger ones move relatively slowly (11). At the end of electrophoresis, different sizes of DNA molecules appear as bands in each lane (12). Negatively charged DNA/RNA migrates through the pores of an agarose gel towards the positively charged end of the gel when an electrical current is applied, with smaller fragments migrating faster (13). High GC content of primers was also an issue because high GC contents cause primer dimer to appear (14). The GC content for the CF Insig and CR Insig are 43% and 53% respectively, which are within the appropriate range for an optimum PCR primer of 40-60%. Parameters such as amplification intensity and specificity were discussed based on the results observed above.

4.2 Amplification Efficiency

The thickness of the band estimated the relative abundance of DNA molecules in a sample where a high intensity of band appeared to indicate plentiful of DNA while low intensity of band indicated lack of DNA present in the samples. These samples contained weak band or band-less PCR products at lower concentrations than 10

ng/µL. The possible reason was that insufficient or low quality of DNA template (DNA A 260/A280 ratio of sample 1 is 1.72 and 1.67 for sample 2, where the best ratio for DNA purity is from 1.7 to 2.0) were used during the PCR reaction, causing the target DNA sequence to be undetectable or not amplification. This was considered low efficiency of the amplification because the DNA cannot be multiplied or the available template was contaminated or degraded, reducing its efficiency. Lack of amplification efficiency can define the process as less reliable because DNA separation was insufficient.

4.3 Amplification Specificity

For the non-specific amplification which was yield of unintended PCR products, additional bands and rather smears on the agarose gel electrophoresis were observed. Primer-dimers, one of the factors for this reaction, were detected for sample 1, representing the primers binding to its complementary primer commonly at ~60bp instead of binding to the target DNA sequence. Since the bands were outside the

DNA ladder, this factor was insignificant to the results. Other factors including insufficient primer specificity. The primers bind to denatured DNA template during polymerase chain reaction (PCR) by hybridising to a complementary target DNA region and providing a free 3′ hydroxyl end (9). But, as primers with a low specificity, they may bind to similar sequence in the genome resulting in the amplification of non-target sequence such as the binding to a different gene instead of the intended INSIG2 gene. By doing so, it produced non-specific binding thus resulting in non-specific amplification and a false-positive results which can be a critical issue in diagnostic applications.

4.4 Troubleshooting

A series of troubleshooting could be conducted for the PCR to be more successful. Troubleshooting are steps to assess quality and purity of DNA template, ensure the absence of contaminants that could inhibit PCR reaction.

The success of PCR reactions can be affected by several factors. Troubleshooting PCR reactions can improve the quality of your results. If the yield of PCR product is insufficient, adding or increasing the quantity of DNA template would help yield better result. Alternatively, using a different DNA polymerase may be beneficial. It is also important to verify the quality of the DNA template before starting the reaction. Contamination can be a major issue, taking steps to avoid it is very important for a more precise and reliable result.

4.5 Optimisation of Concentration & Limitations

From all the results that had been discussed, the most optimal concentrations that produced the highest amplification efficiency and specificity for this DNA samples on Insig gene was a range between 10 ng/µL to 70 ng/µL. The DNA sample 1 might had displayed a more quality DNA template taken from the lab blood sample as it had been extracted with no contamination present during the storage. The DNA sample 2 lacked some of the quality since at concentration 78 ng/µL unlike sample 1, there was no presence of band observed meaning insufficient DNA amplification. The stock concentration might also play a role since for sample 1, the concentration was 100 ng/µL while for sample 2, the concentration was 78 ng/µL. To achieve a more reliable result, the higher concentration of the stock solution was better based on this study. The PCR protocol also determined effectiveness of a reaction. As the PCR annealing temperature increases, the primer annealing stringency increases, leading to more specific and reproducible amplification (9). This will allow the primers to attach a single-stranded template DNA to a specific location by hydrogen bonding. These steps are repeated (“cycled”) 25–35 times to exponentially produce exact copies of the target DNA (15).

Although PCR was a valuable technique, it possessed some limitations. As the test is a highly sensitive technique, just a small amount of contamination or changing in the concentration of the sample by even trace amount of DNA could lead to a misleading result (2). Thus, extensive number of experimental methods were carried out that focused on the relationship between concentrations of DNA template with PCR end results. These findings were contributed to improve broader understanding of PCR optimisation and its application in genetic analysis, disease diagnostics and forensic sciences. The effects on gaining insights of these can reinforce a more reliable results ultimately advancing the knowledge and

proficiency in performing PCR as a prime molecular biology technique.

Another limitation for this study was the quality and quantity of the DNA templates. The extraction of DNA from the blood samples taken from the patient with obesity were probably not optimum, this unable the production of a good quality result as there might be some contamination during the process of extraction and storage. The pipetting technique performed were also a factor as dealing with a micropipette and materials as low as 0.1 µL requires high precision. Thus, repetition of the optimisation of PCR were not able to run as quickly preventing a high percentage assurance of the result reproducibility for the experiments. Investigation of other parameters such as effects of nucleotide and primer concentrations with a wider range of concentration to the standard PCR can help researchers to determine the best optimisation for each DNA target sequence.

5.0 Conclusion

On the basis of the discussed information and results, it was determined that the hypotheses that a high concentration of DNA templates could result in the amplification of non-specific PCR products and that a low concentration of DNA templates could prevent the production of PCR products were supported. The concentration of a template and primers were both critical elements in a PCR process that requires systematic experimentation to amplify the target DNA sequence with high specificity and efficiency. Nevertheless, for this experimental configuration, additional variables including the primer design, quality, and purity of the materials, as well as the DNA template type, were equally crucial in order to enable researchers to optimise the precise DNA concentrations for subsequent study. The determination of the optimal concentration, which was found to be between 10 and 70 ng/µL, would result in a more reliable and accurate amplification.

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Conflicts of Interest

The authors declare no conflict of interest in this present work.

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