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# An Optimization and Common Troubleshooting Solving in Polymerase Chain Reaction Technique

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## ABSTRACT
Many genetic researches now relies on the study of variants in genetic material through different, diverse of universal Polymerase Chain Reaction (PCR) methods. In that context, we are putting a highlighted on the most important fundamental aspects of PCR technology, which help researchers to clarify and reduce the majority problems and difficulties may face them in the laboratory work of any kind of PCR technology in general.
**Keywords:** PCR optimization; primer design; melting temperature; PCR troubleshooting; GC-high; DMSO; genetics.

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## INTRODUCTION

1. Rules of PCR primer design

From a common molecular biology laboratory technique is Polymerase Chain Reaction (PCR) that utilized to build up enough many copies of a definite section of DNA for further analysis in many species (Reece *et al*., 2011)(King, 2010). It is depend basically on the availability of *Taq* polymerase, template of DNA that want to amplify, four different nucleotides (A, T, G, and C) that are essentially structural units for nascent fresh DNA. In another hand, the reaction mixture needs primers, and a solution buffer to supply an unchanging pH (Holden *et al*., 2003)(Barnes, 1994).

PCR primers are short pairs oligonucleotides strand, hybridizing to reverse strands and contiguous the target DNA segment of attention and assists as an initial site for DNA amplification (Rotmistrovsky, Jang, & Schuler, 2004). Successful good and appropriate primer pairs design is crucial for all PCR-methods researches. The important idea designing of primers set to a particular DNA required region for amplification, each primer should be anneal to the unique sequences in opposite strand, which agreement with heading for the (5' → 3')direction (Chavali, Mahajan, Tabassum, Maiti, & Bharadwaj, 2005). In general, poorly designed primers may amplify other, non-intended sequences, so it is as much an art form as a science and preferred criteria should be met as the following guidelines describe the characteristics of a primer sequence:

1. Primer Size: The size had better be long sufficient for acceptable specificity and small sufficient for binding simply to the pattern, and the optimal accepted size of primers is (18-25)bp.

2. Primer Melting Temperature ($T_m$): It is the temperature at which (50%) of the DNA two- stranded will split to single-stranded. The best result produced with Tm range between (52-58)°C. Primer pairs $T_m$'s must be within (1-3)°C of one another to ensure apply three stages (denaturation, annealing and extension) of traditional PCR.

3. Annealing Temperature of Primer ($T_a$): It is the appreciation of the DNA-DNA crossbred constancy. $T_a$ should be neither high nor low to avoiding produce insufficient primer-template hybridization and low PCR product in high temperature, and non-specific products caused by a high number of base pair mismatches in low temperature. The optimal $T_a$ for a set primer pair can be calculated as: Ta = 0.3 x ($T_m$ of primer) + 0.7 x ($T_m$ of product) – 14.9. Most primers require an annealing temperature between (50 – 65)°C. It is normally set at (3-5)°C below the melting degree.

4. GC Content: It represent a number of G's and C's in the primer as a percentage of the total bases and prefer have a content between (45-60)% for each primer.

5. GC Clamp: To promote specific binding at the 3' end of primer, pay attention to the presence of G or C bases within the last five bases of the primer taken in consideration no more than three G's or C's residues due to the stronger bonding of G and C bases.

6. Repeats: Generally, to keep away from misprime should be no repeat consecutive of di-nucleotide such as (ATATATAT) or four of same bases like (AGGGG).

7. Stability of 3'-End: The last five bases from the 3'-end have maximum ΔG value and more stable and vice versa result in less false priming.

8. Template Secondary Structure: Mainly template secondary structure is important in designing primers and its stability depends on their free energy and $T_m$.

There are a few frequent troubles that occur during primer design involving: 1) Primer dimer due to binding primers together rather than template (Figure 1a) ; 2) Hairpin loops formation caused by self-annealing of primers (Figure 1b).

Observation

1. Many of designed software are available to assist primer pairs designing. NCBI Primer design tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/, Primer3plus http://www.bioinformatics.nl/cgibin/primer3plus

/primer3plus.cgi/, and IDT https://eu.idtdna.com/Primerquest/Home/Index are recommended websites for this purpose.

2. It is important and beneficial to engage a blast on NCBI to verify for the intended quarry particularity of the designed primer pairs in order to escape from any related sequence pseudogenes or homologs.

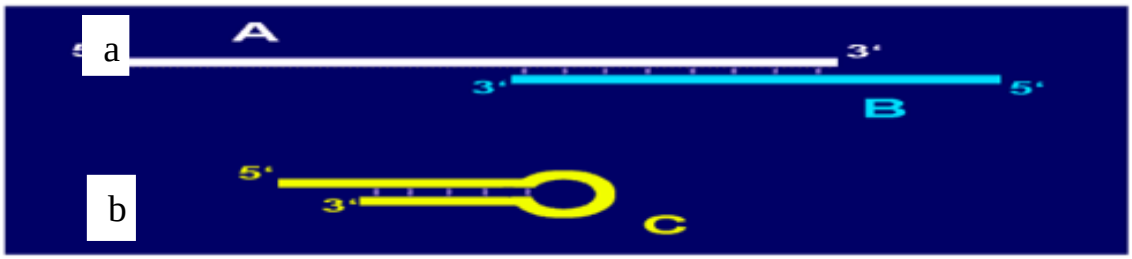


Figure 1: Familiar troubles that occur with primers design (a) Primer dimers (b) hairpin loop structure.

## 2. MATERIALS

Through the working in each experiment of PCR should be taken some considerations that involve the following:

1. To avoid contamination of all reagents of the experiment, laboratory coat, goggle and gloves had better be wore. In addition, it is required to have Material Safety Data Sheets for a right management of equipment and harmful materials that used in experiments.
2. Keeping all reagents secure on ice pail throughout preparation of experiment and leave them to thaw fully before setting up a process.
3. Mainly the reaction components playing an important role in successful amplification protocol which achieved by six critical factors.
   - ✓ Template desired target for replication could be any DNA resource, like genomic DNA (gDNA) and complementary DNA (cDNA) or even plasmid DNA. However, the variable input amounts of DNA for amplification governed by the complexity or composition. For example, up to 25 ng of gDNA may be enough as an initial amount, and on the other hand up to 0.5ng of plasmid DNA is sufficient in a (25-50)µL scale in (200-500)µL microcentrifuge PCR tubes.
   - ✓ A two set of appropriate upstream oligonucleotide primer (F) and downstream oligonucleotide Primer (R) designed specifically to bind with opposite sequence of wanted goal gene or DNA section to be magnified. Approximately 10mM is sufficient for most PCR reactions.
   - ✓ *Taq* DNA polymerase which consider a standard and suitable thermostable enzyme for amplification of most types of PCR. In general, using up to 2.5U per reaction is recommended of *Taq* DNA polymerase in a 25µl whole volume reaction. In addition to sometimes even 0.1U can succeed in 20ul reaction volumes(White, Chiang, Chow, & Broker, 2003).
   - ✓ Building blocks of new DNA strands which are deoxynucleoside triphosphates (dNTPs) that involve four basic nucleotides- (A, C, G, and T). It is significant to maintain each concentration of four dNTP between10 -15 µM. However, the excessive amounts of dNTPs can slow or inhibit PCRs.
   - ✓ A suitable buffer for PCR that occur in appropriate substance setting for action of DNA polymerase. Frequently pH of buffer between 8-9.5 and stabilized by Tris-HCl. Most PCR buffer comes with 10X, and composed from KCl ion (35-100)mM, Tris-HCl 100mM, $MgCl_2$ (0.5-5.0)mM and gelatin 0.01%. (Table: 1) demonstrate most common additive reagents recommended with final concentration that promote PCR.
   - ✓ Finally nuclease-Free Water to avoid any contamination that cause DNA degradation (Pelt-Verkuil EV, Belkum AV, Hays JP, 2008).

Table 1: Most common additive reagent with their final concentration

| Reagent | Final concentration |
|---|---|
| Dimethyl sulfoxide (DMSO) | 1–10% |
| Glycerol | 5–20% |
| Formamide | 1.25–10% |
| Bovine serum albumin (BSA) | 10–100 µg/mL |
| Polyethylene glycol (PEG) | 5–15% |
| Betaine | 0.5 - 2.5 M |

4. Accessories and equipment on bench involve using micro-centrifuge PCR tubes with their rack, set of automated pipettes that dispense from (1-100)µl, and thermal cycler.

5. At the end of PCR experiment you have to evaluate the product through using gel electrophoresis and comparing with standard DNA known molecular weight sizes(Pelt-Verkuil *et. al*, 2008).

3. Making up a Reaction Mixture

Template of DNA

For good amplification, an ideal DNA quantity should be applied. Ideal DNA scales for this process are (0.001–1)ng of plasmid DNA or (1–10)ng of genomic DNA. Excessive DNA can be giving unspecific products in the PCR process. Special attention must be taken in the selection of a DNA isolation process. The presence of any impurities with the isolated DNA, as a trace of (phenol, Proteinase K, or EDTA) can hinder a PCR process. So, A best solving for similar restriction trouble is the diluted of the DNA sample, that provides increases not only DNA dilution but to quantities of impurities also.(Bartlett & Stirling, 2003).

Primers

Primer is small oligonucleotides in range (18-30) nucleotides that elucidated in details as in the previous paragraphs.

*Taq* DNA polymerase

It is one of the majority principle substances for improving PCR process. It stimulates the DNA formation in the (5'-3') direction. The definitive concentrations of polymerase common appropriated is (0.5 –2.5)U in a (50)µL. More than polymerase can lead to a big amount of non-specific amplification outputs showing as 'smear' bands in electrophoresis. Despite that, if there are PCR inhibitors in the reaction solution, utilizing elevated polymerase activity (2.5–3.0)U in 50µl may be needed for the purpose of getting good amplification efficiency(Henry A. Erlich, 2019).

dNTPs Levels

Deoxynucleotide triphosphates (dNTPs) are backings of PCR technique; dNTPs provide the formatting of fresh DNA strands. The ideal dNTPs levels in a PCR solution are generally 800µM; 200µM of each dNTP. It is necessary to keep the equal levels of each dNTP is at the same level. Improper levels of even one dNTP may effect on polymerase to combine the incorrect dNTP into the freshly formed DNA molecules; that is to say, it influences the reliability of the enzyme. The ideal dNTPs levels rely on the size of the amplification products, the concentration of $MgCl_2$, and the concentrations of the primers (Keohavong, Wang, Cha, & Thilly, 1988).

Reaction Mixture Set Up

Initial step of PCR contains mixing the original DNA, primer pairs, DNA polymerase, dNTPs and a buffer. Then the mixture is cycled usually 30 running over temperatures that permit denaturation, annealing and extension. The product is then displayed on an suitable gel and observed for yield and specificity(E. J. Wood, 1983).

4. Necessary PCR Protocol

Basically the best approach to carry out PCR reaction is in a dedicated cabinet, the components and equipments also specific for PCR and the reaction will be prepared either as single tubes or as a plate with (96, 288, 384) well. The tubes placed in an ice container in addition to reagents to avoid the activity of nuclease and unwanted priming. In the other hand pipetting PCR reagents in order in a PCR tube included half total volume of reaction a master mix containing 10X PCR buffer, dNTPs, MgCl2, then primer pairs and DNA template were added, then the total volume completed into desired reaction (25 or 50)µl. A negative control and as possible a positive control preferred in each experiment for helping to set up master mixing reaction. In negative control will be added all PCR reagents except DNA template will be added nuclease free water instead it, to compensate the omitted volume. Also if possible you can use positive control in a separate reaction contains well known DNA template and all other PCR reagents to amplify under the same conditions. Finally, tightly close all PCR tubes caps and transferring them to thermal cycler with a suitable program.

At the end thermal cycler program, transferring the tubes into refrigerator and kept at 4°C. The amplicon can identified through agarose gel electrophoresis with DNA binding staining dye under UV illuminator.

Table2: Recommended ordered added components into single reaction PCR tubes on ice and quickly transferring to a thermocycler

| Component | 25µl | 50 µl | Final Concentration |
|---|---|---|---|
| Matermix | 12.5 | 25 | 1X |
| Forward primer 10 µM | 1 µl | 1-2 µl | 0.2-1µM |
| Reverse Primer 10 µM | 1 µl | 1-2 µl | 0.2-1µM |
| DNA template | variable | variable | <1µg |
| dd$H_2O$ | To 25µl | To 50µl | |

Hint: Total volume reaction up to 25 µl, and 50 µl respectively.

Table 3: Thermocycler program suggested

| | Step | Temperature | Time |
|---|---|---|---|
| 1 | Initial Denaturation | 95°C | 30-10min |
| 2 | Denaturation | 95°C | 15-45sec |
| 3 | Annealing | 45-68°C | 15-69sec |
| 4 | Elongation | 72°C | 1min/kb |
| 5 | Hold | 4-14°C | |

Hint: according to suggested thermocycler program (http://www.mutationdiscovery.com/md/MD.com/screens/optimase/OptimaseInput.html?action=none) website

5. Melting Temperature (Tm) Calculation

As mentioned in a previous paragraph, Tm defined as the temperature at which 50% of the DNA two- stranded will split to single-stranded state in solution. Performance success PCR experiment should be known Tm. A numerous Tm calculators available and it is calculations include measurement the veritable Tm because needed a specific information relative to a particular reaction for the Tm calculators themselves. The calculators compute in addition to Tm of primer, they calculate annealing temperature depending on the primer pair sequence, concentration, and DNA polymerase that used in PCR. In this direction they based on the thermodynamic model and used the following formula: Tm ˚C = 4(G+C) + 2(A+T). Tm calculations not take only base composition, but also based on the precise sequence and base stacking parameters.

There are several websites were recommended for calculating $T_m$ value:

http://www.sciencelauncher.com/oligocalc.html
http://insilico.ehu.es/tm.php?primer=GTATGTGTGTATATATATGT&NearestNeighbor=1&cp=200&cs=50&cmg=0
https://tmcalculator.neb.com/#!/main
https://www.biophp.org/minitools/melting_temperature/demo.php?formula=basic
https://depts.washington.edu/bakerpg/primertemp/
http://biotools.nubic.northwestern.edu/OligoCalc.html
https://www.promega.com/a/apps/biomath/index.html?calc=pmolulugml

Uses one of above websites to calculate and predict Tm of primer pairs, should be type or paste primer sequences and final primer concentration, $T_m$ values, GC contents, length of primers, and suggested annealing temperature are automatically generated.

6. Thermal Cycling Settings Optimization

DNA is magnified by a series of polymerization cycles include three steps: DNA duplex strands denaturation, primer-"single-strand DNA" (ssDNA) template annealing, and DNA extension by a Taq polymerase. Best denaturation temperature or melting temperature Tm ranges from (93–98)°C to separate two DNA strands in percent 50%, while the time range of denaturation step is between (0.5-4) minutes. The GC content of the DNA template order is the important factor that determines an increase range levels of temperature and timing of the denaturation step. When Tm is so low, denaturation will not be good and amplification will be no efficient. A denaturation temperature of 95°C is commonly appropriate. In other side, the excellence of products of PCR reaction depends on many factors, annealing temperature Ta is one of them. The optimal Ta is commonly 5ºC smaller than Tm of the primer-ssDNA template. The Ta depends on the number and order of nucleotides in primer. When the Ta is so high, the primers will be facing high difficulty in ligation successfully with the ssDNA template, which leads to minimizing the efficiency of the PCR process. Conversely, when the Ta is very low, that directs to lower primers specificity binding with a template. At a less temperature, the primers may bind to multiple sections in the ssDNA template will give outputs other than the planned goal, because inner one nucleotide mismatches or incomplete binding may be tolerated. That can cause an unspecific PCR process and will then decrease the wanted output.

Optimizing the Ta will strengthen any PCR reaction that is combined with other additives and/or beside other amendments to cycling requirements(Rychlik, Spencer, & Rhoads, 1990).

Wonderful progress to PCR efficiency has been obtained by utilizing of Hot-Start energizing designs that purpose to avoid DNA polymerase amplification until increased temperatures are achieved. The advantages of using Hot-start are increasing wanted yield amounts, raise the efficiency of the PCR reaction by eradicating primer-dimer and unspecific yields synthesis (Todd C. Lorenz, 2012)(Lebedev *et al.*, 2008).

On the other hand, a good software strategy can minimize unspecific output, which is the "Touchdown PCR" program. This program enhances primer annealing to DNA templates at the best Ta. Touchdown PCR starts at big Ta and progressively drops to lower Ta every three cycles. The highest Ta for primers will be lead to an effective reduced for unspecific amplification. Consequently, a touchdown PCR protocol is extremely active in minimizing a broad spectrum of troubles PCRs which give wrong yields through amplifying with normal PCR cycling settings(abm, 2015)(Don, Cox, Wainwright, Baker, & Mattick, 1991).

Additionally, another type of modification in the additive reagents and programing setting of PCR had named the "Slowdown PCR" method, which lets the excellent PCR-process of highly GC-rich (>83%) DNA templates and for templates with different annealing temperatures. The disadvantage of this procedure requires 5 hours to complete (Frey, Bachmann, Peters, & Siffert, 2008)(T.C. Lorenz, 2012).

7. Essential PCR Troubleshooting and Solutions

Even with simple standard PCR technique that designed to amplify the gene of interest (GOI), problems with experiment components and amplification procedure are analyzed at last step through agarose gel electrophoresis (Aitken, 2012). Despite the exclusion of the desired PCR products, malfunction of magnifying under the best circumstances may produce generation several vague and unwelcome products. Problems of PCR technique may be disappointing attempt at times. However, to minimize time and trials needed to get the wanted result should be understand and analyze the regents used in PCR experiment(Heller *et al.*, 2019). Most troubleshooting that occur during PCR reaction involve template DNA sequence like high GC content, hairpin loop of primer, primer dimer, primer concentration, cycle conditions, no amplification (no-band), and other effectors related to $Mg^+$ and $K^+$ ions. So, first be sure that error(s) not due to human. Then, exclude the reagents contamination through checking wholly components needed(Bustin & Huggett, 2017).

Therefore, to identify the major causes of PCR failure and improve PCR efficiency, guided in troubleshooting bellow(T.C. Lorenz, 2012).

1. Reagents: Prepare new fresh working reagents; next regularly adding of a single fresh chemical agent to PCR eppendorf tube. This way will determine any one of substance was the reason for the unsuccessful test.

2. DNA template: It present mainly in two forms target sequence to be amplified and non-target DNA. Template DNA concentration should be balanced with cycle's number in the reaction. Fail the reaction with PCR product is very common due to reaction state. No amplicon of the target sequence for one or both primers due to not existing in the pattern DNA. In this case, the verifying accurate arrangement design of primer and primers complementary to the right strand of target is required. If complete quantity of the genomic templet in a solution is tiny, this will increase chance of loss or contamination from impurities that can contact with the DNA solution(Bernstein, 2017). In general DNA degradation in PCR protocol comes from nucleases which abundant on human skin and can be present everywhere else too. In this side, mild autoclave of the DNA diluents and everything used in the experiment in addition to washing working bench with 6% $H_20_2$ enough to resolve that. Another cause related to template is too much DNA secondary structure and can overcome this obstacle by adding dimethyl sulfoxide (DMSO) for a final volume (5-10)% V/V to enhance PCR reaction(Technologies, 2011). However, the weakness or faintness of amplicon can be use many keys like reduce the annealing temperature, increase the annealing time, use touch-down PCR, increase number of cycles, and re cleanup of isolated target(Yuryev, 2007).

3. Primer dimer formation: It is formed in the reaction when primers are extra levels over the template concentration and liable to self-anneal or anneal the further primers to appear as small product in agarose gel <100bp. If this happen, alteration of template to primer concentration is the best choice. However, using optimal primer concentration between (0.1-1)$\mu M$ of each primer, supplement DMSO and/or utilizing a hot-start process could dispel the trouble. Finally, as the last solving-step, can go to restructuring unprecedented primers (Roux, 2009).

4. $[Mg^+]$ and $[K^+]$ ions: Magnesium is essential of PCR enzyme activity and increase stabilization of dsDNA in PCR reaction. Potassium normally used in PCR amplification to improve of DNA fragments, especially fragments in the size range (100-1000)bp (Green & Sambrook, 2019). The optimum concentration recommended for $[Mg^+]$ and $[K^{+]}$ are ranged (1-4)mM and (70-100)mM respectively. Excess magnesium will lead to reduce polymerase activity and may generate unwanted products. Lower [K+] is recommended for amplification longer product and vice versa. Since the adjustment of $[Mg^+]$ in reaction may require in any changing in component of the PCR mixture or $[K^+]$ based buffer concentration.

## 8. PCR Reagents and manipulation

Typical PCR components contain a set of suitable primers, DNA polymerase, buffer solutoin for the particular DNA polymerase, dNTPs, DNA template, and DNAase, RNAase free water(Rahman, Uddin, Sultana, Moue, & Setu, 2013). The DNA polymerase is the main enzyme that binds separate dNTPs together to procedure the amplicons. The dNTPs contain four nucleotides – adenine (A), thymine (T), cytosine (C), and guanine (G) – which have created DNA. These do as the structural units that are utilized by the DNA polymerase to produce the outcome amplicon. The primers recognize the exactly meant DNA output to be amplified. Primers are teeny DNA parts with a fully known sequence harmonious to the part of DNA templet that is to be studied and amplified. These work as a protraction dot for the DNA polymerase to create the new amplicons(Garibyan & Avashia, 2013).

Supplementary chemicals may contain Magnesium chloride MgCl2, Potassium chloride KCl, dimethylsulfoxide DMSO, formamide, bovine serum albumin and Betaine. Appreciative of the purpose of substances utilized on normal PCR is important when fundamental choose how finest to change reaction status to get the required product. The favorable outcome plainly may depend on modifying the following: $[Mg^{+2}]$, $[K^+]$, [dNTPs], [pair of primers], [target DNA], or [PCR enzyme]. Nevertheless, the mistaken amount of any substances can result in fake outcomes, reducing the rigor of the processing. During the time of crisis management of PCR, exclusively single substance has to be maneuvered it at one PCR test (Innis & Gelfand, 1999)(T.C. Lorenz, 2012).

Nevertheless, to develop excellent PCR harvest and decrease unspecific yields, the DNA concentration, in addition to its purity will have a considerable effect. [DNA] and [RNA] levels can be determined by employing the optical density evaluations at 260 nm ($OD_{260}$) for them (T.C. Lorenz, 2012).

Contaminations of DNA isolating are usual PCR spoilers. Therefore, workers should be very careful to prevent that. The most public contaminations of PCR processing are contain organic substances such as; proteins, RNA, and solvents, besides to chemical substances such as detergents. Utilizing the extreme $OD_{260}$ of nucleic acids paralleled to that $OD_{280}$ of proteins ($OD_{260/280}$), it is promising to control an evaluation of the pureness of DNA isolation. Preferably, the ratio of $OD_{260/280}$ is in the middle of (1.8-2.0). Lesser $OD_{260/280}$ is the sign of organic contamination (proteins and/or solvents pollution), which in all chance, lead to a poor PCR processing. Furthermore, to the fitness of DNA target, optimization of the amount of DNA can so helpful for the end results of PCR processing. In spite of it is suitable to evaluate the amount of DNA in Nano or Pico these are usually result by modern spectrophotometers. However, the best templet number of molecules are between 104 to 107 molecules(T.C. Lorenz, 2012).

Magnesium salt or $[Mg^{+2}]$ works as a cofactor throughout process and it is utilized at last PCR reaction levels of (0.5-5.0)mM. Altering $[Mg^{+2}]$ level is one of the calmest substances to control with possibly the highest influence on the accuracy processing. Overall, the produce will rise at an adding more of $[Mg^{+2}]$. On the other hand, enlarged of $[Mg^{+2}]$ will also reduction the efficiently of the PCR enzyme. Greatest producers involve a mixture of $[Mg^{+2}]$ as ($MgCl_2$) besides the PCR buffer (10X) concentration and polymerase enzyme. The 10X PCR buffer concentration can contain 15mM $MgCl_2$ that is sufficient for a regular the reaction. The $[Mg^{+2}]$ is not actually depleted at the reaction, but whereas no sufficient level of it, subsequent no PCR result. Whatever times there is abundant $[Mg^{+2}]$, it can stop the whole denatured of the pattern through calming the duple strands. Furthermore, $[Mg^{+2}]$ may steady false annealing of primers to unfitting target locations and reduction specificity yielding in not wanted PCR amplicons at high levels(Innis & Gelfand, 1999).

Potassium chloride (KCl) is typically utilized in a PCR processing at a final level of 50 mM. Particularly segments in the dimensions range in the middle of (100-1000)bp, while a KCl level on of concerning (70-100)mM is suggested. Conversely, amplification of extended yields ranges between (10-40)kb, advantage from dropping it from the standard 50mM level, often via combination with the adding of DMSO and/or glycerol. Therefore, a depleted $K^+$ seems to be superior that is maybe for the reason that a rise salt concentration leads to smaller DNA molecules to denature specially to lengthier DNA molecules. Smaller molecules are consequently amplified well at upper salt levels. It has to be known but that a salt level above 50mM may hinder the *Taq* polymerase(T.C. Lorenz, 2012)(Cheng, Chen, Monforte, Higuchi, & Houten, 2016).

9. Enrich GC Templates additives

Although PCR actuality an essential and routine technique, but in sometimes it passes through numerous problems that arise during the progression of the work, for instance, big guanine-cytosine (GC) amount (was defined as parts have (60% and up GC) in the targets of DNA strand(Rex & Grayson, 2018). By utilizing, the PCR technique, DNA amplifies to GC wealthy parts is generally hard, in the parallel to other DNA regions consequent to three reasons. The first reason, GC loaded targets give rise to a stabilized secondary forms output. Second reason, that hinder or minify the DNA polymerase headway and leading to secondary annealing position contribute to show the nonspecific amplified bands, and this is the third reason. Usually, secondary forms give two different products(Moreau, Duez, & Dusart, 1997). The first yield has the folding between DNA strands (inter-strands), and the second yield (intra-strands) has a cross-linking of DNA folding (hairpin and/or twist), which attributable to greater hydrogen bonding with adjacent GC(Mohan *et. al.*, 2014). Development of those secondary shapes at the primary amplification rounds, deny a template DNA from amplified appropriately in following rounds.

Various common advices on what a way to modulate PCR amplification, are pointing. For PCR primers the significant point that confirms that primers own border on GC amount of (50-60)%, which will block secondary structures shaping. Extra that, regarding the primer longitude at the border (18-22)bp, in the generality situations, with optimize the conditions(Frey *et al.*, 2008).

From the 30,000 genes in an individual DNA, about 28% have GC-high zones(Saccone S, De Sario A, Della Valle G, bernardi G 1992), together with regulative genes that existing within those parts(Hapgood J, Riedemann J, Scherer S.,2001). So GC-rich sections may be strongly catching the attention for cloning and sequencing investigations(Ralser *et. al.*, 2006)(Zhang *et al.*, 2009).

Gratefully, one proven development to solve those problems is by the support of additives substances to the PCR buffer. The additives compounds act for reducing the formation of primers secondary structures, which in turn enhance target amplification and minimize appearance non-specific bands(Musso M, Bocciardi R, Parodi S, Ravazzolo R, Ceccherini I, 2006).

Through the previous concepts, the additives are divided into four main types depending on their effect:

1) Reducers of Secondary Formations: It is the most common type of additives. Includes 3 chemical compounds, which are:

   A) *Dimethyl sulfoxide (DMSO)*; utilized at a percentage in the middle of (2-10)% (Sarkar, Kapelner, & Sommer, 1990) (Coutlée F & Voyer H 1998) . Utilizing higher than10% DMSO may lower *Taq* polymerase action into more than 50% (Chevet E, Lemaître G, Katinka M 1995).

   B) *Gycerol*; utilized at (5-10)% percentage(Rex & Grayson, 2018).

   C) *Betaine*; utilized at a concentration of (3.5-0.1)M. Additionally, it can improve specificity(Henke, Herdel, Jung, Schnorr, & Loening, 1997).

2) Non-Ionic Detergents: This group utilized to amplify the amount of product; it minifies of nonspecific amplification; helps in neat output via fight SDS impurity that comes during DNA isolation. Includes 3 chemical compounds, which are (Wahyuningsih *et al.*, 2017):

   A) *Triton X-100;* utilized at a percentage of (0.1-1) %.

   B) *Tween 20;* utilized at a percentage of (0.25-1) %.

   C) *NP 40;* utilized at a percentage under 0.1%, up 0.1% *Taq*

   polymerase converts weakened( Kreader CA, 1996).

3) Reducers of Nonspecific linking: Includes three chemical compounds, which are:

   A) *Formamide;* utilized at a percentage of (1-10) %( Kreader CA, 1996).

   B) *7-DEAZA-2'-Deoxyguanosine*; successfulness averages have been publicized to be above to 83% for very GC privileged targets(Moreau *et al.*, 1997).

   C) *Tetramethyl ammonium chloride (TMAC)*: utilized at (10-100) mM concentration.

4) PCR Buffer Additives: Includes two additives, which are:

   A) *Magnesium ($Mg^{2+}$):* the $Mg^{2+}$ concentration in the PCR buffer must be in (1-4) mM (Chevet E, Lemaître G, 1995).

   B) *BSA (Bovine Serum Albumin):* BSA is a so prevalent increment for restriction enzyme digestion. Moreover, it is good for blocking the impurity of phenolic compounds plus to pause reaction substances of adsorbing to the tube wall (0.8) mg/ml (Farell & Alexandre, 2012).

10. Promotion of PCR in the presence of inhibitors

A common problem and challenging task with non successful PCR reaction is the presence of variety inhibitors. It is representing a group of substances that include chemical and biological materials that accompanying with DNAs extraction and affect downstream processing. PCR inhibitors may be stop off polymerase, or compete with reaction components. Some inhibitors found predominantly in particular kinds of samples, thus lead to a suitable strategies protocol for preparing DNA before continue to PCR reaction. They are many familial protocols have been founded to eliminate or minimize the effect of PCR inhibitor.

Extraction procedure that use phenol–chloroform fail to remove water-soluble inhibitors like urea or humic acids, and salt such as $Fe^{+2}$, similarly on Chelex that can be

ineffective removing inhibitors from blood samples like heparin and heme(Wahyuningsih *et al.*, 2017). A lot of nucleic acids purification methods are accessible and allow PCR-inhibitory substances go away. From these methods, using alternative procedures prior PCR to increase DNA yield or overcome inhibition like selective precipitation, silica membrane spin column, immune-magnetic DNA separation, and others. Additionally evaluate DNA purity through calculate the $A_{260}/A_{280}$ ratio, and absorbance at (230)nm to detect presence phenol. In addition to some inhibitors comes from consumable materials and $H_2O$ that used in reaction, and best way to eliminate this problem using sterile accessories and nuclease free water.

Table 4: Selected additive materials with their optimal concentration to enhance PCR

| Enhancer | Function | Optimal Quantity |
|---|---|---|
| Non ionic detergents (Triton X-100, Tween 20) | 1.Suppress secondary structure<br>2.Help stabilize the DNA polymerase<br>3.Neutralize the inhibitory affects of SDS | 0.1 - 1% |
| Formamide | Reduces secondary structure and useful for GC rich templates | < 10%. |
| DMSO | Reduce secondary structure and useful for GC rich templates | 2-10% |
| BSA | Reduce melanin inhibitors in templates | 0.01- 0.1µg/ µl |

11. Cycling Conditions Adjustment

The conditions of PCR reaction involve total number of cycles, temperature, and duration of cycles. The number of cycles depends on the DNA target, number of desired copy of amplicon and the efficiency of primer extension. If the DNA target is less than (10)copies, up to (40)cycles may be required to produce a sufficient yield. More than (45)cycles is not suggested as nonspecific bands start to appear with higher numbers of cycles(Kainz, 2000)(Breslauer, Frank, Blöcker, & Marky, 1986).

At least (25)cycles are required to reach acceptable levels of amplification like in case of 105 copies of DNA template and Taq DNA polymerase with efficiency (0.7). Also, if the products with high amounts and the reaction components is exhausted so the PCR efficiency will be lower extremely, consequently in a characteristic plateau phase for a PCR amplification curve ( Figure 1)(Rychlik *et al.*, 1990).

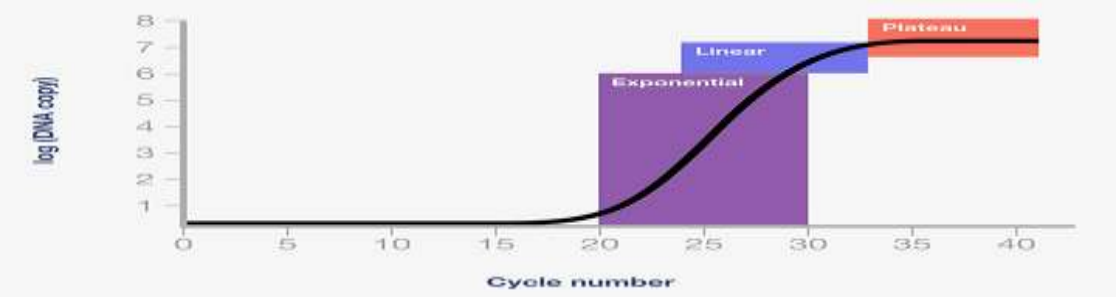


Figure 2. PCR amplification curve showing product accumulation over the number of cycles.

The number of standard cycles PCR reaction is about (25 - 35), this results in from approximately (34 million) to (34 billion) copies of the chosen sequence using (25) cycles and (35) cycles respectively. After the number of cycles is adjusted, it is essential to select the temperature and duration of each step in the cycles(Donald M. Coen, 2006).

Initial Denaturation Step

The denaturation of genome template on the beginning of the PCR amplification is one of three main significance steps. If genome melting has not fully, therefore, the outcomes in the incompetent applying of a target in the earliest cycle and PCR output will be small quantities(Douglas & Atchison, 1993). The first denaturation must be perfect through a period range between one to three minutes at 95°C when the GC amount is 50% or lower. That period necessity be prolonged up to ten minutes for GC rich patterns (Baskaran *et al.*, 1996). If the primary denaturation is no more than three minutes at temperature equal to 95°C, PCR polymerase enzyme may be supplementary into the solution of original reaction. When lengthier primary melting or a upper temperature is needed, PCR polymerase enzyme must be supplementary single next the primary denaturation, as the *Taq* DNA polymerase strongly reduction its activity at heat degree more than 95°C(T.C. Lorenz, 2012).

Denaturation Step

Denaturation or melting of duplexed desoxyribonucleic acid to produce dual single strands typically on the period range (0.5-2)min at (94-95)°C. As the PCR amplicon at the start of the amplification process, it is significantly lower than the target segment and is fully melted at these settings (Kovarova & Draber, 2000). Denaturation period have to be augmented up to (3-4)min if result has a rich GC sequences. Ta must be set empirically particularly in the existence of additives, because while these additives are utilized, the Tm will considerably reduce(Keith *et al.*, 2004).

The melting period equal to 45seconds at (94-95)°C is typically utilized for magnify lined genome molecules

which have GC-low (less than 55%) and upper temperature for target DNAs which have GC-high(extra than 55%)(Lawyer *et al.*, 1993).

Annealing Step

It is critical and ideally usually is a 5°C bellow Tm of primer-pattern of genomic. It is sufficient that the incubation be (0.5-2)min. The Ta need to improve by rising it gradually if DNA products and nonspecific PCR products appearance. When it is too high, primers poorly anneal, at all to the pattern and the produce of amplified reaction is too small. In other hand when the annealing temperature is too low, nonspecific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA(Schochetman, Ou, & Jones, 1988).

Extending Step

This step is usually carried out at (70-72)°C. The genomic composition made by the PCR enzyme is maximum at this heat degree. Nevertheless, the production of large PCR yields size in rang to 2 kb or more. This needs to expand the time in range 1min. As soon as bigger genomic segments are needed, the period is generally enlarged in the range 1min for each 1kb (Dieffenbach & Dveksler, 2003).

Final Extending Step.

After the final cycle, the mixture is commonly keep at 72°C for 5-15min to complete the prolonged segments of fresh amplicons. Additionally, the final transferase action of polymerase enzyme increases the number of bases to the 3'-terminations of PCR products with this step (Bustin & Huggett, 2017) (Dieffenbach & Dveksler, 2003).

12. Performance Outcome

Traditional PCR is a needed tool for molecular biology studies in the utmost research laboratories. Nonetheless, the information gained may only be explained as either good (observable) or bad (unobservable). PCR examines a terminal point check whereby the magnified PCR outcomes (amplicons) can merely identify the existence of these at the end of electrophoresis utilizing any nucleic acid stains(Antiabong, J.F., Ngoepe M.G., Abechi A. S., 2016).

Investigations planned to match the clinical precision of PCR check to diverse "model" characteristic approaches normally examine four results (Table:5). While the outcomes of amplification and "common" test results are identical, (both outcomes are the same (+)ve or (-)ve). So the results are perfect. Queries usually appear while the outcomes of PCR check do not support regular isolated outcomes. PCR tests are inclined to get fake-positive resulting when solution pollution takes place throughout any previous processing at the samples.(Paxson, 2008)(Cohen, Martin, Simpson, Wallis, & Neibergs, 1996) .

Studies of the origin of random fake(-)ve PCR result revealed that internal glove dust. Accidentally internal glove dust scattered to eppendorf tube while the worker are altered their gloves in an attempt to lower fake(+)ve effects. This may un-specifically prevent each of the important three stages of the PCR reaction(Sellon DC, Besser TE, Vivrette SL, McConnico RS, 2001)(De Lomas JG, FJ Sunzeri, MP Busch, 1992).

Table 5. Assessment of PCR and Gold-Standard Disease-Producing Detection Outcomes

| PCR Outcome | Typical Outcome | Common Clarification | Different Clarification |
|---|---|---|---|
| (+)ve | (+)ve | Disease-producing agent existing | — |
| (+)ve | (-)ve | Disease-producing DNA existing, but without sign of live disease-producing agent | Fake(+)ve PCR outcome (because of polluting DNA)<br>*Or*<br>Fake(-)ve typical outcome (because of disease-producing being existing but gone or very rare to detect, faults in management the sample, or previous antibiotic take) |
| (-)ve | (+)ve | Disease-producing agent existing | Absence of disease-producing DNA in PCR sample or fake (-)ve PCR outcome (because of the existence of inhibitory materials, bad DNA isolate, or bad reaction running) |
| (-)ve | (-)ve | absence disease-producing agent existing | — |

Agreement with Moral Principles

- Funding: This work done with no fund by any institutions.
- Conflict of Benefit: Authors declares there is no conflict of benefit for this work.
- Moral endorsement : This work does not have any handling with a human being.

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## CONFLICTS OF INTEREST

I would like to declare there is no confliction in this work.

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