Optimisation of Standard PCR Programme.

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Abstract:

PCR is a very sensitive technique that gained its significance through the high contribution on a variety of bioscience fields. It is not surprising that the most cited scientific paper in biosciences is related to the PCR programme. The optimisation of the PCR is very important to achieve amplification with reliable results. Variables like Mg^{++} concentration, template DNA dilution, annealing temperature, and primers' concentration were optimized using classical and touchdown amplification methods. Other PCR methods like hot-start can improve the quality and the quantity of the PCR yield. The utilization of mathematical and statistical ways in designing a PCR programme like Taguchi or chessboarding methods can efficiently enhance the PCR reaction. Also the usage of very pure reagents and the utilization of a suitable kind of the *Taq* polymerase would highly improve future experiments. This work was performed to find the optimal reaction conditions for the utilized standard PCR programme.

تكامل برنامج تفاعلات الكوثرة القياسي.

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مفتاح البحث: تفاعلات الكوثرة القياسية، تركيز ايونات المغنسيوم، *Taq* polymerase .

الخلاصة:

تفاعلات الكوثرة (PCR) هي تقنية حساسة جدا و التي كسبت أهميتها من خلال المساهمة العالية على مجموعة متنوعة من مجالات العلوم الحيوية. فإنه ليس من المستغرب أن الورقة العلمية الأكثر ذكرا في العلوم البيولوجية هي المتعلقة ببرنامج PCR. وتعظيم الاستفادة من PCR مهم جدا لتحقيق التضخيم مع نتائج يمكن الاعتماد عليها. المتغيرات مثل تركيز آيونات المغنيسيوم ، تخفيف قالب الDNA ، ودرجة الحرارة التصلب، وتم تكامل تركيز البوادئ باستخدام الأساليب الكلاسيكية والهبوطية للتضخيم. طرق اخرى لل PCR مثل البداية-الساخنة يمكنها تحسين نوعية وكمية الناتج للPCR. استخدام الطرق الرياضية والإحصائية في تصميم برنامج PCR مثل طريقة تاجوشى (Taguchi) أو طريقة الشطرنج (chessboarding) يمكن أن تعزز كفاءة تفاعلات الكوثرة. أيضا استخدام كواشف نقية جدا واستخدام نوس مناسب من انزيم (Taq polymerase) يمكنها أن تحسن بشكل كبير التجارب المستقبلية. تم تنفيذ هذا العمل للعثور على ظروف التفاعل الأمثل لبرنامج PCR القياسي المستخدم.

Introduction:

It has been proved that the polymerase chain reaction (PCR) is a laboratory tool that has a high impact on the progress of the molecular biology and related disciplines. This technique encompasses the amplification of a very tiny amount of DNA templates (could be even one DNA template). The amplification can be performed by the implementation of the thermal cycling processes that involves the melting of the DNA (separation of the DNA strands) and the DNA replication with the ease of heat stable biological catalysts [(1)].

Therewith, the setup of a basic PCR has been revealed as it involves the usage of many chemicals, which are: 1) The template (the template could be either DNA or cDNA that has been copied from an RNA; in such case it necessitate the involvement of reverse transcriptase

to produce the cDNA from its parent RNA. 2) The primers. 3) Specific heat-stable DNA polymerases. 4) Deoxynucleotide triphosphatess (dNTPs). 5) Buffers containing singly and doubly charged cations (potassium and magnesium (or to a less extent manganese) respectively) [2].

Also, PCR process is widely employed in different laboratory experiments to create a high-throughput of a required DNA sequence. Unfortunately, there is no single optimal condition to be used to generate such high-yield product for different templates. This can be explained as that the usage of different templates means the usage of other primers. The latest usually requires changing the annealing temperature, cation concentration and or other factors (will be explained later on in details). Such changes and variations must be done for any new PCR. Not only to ensure that this particular PCR will work but also it is very important to get pure and high yield PCR. Such changes in the PCR variables are called PCR-optimisation [3, 4]. To elucidate the effect of such variables on the efficiency of a PCR, each one of the following variables will be explained briefly. These variables are: 1) Magnesium concentration variation. 2) The influence of the DNA template concentration. 3) Annealing temperature effect. And 4) Using different programmed annealing temperatures, which is called "touch down" process.

1. Magnesium Titration:

It has been stated that Magnesium ions have a potential role of a range of cellular functions. For instance they are responsible for sustaining many of proteins, lipid bilayers membranes and nucleic acids structures. This is achieved through the binding of the divalent cation to a specific binding site on the macromolecule. This specific binding is capable to give the macromolecule its particular structure or catalytic activity. In the case of the DNA polymerase (which is responsible for repairing and replicating the DNA), the roles of magnesium ions are studied by scientists to be: The production of Mg^{2+} –substrate complex to which the enzyme is attached; the alteration of the enzyme structure via the direct binding to them. Moreover, each DNA polymerase molecule requires two-bounded Mg^{2+} cations, one of them act as nucleotide binding and the other play a catalytic role as illustrated in figure 1 below [5]:

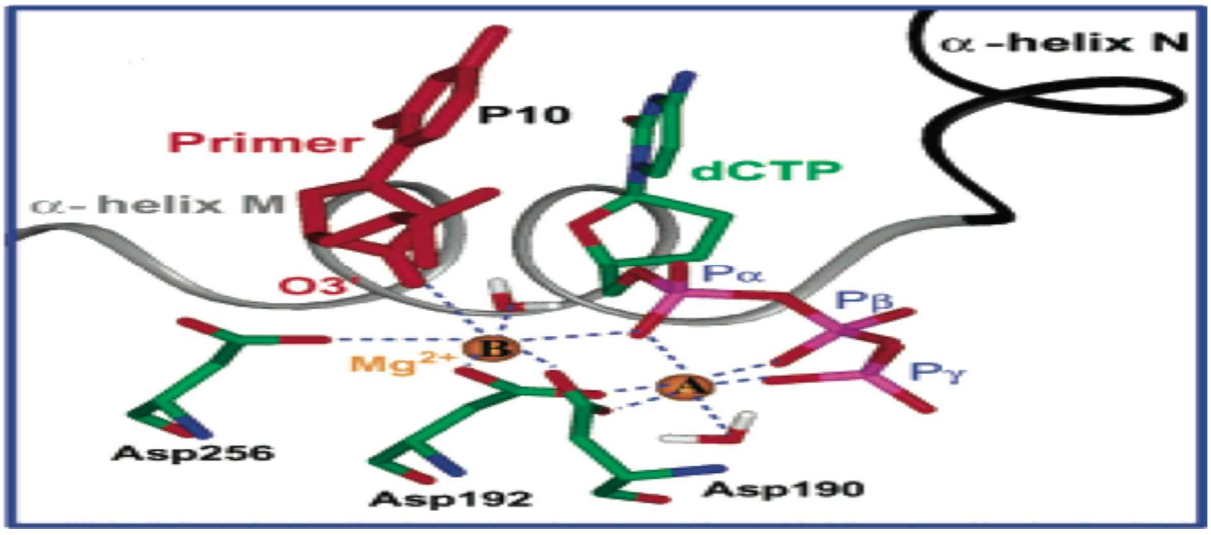

Figure 1. A general DNA polymerases-nucleotide binding (labeled as A) and catalytic (labeled as B) sites, which shows the coordination of the nucleotide- binding Mg^{2+} to the oxygen molecules of alpha, beta and gamma phosphate molecules of the dCTP also to Asp192, Asp190, and lastly to a single H_2O molecule; and, the coordination of the catalytic Mg^{2+} to the oxygen molecule of the alpha phosphate group of the dCTP, also coordinates with the primer P10:O3′, the same previous aspartates, Asp256, and another H_2O molecule [5].

Mg^{2+} has a crucial role in the PCR amplification process and it affects the fidelity and the specificity of any individual PCR reaction. This is believed to be dependent on the magnesium concentration due to its function during primer-binding process. Moreover, Mg^{+2} cations could influence the dissociation temperature of for the PCR output, which in turn is capable to produce a primer-dimer artefact. Low Mg^{+2}concentrations may result in no reaction because of the inadequate performance of the DNA polymerase; also it might result in preventing any annealing process. In contrast, high Mg^{+2} concentrations are capable of push the DNA polymerase to be over-reactive and this consequently leads to non-specific unwanted PCR products that might appear in the run gel [6, 7].

The existence of divalent cationic chelators like ethylene diamine tetra acetic acid (EDTA) and /or citrate can directly affect the level of free $Mg^{+2,}$ which in turn influence the activity of the DNA polymerases as explain earlier. Also the concentration of the DNA template; dNTPs concentration; and the presence of impurities (especially proteins), all could affect the apparent free Mg^{+2} level, which is available to be utilized in the reaction buffer. Also the type of the DNA polymerase utilized in the reaction has different requirements of the free Mg^{+2} concentrations. For instance, *Pfu* DNA polymerases are more sensitive to the free levels of Mg^{+2} cations than the Taq polymerase and hence require less Mg^{+2} concentration in the buffer solution. Thus, the optimal Mg^{+2} concentrations must be experimentally obtained for each single reaction. The Mg^{+2} targets are that associated with the highest amplification and lowest non-specific PCR products. The classic concentration of the free Mg^{+2} cations usually used is scaled between 1-4 mM; nevertheless, it is not quite easy to decide which is the best unless a serial solutions are made with small difference in-between to reach the best concentration of the magnesium cations for this specific PCR reaction [7, 8].

2. The quality and the quantity of the DNA template influences the PCR reaction:

PCR amplification also depends not only on the concentration of the DNA template available but also on the purity of such template. The amplification on the PCR can be performed over a wide range of template concentration. Tiny concentrations of the DNA template might not be able to deliver PCR product. In contrast, very high concentration of the DNA template may result in steric hindrance for the reaction, as the active site of the polymerase enzyme would be over-crowded that would result in decreasing the rate of amplification. Hence, the DNA template optimization is critical for observing the limit of detection (LoD).

In addition to the quantity of the DNA template, the quality of the used template is also very significantly affects the PCR output. There could be chemical reagents and impurities that might exist within the DNA template and have a potential inhibitory effect on the DNA polymerases. Almost all the impurities are chemical reagents that have been used to extract and purify the DNA. For example sodium dodecyl sulphate (SDS) and the organic solvents are the most frequently encountered [9]. Accordingly, it is highly recommended to run a positive control (well-recognised PCR template that characterised to deliver a positive result using the same reaction environments) with the other samples [7, 10].

Since PCR is a quite sensitive exponential throughput technique, therefore, single DNA template that might contaminate the reaction could result in a very large amount (could be millions). That is why a negative control (a blank mixture of the whole components but not the target DNA template), is highly required to run. As a result if there is positive signal with

the negative control then it means that there could be a contamination in at least one of the used PCR components and the results obtained with such PCR are untrusted anymore [2, 11].

3. The annealing temperature of the primers:

The annealing temperature used with the primers is highly dependent on the melting temperature (Tm) of the target. The melting temperature also relying on the concentration, nucleotide bases contents and the length of the selected primers. It is believed to apply annealing temperatures that are less than the melting temperature with about (3-5) °C [12].

Many studies demonstrated that the annealing temperature is obtained between (55-72) °C; however it is critical to optimise the annealing temperature for each PCR procedure. This can be explained as if the temperature is low a high yield could be obtained with a loss in the specificity of the polymerase enzyme resulting in higher non-specific product as well. On the other hand, if the annealing temperature is higher than the melting temperature, then the specificity is highly enhanced as a firmer binding can be obtained. However, very high annealing temperatures should be avoided as these can reduce the stability of target DNA-primer binding, which can significantly reduce the PCR output [7, 11].

4. Forward and reverse primers' concentration:

The optimisation of the utilized concentration of each one of the primers is one of the most important parameters in the PCR process. Usually, the forward and the reverse primers have different level of activity. This recommends the usage of different primers' concentrations to get the maximum activity. The optimisation can be performed using the chessboarding method [13]. This method can be revealed by getting 5 different double dilutions of the forward and the reverse primers and put them in a table just like the chessboarding one. For example if the concentration of the primer was 200 pmol/µL then the concentrations that would be made can be seen in table 1 below:

Table 1: primer optimisation using chessboarding method. This table explains the use of the chessboarding method for the primers optimisation. As shown below half the volume (10ul) comes from the forward primer and another half comes from the reverse for optimizing the PCR reaction using primer chessboarding method [13].

			Forward pmol/µL					
			A	B	C	D	E	F
			200	100	50	25	12.5	6.25
Reverse pmol/µL	1	200	20 ul	20 ul	10 ul	10 ul	10 ul	10 ul
	2	100	20 ul	20 ul	10 ul	10 ul	10 ul	10 ul
	3	50	10 ul	10 ul				
	4	25	10 ul	10 ul				
	5	12.5	10 ul	10 ul				
	6	6.25	10 ul	10 ul				

5. Touch down reaction:

It has been referred to the "Touchdown" PCR as a gradual reduction of the PCR annealing temperature during the PCR cycling period to reduce the random PCR products by starting with a temperature that is higher the melting temperature by few degrees and ending with a temperature which is lower the melting temperate by also few degrees. This can be explained as the high temperatures can afford more specific binding for the primer while the low temperatures can efficiently amplify the high specific products resulted from the previous cycles. The story behind the touchdown can be illustrated as that the existence of non-specific bands in the PCR products is one of the major problems that has been encountered with the standard PCR. This has been reported as a result of mispriming of the forward and/or the reverse primer to the target DNA segment. These non-specific products could be formed because of the less length of the misprimed segments prefer to be attached to the required PCR bands through the reaction period. This can be solved by using a step down "touchdown" in the annealing temperature to be reduced within each cycle to reduce the non-specific band by significant exponential folds, which in general could be 5-folds for each degree Celsius [(14)]. Scientists stated that the "touch down" PCR-amplification method is use when it is difficult to amplify some kinds of the tissue specific genes that are rich with GC bases. Touchdown was reported to be successful, rapid, and low cost in the amplification of more than 70% contents of the GC bases [(15)].

The analysis of the PCR bands is performed using agarose or polyacrylamide gel electrophoresis. While for the visualization, Ethidium bromide or SYBR® Safe DNA Gel Stain maybe employed [(16)]. The size of the resulted PCR output can be estimated by the usage of a known molecular weight DNA ladder that has to be run alongside with the PCR products. The ladder will give bands with already known molecular weights which then to be compared with the PCR products to estimate their weights [(2)].

The aim of this work is to find the optimal reaction conditions for the standard PCR programme

Materials and methods: [(4, 17)]

Section1: optimal PCR mastermix reaction:

The amplification of the target DNA pieces was achieved using an already made PCR mastermix. The optimization process included the labeling of three 0.2ml-PCR tubes; the content of each tube was 9.5ul H_2O, 12.5ul 2xPCR mastermix and 1 ul of each of the forward and reverse primers. In contrast, 1ul of neat DNA sample, 1 ul of 1/10 diluted DNA sample and 1 ul of H_2O were pipetted into the tubes 1, 2, and 3 respectively.

These three PCR tubes were allocated in a pre-set "standard PCR programme" as 2 minutes at 94˚ C for *denaturation* (i.e. separation of the double strand DNA into two single strands). Then the *amplification* step was performed for 30 seconds for both 94˚C (for denaturation) and 55˚C (for annealing), and 1 minute at 72˚ C (for extension). This step of amplification was repeated 35 cycles. Last step was the final extension step that was performed for 3 minutes at 72˚ C.

The PCR outputs besides the molecular weight marker were then run on freshly made agarose gel (2% gel for PCR analysis) and seen in the visualizer using the Ethidium bromide staining.
The agarose gel was prepared by carefully weighing one gram of agarose powder and placed in a 50-ml conical flask. Then a freshly prepared 50ml of 1xTBE buffer was added (by diluting down 10xTBE to 1xTBE). The conical flask then placed in a steamer for a 30-minutes. The completely dissolved agarose was cooled until the flask is touchable by hands without sensation of any overwarming and a 2ul of Ethidium bromide was added and was poured in agarose gel tray in which a comb was allocated and was left for the lunchtime. Then the comb and the rubbers were removed and the 1xTBE buffer was added to the mark on the casting tray.
The agarose gel was run by the addition of 5 ul aliquots of the gel-loading buffer to each tube of the three PCR tubes after finishing the PCR programme. Then a 10 ul of each sample alongside with the molecular weight marker were added to the wells of the gel and the gel was run for 45 minutes and then an extra 30 minutes to a total of 75 minutes at 79-81 volts.

Section2: PCR optimization of the reaction-variables:

The optimization that was performed to get the best reaction handled the following parameters: the magnesium titration, the temperature of the annealing process, primers and template concentrations. In addition to the "touch-down" PCR methods that were performed that will be explained below.
Each amplification process of the PCR had the exact preparation steps of: 0.5 ul of Taq polymerase; 0.5 ul of dNTPs and 2.5ul 10x reaction buffer, to which the remaining of PCR contents were inserted in regarding to the required optimization step.
For the optimization of the annealing temperature, the following volumes were multiplied by 6 with careful mathematical calculations and mixed together; 17.75 ul of H_2O; 0.75 ul of $MgCl_2$ (50mMol); 1ul for both of the primers (For. and Rev.) and 1 ul of DNA template were mixed with previous prepared solution. 25ul aliquots were allocated in a five PCR tubes and run on a programmed gradient PCR block. The annealing temperatures were as follows: 46˚C, 52˚C, 55˚C, 58˚C and 65˚C. The PCR programme was run as 2 minutes at 94˚ C for denaturation. Then the amplification step was performed for 30 seconds at 94˚C (denaturation) and 30 seconds for stepped increased range of temperatures from 45˚C to 65˚C (annealing), and 1 minute at 72˚ C (extension). This step of amplification was repeated 35 cycles. Final extension step was performed for 3 minutes at 72˚ C.
While for the optimisation of the $MgCl_2$ concentration, 6 times the following volumes were mixed together, providing special attention to the mathematical calculations that were double-checked; 16 ul of H_2O; 1ul of each of the forward and reverse primers and 1 ul of DNA template were mixed with the previous prepared solution. Five 22.5ul aliquots were inserted into the PCR tubes and mixed with 2.5ul aliquots of the following $MgCl_2$ concentrations (0.0, 0.5, 1.5, 2.5 and 5 mMol) and run on a PCR block as in section1.
However, for the optimization of the primer concentration, it is a bit differs even the same principal was used the diluted concentrations of the primers was slightly different. As mentioned above 6 times the following volumes were carefully prepared; 17.75 ul of H_2O; 0.75 ul of $MgCl_2$ (50mMol); and 1 ul of DNA template were pipetted to the freshly prepared solution. Five aliquots of a 23ul were poured in the PCR tubes and mixed with 2ul of the

following combinations; (1ul of the forward plus 1ul of the reverse with exact concentrations that were representing (forward: reverse) as listed: (neat: neat), (1/3: 1/3), (1/10: 1/10), (neat: 1/10), and (1/10: neat). These five tubes were run on a PCR block as in section1.

Moreover, the optimisation was performed for the DNA template, the following volumes were multiplied with a factor of six; 13.75 ul of H_2O; 0.75 ul of $MgCl_2$ (50mMol); and1ul of both of the utilized primers (forward and reverse) were added to the previous preparation. From the final volume of 120ul, five aliquots of 20ul were taken and poured in 5 PCR tubes and mixed with 5ul of the DNA templates with the following volume-mixtures;(DNA template ul: PCR grade water ul): (5: 0), (2: 3), (1: 4), (0.1: 4.9), and (0.01: 4.99), the five 0.2ml-PCR tubes were put on a PCR block as in section1.

Lastly, the same steps used to prepare the five PCR tubes for that of the template optimization were used to prepare other five tubes those were run on a touchdown PCR block, which was programmed as 3 minutes at 94˚ C for denaturation (i.e. separation of the double strand DNA into two single strands). Then the amplification step was performed as for denaturation at 94˚C for 30 seconds and for annealing at 64˚C for 30 seconds, and extension 1 minute at 72˚C. This step of amplification was repeated for 3 cycles. This three cycles amplification step was repeated for three more times with changing only the annealing temperature as 61˚C, 58˚C, and 55˚C. The last amplification part was by denaturation at 94˚C for 30 seconds and for annealing at 53˚C for 30 seconds, and extension 1 minute at 72˚C but this time repeated for 30 cycles. Last step was the final extension step that was run for 3 minutes at 72˚ C.

The PCR throughput of each single optimized step was run alongside with the known MWT ladder on another polyacrylamide gel. These were prepared relying on the standard operating procedure (SOP) listed in the lab handbook of Sheffield Hallam University (2012) and can be illustrated as the freshly well-cleaned plates and rubber were constructed together and examined for leaking with deionised water. Then two polyacrylamide gels were prepared by gently swirling the following volumes; 13.8ml, 2ml, and 4ml of water, 10xTBE, and polyacrylamide/ bisacrylimide (40% w/v) respectively. Immediately after that a 200ul of 20% w/v of prepared ammonium persulphate was added and then swirled with 10ul of 10ul of TEMED. Afterward a careful and quick pipetting was performed into the prepared gel-plates and a plastic-cleaned comb was placed at the top, which was removed after the gel setting in approx. 30 minutes period. The gel plates were fitted in the plate jar that was filled to the mark with 1xTBE buffer.

After that the gel was performed by pipetting of 5ul of the gel-loading buffer within each prepared PCR tube. 5ul of the final mixture besides a 5ul of the DNA ladder was pipetted into the wells of the gel and run a101-103 volts for 40 minutes and then the voltage was increased to 116 volts for further 15 minutes to compose a total running time of 55 minutes. Then the gel was carefully removed and placed in a plastic boat rich with 1xTBE buffer to which a 5ul of Ethidium bromide was placed and gently swirled and left for 10 minutes to be ready for visualization with the gel-visualizer were the gel pictures were printed.

Results:

PCR master mix positive control:

Three different template-DNA concentrations alongside with DNA ladder were run in agarose gel electrophoresis. The utilized DNA ladder starts with 100 base pair (bp) with a consistence increments of 100bp. The 223 bp neat template-DNA delivered an intense band while it is not very clear (although it is still there) in the 1/10 dilution and as expected it is absent in the non-template addition sample (blank) as shown in figure2 below:

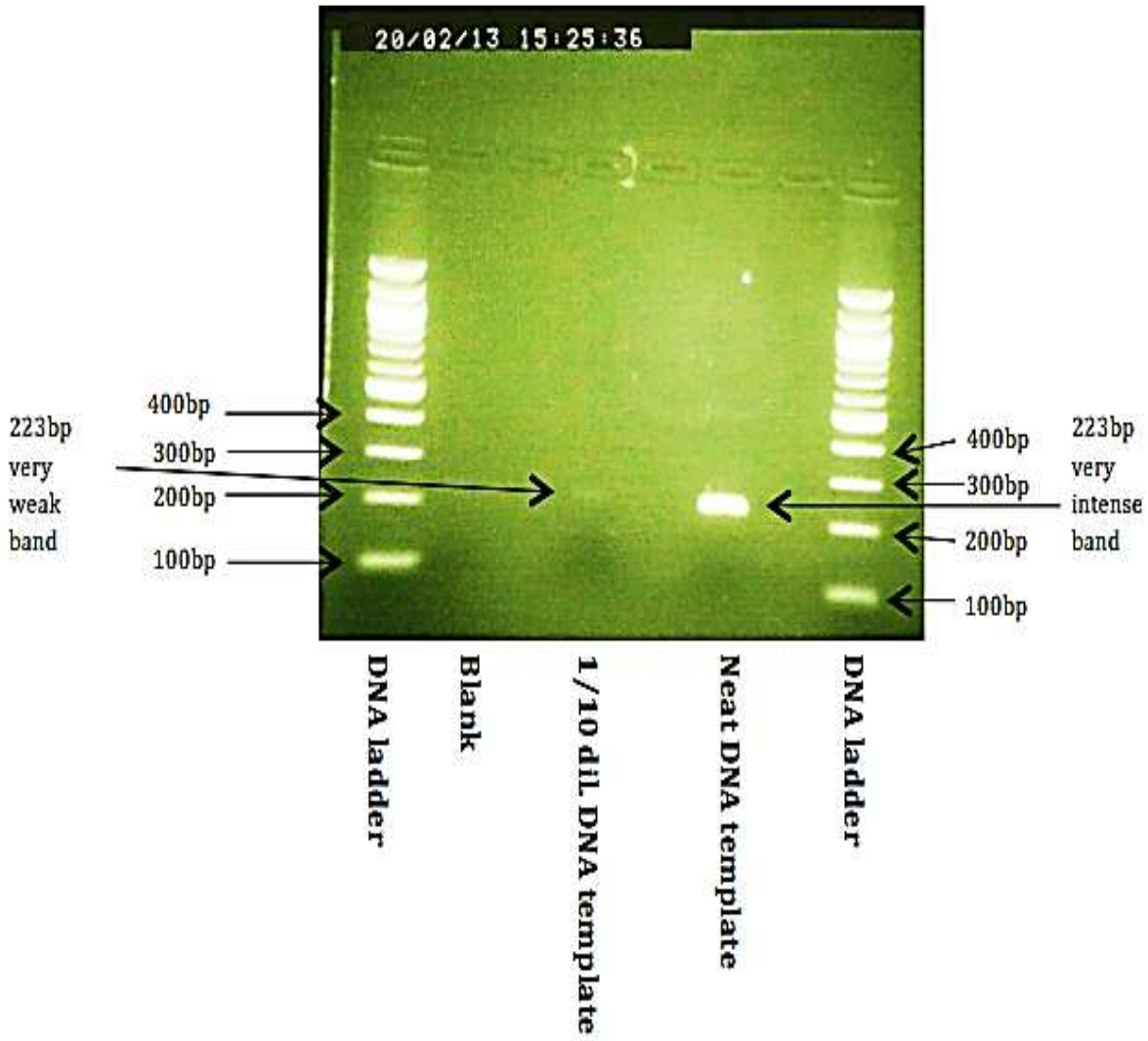

Figure 2: A visualized agarose-gel photograph of the PCR positive control. In this representative photograph the first and the last lanes show the DNA ladder. Whereas, the 4^{th,} 5th, and the 6th lanes 2 shows the blank (only PCR grade H_2O was added), 1/10 of the DNA template, and the neat template DNA of the 223bp that was added to the mastermix. the gel was visualized by the addition of Ethidium bromide.

The second and onward gels used were polyacrylamide gel electrophoresis (PAGE). This PAGE utilizes the effect of Mg^{++} on the amplification of the PCR. Different $MgCl_2$ concentrations were used starting with the blank concentration till reaching a maximum concentration of 5mM (0.0, 0.5, 1.5, 2.5, and 5 mM of $MgCl_2$). The best amplification within the given conditions was observed with the 0.5 mM of $MgCl_2$ as can be seen in figure 3 below:

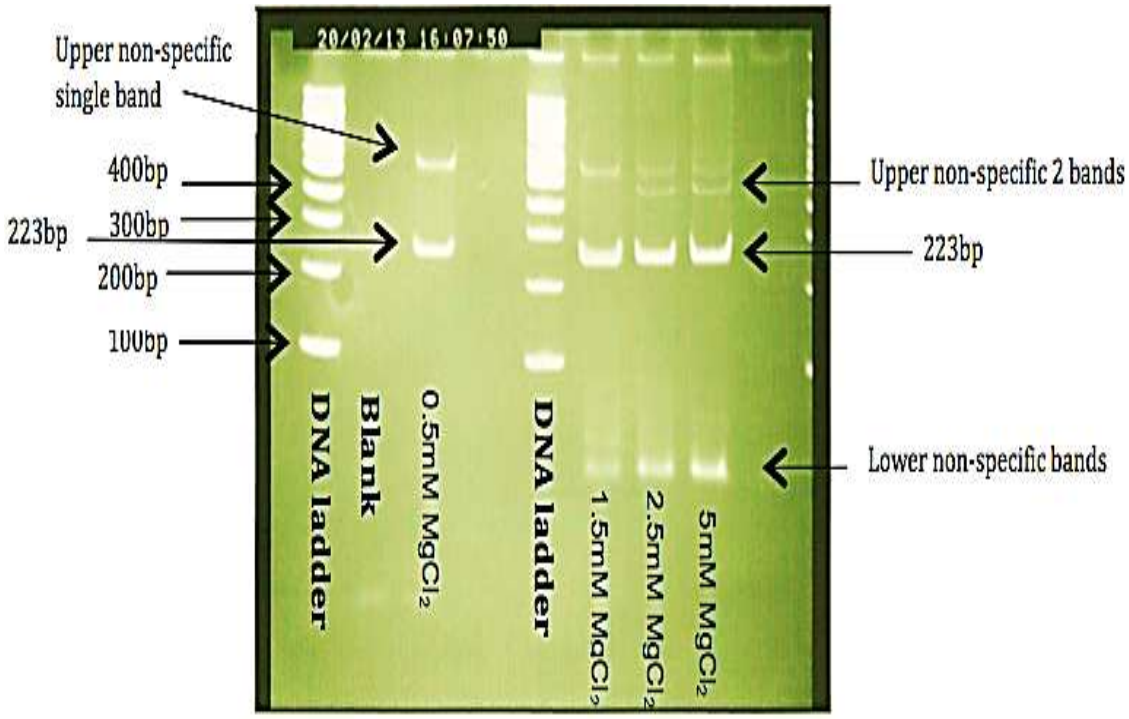

Figure 3: **Effect of altering the concentration of Magnesium on PCR amplification.** This photograph represents a polyacrylamide gel, which shows the effect of changing the Mg^{++} concentration on the utilized 223bp DNA sample. The titration was from 0.0 to 5mM of $MgCl_2$ as clearly labelled above. This gel was visualized with the addition of Ethidium bromide.

Magnesium titration was performed also using touchdown method of the PCR. In this part of the PCR different magnesium cations concentrations were used at the same time using different annealing temperatures as can be seen in figure 4.

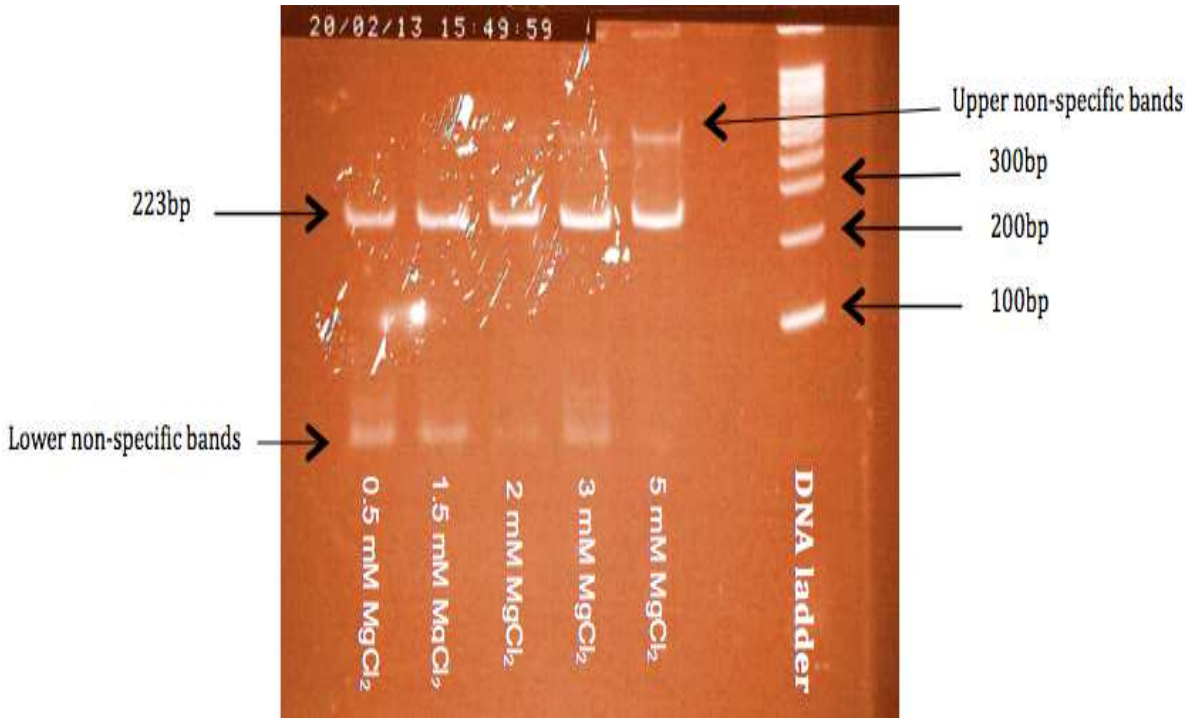

Figure 4: Effect of altering the concentration of Magnesium using the touchdown PCR amplification. This representative PAGE photograph was visualized using Ethidium bromide and reveals the effect of using multiple Mg^{++} concentrations (from 0.0 to 5mM as assigned above); this was performed alongside with the utilization of the touchdown PCR reaction using different annealing temperatures from 65 to 45^0C.

Altering the annealing temperature has an effect on the amplification of the PCR. Using high annealing temperature (e.g. 65ºC) resulted in high specific PCR product (where the non-specific band associated with the other temperatures' lanes is absent in the 65ºC lane), however this decreases the PCR throughput. In contrast using relatively low annealing temperatures resulted in high throughput, which is also associated with low specificity as shown in figure 5 below:

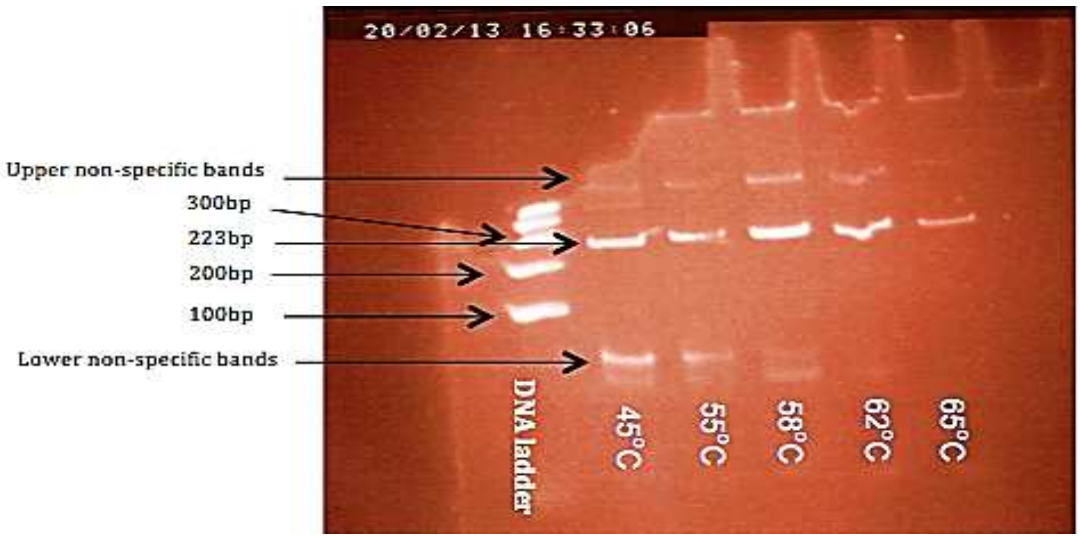

Figure 5: The effect of utilizing different annealing temperatures on PCR amplification. This polyacrylamide gel shows the influence of increasing the annealing temperature from 45 to 65ºC on the PCR product. As can be seen with increasing the annealing temperature, the amplification decreases while the specificity increases. The wired shape on the 62ºC band is related to the well-malformation which is most probably due to air bubble. Ethidium bromide was used to facilitate visualization.

The template DNA used in the PCR programme also was optimised. The wide range of the template concentrations were utilized shows a significant impact on the PCR product. This can be fluctuated from inhibitory effect on the PCR amplification observed with the 5 ul template DNA till reaching the non-detectable 0.01 ul of the template DNA as shown in figure 6.

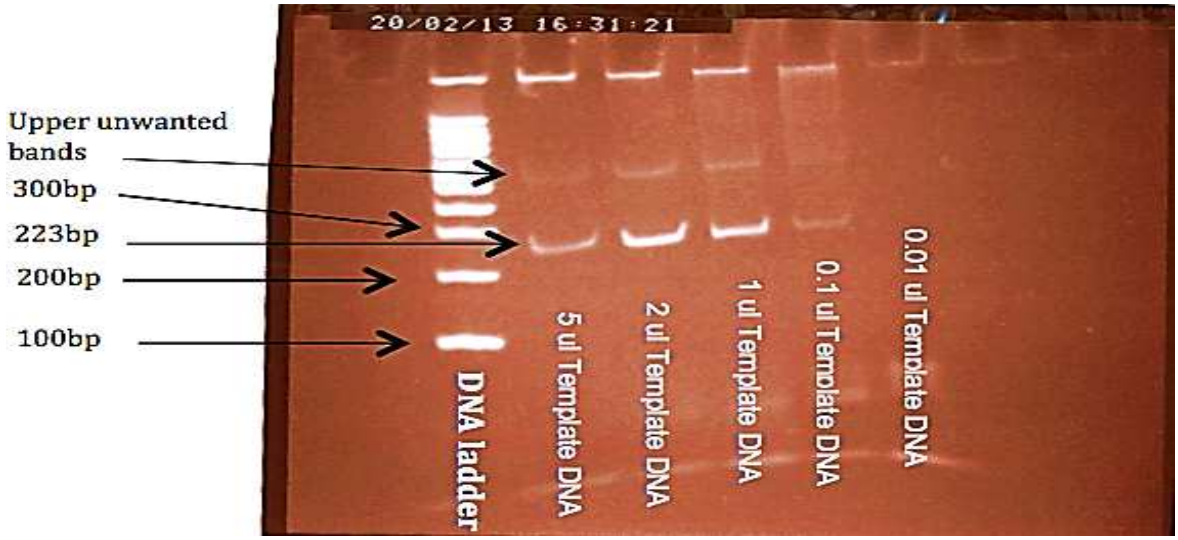

Figure 6: The template DNA optimisation. This polyacrylamide gel reveals the effect of using different DNA template concentrations on the PCR amplification product. Also it can provide an approximate detection limit, which is in this case is the 0.1 ul DNA template. Ethidium bromide was used for visualization.

In addition, the used primers in this PCR programme were also optimised and were proven to have different activities. The forward primer is less active than the reverse one (c.a. 10 folds less active). This can be clearly seen in the sixth lane in figure 7 where neat forward used with 1/10 of the reverse primer.

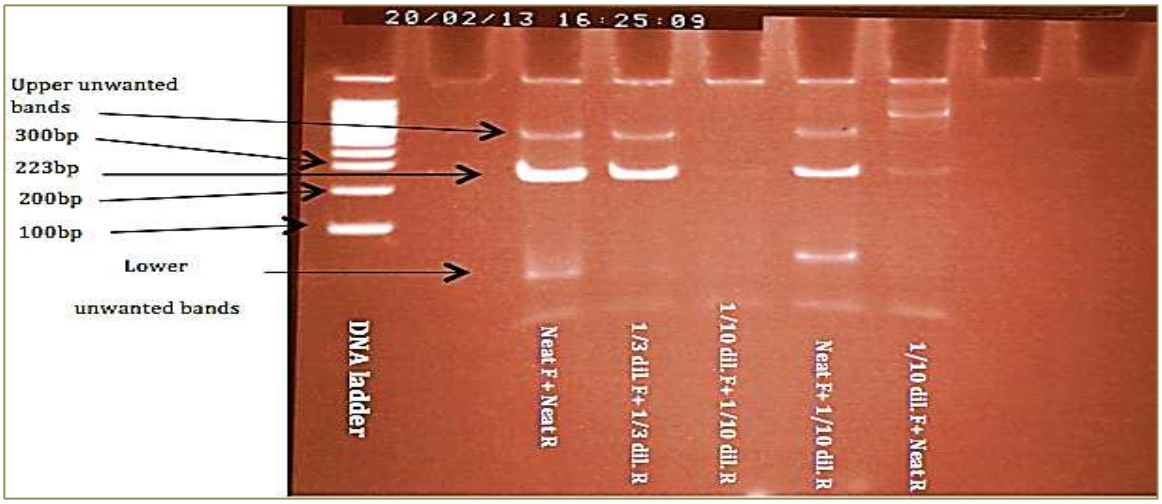

Figure 7: The effect of using different concentrations of the forward and reverse primes on PCR optimisation. This polyacrylamide gel was visualized using Ethidium bromide and reveals the process of PCR optimisation using the shown combinations of the forward (F) and the reverse (R) primers. The graph clearly shows that the neat forward primer used with 1/10 of the reverse primer was the best combination within the given condition.

Discussion:

The sensitivity of the PCR technique requires the application of control to be run alongside with the amplified sample. Few strands of a contaminant (or could be even single DNA contamination) can result in thousands or millions of unwanted products. The PCR performed using the agarose gel in figure 2 shows that the PCR program was successful in producing the expected band (i.e. the 223 bp) with no non-specific bands. The circled 223bp band for the neat DNA was very clear and intense than the 1/10 DNA dilution, which is very sensible and the absence of any signal within the blank lane indicates the reaction was free from contaminations and it is quite true to say the resultant band is due to the expected PCR product not something else, otherwise it would appear in the blank lane. Accordingly, it is so important/ easy to run a blank/control sample to obtain trusted results [(18)].

There are few differences between the utilized types of gels. For the agarose gel it is easy prepared and relatively cheap, however agarose gel has low-resolution power as compared with the polyacrylamide gel. In contrast the good resolution of the polyacrylamide gel besides the different types can be prepared of the PAGE depending on the percentage of the bisacrylimide content make it more favorable despite the fact that polyacrylamide has potential carcinogenicity [(10)].

The reason behind using more than one DNA ladder is that sometimes due to the utilization of high voltage (due to the shortage in time) or might be other reasons the shape of the gel can be curved or irregular, in such case it is very hard to allocate the resultant band in respect to the utilized ladder, while if two or more ladders were used then simply a virtual line can be drawn and the resulted band can be confidently allocated [(18)].

The optimal observed condition is that with an intense brightness of the expected band (in this case the 223bp band) and of course the absence of the non-specific bands. The non-specific bands can be classified into two major types: the upper and the lower bands regarding to the

target PCR product. The lower bands might result from dimer formation due to primer-primer binding when the 3' termini express partial complementary or due to relatively low annealing temperature or even to the existence of high concentrations of the utilized primers. On the other hand, While the upper unwanted segments is most properly because of the non-specific binding of the products ends or the mispriming performed by the polymerase enzyme due to high Mg^{++} concentration or relatively the usage of low annealing temperatures. Accordingly, the presence of upper non-specific bands has a negative impact on the quality of the observed PCR product [10, 18].

To achieve an optimal PCR product each single PCR-component has to be optimized.

Optimization of Mg^{++} concentration:

By returning to figure 3, the exclusion of the divalent cations (i.e. Mg^{++}) in the blank lane resulted in the diminishing of the amplification, while as expected the bands' intensities were increased parallel to the increase in the Mg^{++} concentration, also there were increases in both upper- and lower- unwanted bands. In this specific gel in figure 3 the best result was achieved with the 0.5 mM Mg^{++} concentration, even it is too hard to explain why in the third lane where the 0.5 mM concentration was used, why the upper non-specific band is more intense than the others in lanes 6, 7, and 8 respectively. However, in my opinion, the 0.5 mM of the Mg^{++} concentration is the best especially the absence of the lower non-specific band whereas it is present on the other lanes and more importantly the appearance of *two* (instead of one in lane 3) upper non-specific bands that shows increased intensities with the increased Mg^{++} concentration in lanes 6, 7, and 8 respectively. If I would do the same protocol in future, then I would use more lower concentration ranges and not to go too high i.e. 0.0 mM Mg cations (control, very important), 0.1, 0.3, 0.5, …, 1.5 mM Mg^{++}. Unfortunately, the photograph that represents the touchdown method used with various Mg^{++} concentrations is not quite clear to make a confident comparison with the previous non-touchdown one. Generally speaking there is no big difference between these two gels accept the upper unwanted band in figure 4 supposed to be gradually decreased until absence with the reduction of the Mg^{++} concentrations. Even it is not quite clear but at least it is logic and it is the case that can be seen in the 5 and 0.5 mM Mg^{++}.

Annealing temperature optimization:

The annealing temperature optimisation is considered as one of the significant factors to achieve a successful PCR product. As can be seen in figure 5, different PCR bands in terms of fidelity and strength were observed when the annealing temperature was changed. As the temperature was increased from 45 ^{o}C to 55 ^{o}C then 58 ^{o}C, the 223bp band shows increasing in strength, while it starts to decrease at the 62^{o}C to be its minimum at 65 ^{o}C. This is logic and already expected and can be justified as when the temperature is low (e.g. 45 ^{o}C and 55 ^{o}C), a loss in the specificity of the polymerase enzyme resulting in higher non-specific product. In contrast, when the annealing temperature becomes higher than the melting temperature (e.g. 58 ^{o}C and 62 ^{o}C), the specificity will be magnified and a stronger binding can be observed. Nevertheless, *very high annealing temperatures should be avoided* as these can reduce the stability of target DNA-primer binding, which can significantly reduce the PCR output (e.g. 65 ^{o}C) [7, 11].

Many complicated equations were utilized to estimate the optimised annealing temperature of a corresponding PCR programme for example, "$T_m = 81.5+16.6(\log [Na^+])- 0.41(\%G+ \%C) +$

675/(probe length in bases) -1.0 (%mismatch - 0.65 (% formamide)" [19].

Or,"$T_m^{Product}$= 81.5+0.41(%G+ %C)+ 16.6log[K^+]-675/I" [20]. Moreover, freely accessed online biomath-tools can be utilized to calculate the annealing temperature required for a particular PCR reaction [21].

The DNA template optimisation:

The DNA template concentration has to be within the linear region of the detection limit. Very low concentrations resulted in significantly reducing or even losing the amplification so that the PCR-target band cannot be detected. This can provide an explanation for the absence of the 223bp band in the 0.01 ul DNA template used in figure 6. The band intensity was increased as the volume of the DNA template increased till reach its maximum on 2 ul and the intensity decreased again at the utilized 5 ul DNA template. This can be explained as the usage of too high DNA template volumes can negatively affect the PCR amplification as the access of the template can significantly reduce the efficiency of the polymerase enzyme. This could be due to the overcrowded substrate that would take a spatial non-favorable location in respect to the taq-polymerase.

Optimisation of the forward and reverse primers' concentrations:

Usually, the used primers in the PCR programme have different efficiencies at a given/fixed temperature. To apply a balanced reaction the forward and reverse primers' concentrations have to be optimised also. This can be seen very clearly in figure 7 where the best combination was the usage of the neat forward primer with the 1/10 of the reverse one. Generally speaking this can be discussed, as the reverse primer is 10-folds more active than the forward primer so that 1/10 dilution of the reverse primer was enough to produce good intensity PCR product. In this case the rate limiting concentration would be the concentration of the forward primer as shown in figure 7 the reverse primer can produce PCR product in the tree used concentrations (i.e. neat, 1/3 dilution, and 1/10 dilution; in the 3rd, 4th, and 6th lanes respectively), while the forward primer can produce product in neat and the 1/3 dilution only and it is absent in the 1/10 dilution (5th and 7th lanes).

Other contents of the PCR reaction such like the dNTPs are important to obtain a successful PCR by maintain their concentration equipoised and more than the expected Km (the substrate concentration at which the maximum velocity of the reaction is at its half value) of any one of them [22].

Buffers:

Some of the salts (e.g. KCl) and buffers (e.g. Tris which is widely used in TBE buffer) concentrations are kept constant. For instance it has been reported that KCl was found to be optimal at 50mM [23]. In contrast scientists have stated that the decreasing in the [K^+] by 10-40 per cent can result in more efficient amplification [24].

PCR reaction-enhancers:

The use of certain additives such as betaine (1-2 M), formamide (1.25%-10%) and dimethylsulfoxide (DMSO, 1%-10%) promotes similar melting temperature for both GC- and AT-rich segments and enhance the optimization of the PCR [25, 26, and 27]. Some agents are used to stabilize the DNA polymerase and reduce the losses of the reagents by sticking to the walls of the used tubes. For example, non-ionic detergents (0–0.5%), gelatin (0.1–1.0%) and Bovine serum albumin (BSA, 10-100 μg/mL) may overcome the amplification failure in some cases [27, 28].

Hot-start PCR:

Besides the already discussed touchdown PCR (TD-PCR) the use of the *hot-start PCR* can significantly enhance the fidelity of the PCR reaction [25, 27]. The problem arises when PCR reaction components are exposed to temperatures lower than T_m even for a short period it can produce unwanted bands especially for the primer-dimer ones. Hot-start method can provide a solution for this by preventing one significant component from sharing in the reaction until it is assured that the temperature exceeds the T_m of the reactants. This can be performed by different ways. For example a drop of oil can be added as a layer to prevent a component (usually the *Taq* polymerase enzyme) from reacting with the other components. Finally the whole components can be mixed together when the temperature reach the required value above the T_m and the oil can no longer prevent mixing due to the high temperature. Other method relaying on the utilization of waxes separating the reactants and also the wax will be melted at the required temperature and the components will be mixed through the heat convectors. Alternatively, a kind of *Taq* polymerase antibody can used to inhibit the polymerase from its activity; eventually the antibody will be denaturized at the required high temperature leaving the *Taq* polymerase to perform its activity.

***Taq* polymerases types utilized in PCR amplification:**

Different types of taq polymerases can be used to enhance the reaction conditions and obtain a good quality/quantity PCR amplification. For example the AmpliTaq Gold® DNA Polymerase is highly purified polymerases that is inactive at low temperatures which can provide an effect similar to that of hot-start reaction. Whereas AmpliTaq® DNA Polymerase, Stoffel Fragment is more thermostable polymerase which can be used with wider range of Mg cations (2-10mM) as well. The most important type of the polymerases is the *rTth* DNA Polymerase "Recombinant *Thermus thermophilus* (*rTth*) DNA Polymerase" in addition to the ultrapurity and the thermostability of this enzyme its significance is due to that besides its activity as DNA polymerase, it can act like reverse transcriptase in the existence of the Mn^{++} cations. This makes it suitable to be utilized in the RNA-PCR by reversely transcribing the RNA to cDNA [1].

The optimized variable can affect the performance of another variable for example changing the Magnesium concentration might alter the annealing temperature. To resolve this Taguchi method can be used. The core of this method is that the observation of each variable effect on the process bearing in account the intra-variable interaction or in other words what is the effect of a particular component on the whole process and on each other component as well [30].

The use of stepwise variable-optimization was studied. In regarding to what is required to be enhanced; the specificity (good fidelity) or the sensitivity (more product). To enhance the PCR specificity all the following variables have to be optimized and the steps have to be in this order: perform the hot start-PCR, touchdown-PCR, use optimal primer design, magnesium concentration, dNTPs, pH, *Taq* polymerase, cycle segment lengths, cycle number, annealing temperature, inhibitors, enhancers, primers [29, 31].

Conclusion:

The optimization of any new PCR reaction is quite important because there is no magical protocol to be used directly everywhere and delivers the best results from the first run.

The use of stepwise PCR-components optimization rather than random selection will produce faster and more robust results. For instance the use of Taguchi or chessboarding methods can perform easier and more trusted statistical processes.

For the performed PCR reaction in this work and according to what has been achieved the optimized conditions were as follows: O.5mM of Mg^{++}; neat forward primer; and 1/10 diluted reverse primer concentrations, 1ul DNA template concentration at the annealing temperature of 58 ˚ C.

Based on what has been achieved/ studied, in future if I would be requested to do similar work and if it is feasible, I would use the Hot-start method or at least stick to the Touchdown PCR, as I believe these methods can improve both quality and quantity of the resulted product. Also if there is a good budget, then using ultra-pure reagents (especially for *Taq* polymerase) will highly improve such sensitive technique product.

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