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Potential Panacea to the Complexities of Polymerase Chain Reaction (PCR)

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Abstract
The polymerase chain reaction (PCR) allows the exponential amplification of target DNA sequences, and has greatly impacted the world of scientific research. Although the reagents, equipment needed and the process (denaturation, annealing and elongation) appears simple and straightforward, PCR is adorned by complexities. Hence this paper seeks to discuss the complexities often encountered whilst performing PCR. Findings showed that primers, annealing temperature, wholesomeness and purity of template DNA, PCR contaminants and inhibitory substances, choice of polymerase, Magnessium chloride concentration, and number of cycles influence the outcome of a PCR. These factors sometimes enhance quality but compromise yield and vice versa. A high amount of Magnesium chloride or number of cycles in a PCR would result to a good yield but compromises quality and vice versa. In contrast, a high quality but lower yield of PCR is attained where the annealing temperature is either close to or higher than the melting temperatures of the primers. Thus performing PCR is more or less choosing between quality and yield, and this paper would enable researchers strike the needed balance to overcome some of the associated complexities.
Keywords: PCR, DNA, Taq polymerase, PCR inhibitory substances, primers

Introduction

The Polymerase chain reaction (PCR) has greatly transformed the entire world of scientific research, especially the biological sciences and its related disciplines; having a tremendously wide application in diverse fields of study. (Saiki *et al*., 1985, 1988). The wide acceptability and applications of PCR has positioned the technique as the bride of modern day scientific research courted by researchers all over the globe. PCR is sometimes called molecular photocopying because it allows the exponential amplification of target DNA sequences (Saiki *et al*., 1988). The usefulness of PCR in research and other areas of application stems from the important role DNA play in the sustenance of life processes in all living entities. Living organisms are defined by the type and quantity of proteins they produce via series of information contained in their individual DNA (Albert *et al*., 2007; Pierce, 2000). These sets of information are usually transcribed from DNA to an intermediary molecule called the messenger RNA (mRNA) in the form of codes (genetic codons). The genetic codons passed on to the mRNA by the DNA are deciphered in the ribosomes of the cell by yet another type of RNA called the Transfer RNA (tRNA), often nicknamed the dictionary of life, being able to decode the genetic code contained in the mRNA. The process wherein tRNA decodes the genetic code passed on from the DNA to the mRNA is called translation. The deciphering of the genetic code ultimately culminates in the formation of the required protein (Albert *et al*., 2007; Chapeville *et al*., 1962; Crick, 1958;, Pierce, 2000).

Thus the function and role of DNA in the formation of protein makes it a very crucial and important biomolecule. Furthermore the sequence of nucleotides in a DNA molecule is unique to the individual. This awesome uniqueness of an individual's DNA makes the nucleic acid a reliable marker targeted in PCR-based molecular biological researches and investigations. Although the importance of DNA has long been known, up until the advent of PCR, its study had been hampered because target DNA sequences in living cells occur in quantities insufficient for detail studies. As a result, efforts have been made to seek ways to amplify target DNA sequences. To this end, the popular Recombinant DNA technology came into vogue. DNA sequences of interest were often cut with restriction endonuclease enzymes and ligated unto vectors especially plasmids to form recombinant DNA molecule which are then replicated or cloned in appropriate competent cells after transformation of the latter (Lederberg 1994; Wirth *et al*., 1989). Although cloning a Recombinant DNA molecule in competent cells allowed the exponential amplification of target DNA sequences, the process is nonetheless laborious and time consuming (Hill and Stewart, 1992). For example, *Escherichia coli* which is widely used in molecular cloning (Brown, 2006) requires about one hour to complete one generational cycle (Pierucci, 1972). A single cell would therefore multiply to become only 64 cells after six generations (6 hours). Since DNA replication usually corresponds to cell replication, it follows that a single DNA sequence of interest ligated into plasmid for cloning in a competent *E. coli* cells would also increase to a paltry 64 copies of the target DNA sequence, after 6 hours. In contrast, over a billion copies of a target DNA sequence would be attained through PCR amplification, just after two hours depending on the cycling regimes. PCR amplification is

relatively simple, and it entails a sequence of three phases – denaturation, annealing and extension (Hill and Stewart, 1992; Saiki *et al*., 1988).
However, notwithstanding its wide applications, acceptability and apparent simplicity PCR technology is bedeviled by a number of complexities (Yeates et al., 1997). This paper seeks to discuss the different array of limitations and complexities that are often encountered in the application of PCR technology. It is hoped that this paper would enable researchers avoid some of the pitfalls associated with PCR and give directions on ways to overcome its concomitant complexities.

Performing a typical PCR

Although, prior to the birth of the PCR technology, tDNA sequences were successfully amplified through the construction of recombinant DNA molecule and cloning in competent cells, it was not yet hurray with amplification of target DNA sequences of interest, because of the attendant hiccups of delay and labor intensity (Hill and Stewart, 1992). Thus the search for alternative ways of DNA amplification continued until April, 1983 (Bartlett, 2003). On one fateful day of April that year Kary B. Mullis, an American is said to have closed from work and was driving his car down a California highway from San Francisco to Mendocino in the United States of America when an idea formed within him (Bartlett, 2003). That idea is what has metamorphosed into Polymerase chain reaction or simply PCR. Kary Mullis may not have known that the PCR technology would revolutionize the entire world of scientific research, but that is what it has done in leaps and bounds.
To set up a typical PCR, certain chemicals and reagents has to be pooled together to form the PCR mix, and run on a thermocycler. A typical standard PCR mix requires a DNA template containing the region of interest to be amplified. This could be crude cell extracts, total genomic DNA, Plasmid DNA etc. Aside the DNA template, a number of other constituents are also required. These are two primers, magnesium chloride, amplification buffer, deoxynucleoside-5'-triphosphate (dNTP) molecules and DNA polymerase enzyme (Table 1).
The two primers (forward and reverse primers) are very vital to run a PCR. Primers are short single-stranded oligonucleotide probes usually between 18 – 30 base pairs long. The primers determine the section and length of DNA sequence that would be amplified during a PCR. They achieve this by flanking the opposite ends of the region of interest of a given DNA prior to amplification. The amplification buffer is most often Tris-based; this regulates the pH of the reaction, which in turn affects the DNA polymerase activity and fidelity. Magnessium chloride functions as a cofactor for the DNA polymerase enzyme whilst the thermostable DNA polymerase enzyme amplifies the target DNA sequence by extending the primers bound to the template DNA via a process in which the enzyme uses the deoxynucleoside-5'-triphosphate (dNTP) molecules available in the reaction matrix to build an oligonucleotide chain complementary to the template DNA, forming a double stranded DNA molecule again (Hill and Stewart, 1992; Mullis and Faloona, 1987; Saiki *et al*., 1988).
A typical PCR cycling regime applied by Etebu and Osborn (2009) was initial denaturation at 95°C for 5mins followed by 35 cycles of 94°C (1min), 57°C (1min), 72°C (1min) and a final extension at 72°C for 10mins. In another related work Etebu and Osborn (2011) used the following PCR amplification conditions. Initial denaturation 95°C for 5mins, 5 cycles of 94°C (45secs), 55°C (1min), 72°C (30secs) followed by 30 cycles of 94°C (30secs), 55°C (45secs), 72°C (30secs) and a final extension at 72°C for 10mins.
As simple as the PCR process appears to be, it took some efforts and time to arrive at these optimal amplification conditions for the specific regions of pea pathogenicity genes that were studied. It is almost certain that some sort of tweaking would be required to arrive at the optimal conditions required to attain a desired product in any given PCR. It is therefore imperative that certain stages be given due attention with respect to the different constituents of the PCR master mix. Such stages and constituents are hereunder highlighted and potential panacea discussed.

Primer and primer design: The application of PCR in detecting and investigating human, animal and/or plant disease agents in the environment is basically centred on the use of species-specific oligonucleotide primers which hybridise with DNA extracted from a sample under investigation. These primers facilitate the selective amplification of specific region(s) in the genome of the organism(s) of interest. To this end, primers determine the success or failure of any given PCR to a very large extent (Mullis and Faloona, 1987; Saiki *et al*., 1988; Wilfred *et al.,* 2005; Etebu, 2008). As a result, some measures have been proffered as conditions that should be adhered to in the design and selection of primers. Amongst other considerations, some workers have advanced that the following measures are required in the design and choice primers to perform a successful PCR. These are (i) The primers should not be complementary at their 3' ends, (ii) The possibility of primers forming a hairpin structure (2° structure) should be avoided (iii) Ensure that the melting temperature (T_m) of both primer should be about the same. Melting temperature ($T_{m)} = 2\times(A + T) + 4\times(G + C)$; where A, T, G and C represents

the four nucleotides with adenine, thymine, guanine and cytosine respectively, (iv) The possibility of primer-dimer formation be avoided as much as practicable. Primer-dimers occur when primers anneal themselves instead of the target DNA sequence. (v) Primer should be as specific as possible. The use of BLAST Analysis is recommended to ascertain potential specificity, especially if template DNA was extracted from an environmental sample such as soil and water (Wilfred *et al.,* 2005; Etebu, 2008)

Annealing temperature: Polymerase chain reaction entails three basic steps repeated over a number of cycles. The three basic steps are denaturation, annealing and extension. Simply, double stranded DNA template is denatured by heat at > 90°C, thereafter the forward and reverse primers anneal to complementary bases on the template DNA flanking opposite ends of the region of interest of the template DNA. Once the primers anneal, the heat stable Taq polymerase amplifies the specific region whose opposite ends are flanked by primers. The temperature for this third step is usually set at 72°C being the optimum temperature for polymerase activity (Saiki *et al*., 1988). The temperature for the annealing step is to be determined by the researcher. Setting a wrong temperature would result to one of two possibilities. The primers would hybridize to regions of template even if there are many mismatches in base sequences resulting to non-specific PCR products (low quality) or the primers would hardly hybridize to base sequences in the template DNA resulting to low yield of PCR product. Generally, applying a higher annealing temperature than the primers' melting temperatures would increase the annealing stringency of the primers, and minimize the formation of non-specific products (Don *et al*., 1991). This would mean a high quality PCR product but yield may be compromised (low). On the other hand, a low annealing temperature (lower than the primers' melting temperatures) increases the annealing efficiency of the primers and this would result to high yield of PCR product but quality in terms of specificity would be greatly compromised (Don *et al*., 1991). The usefulness of PCR over traditional cloning methods lies in its power to produce high yield of DNA sequence of interest within a short time. The usefulness, however, would be defeated if unwanted or undesired DNA sequences are co-amplified. This is the crux of the complexities of PCR which must be resolved by the researcher. The annealing temperature is one of the crucial conditions to adjust, among the array of so many others, in figuring out the optimum PCR conditions needed to achieve a desired goal. In optimizing annealing temperature, one would have to take the melting temperatures (Tm) of the primers into consideration since using an annealing temperature lower than the primers' melting temperatures would generally result to high yield of PCR product but quality in terms of specificity would be greatly compromised and vice versa (Don *et al.*, 1991; Hill and Stewart, 1992). One way to overcoming complexities arising from deficiencies in annealing temperature in a given PCR is to perform a touch-down PCR. The cyclic programme in a touch down PCR is set in such a way that, as cycling progresses, the annealing-segment temperature, which is usually selected to be initially above the primers' melting temperature, gradually declines to, and falls below, this level (Don *et al*., 1991). A downward temperature gradient from 5°C above the primers' melting temperature to 5°C below the primers' melting temperature is advisable. Non-specific PCR products are easily discerned by the number of DNA bands and their amplicon sizes on electrophoresis gel. The acceptance or otherwise of a PCR showing multiple bands after electrophoresis would depend on the source of DNA sample, the primer target region, nature of the bands and purpose of the investigation (Etebu, 2008).

PCR Inhibitors: Of crucial importance to PCR is the exclusion of substances that are capable of preventing or inhibiting PCR. Substances such as proteins, bilirubin, bile salts, urobilinogens, polysaccharides, humic acid, fulvic acids etc are capable of unfavourably interfering with PCR (Matheson et al., 2010; Khan, et al., 1991; Monteiro, et al., 1997; Watson and Blackwell, 2000; Wilson, 1997), and these must be are excluded, as much as is practicable, from bulk DNA during nucleic acid extraction. Excluding PCR inhibitory substances from genomic DNA during extraction is indeed an uphill task because these substances, humic acids in particular, form complexes with extracted DNA (Crecchio and Stotzky, 1998). Notwithstanding, a number of reagents have been used by various workers to exclude, or substantially reduce, PCR inhibitory substances while extracting genomic DNA from some soils; resulting to improved DNA quality and subsequent PCR performance. These, amongst others, include Calcium chloride, polyvinylpolypyrrolidone, polyethylene glycol 8000, commercial glass milk, ionex, membrane-based commercial purification kits, washing DNA embedded in low melting-point agarose, desalting on Sephadex columns, etc (Sagova-Mareckova et al., 2008; Kauffmann, et al., 2004; Miller et al., 1999; More´ et al.,1994; Moreira, 1998).
More often than not, genomic DNA of some organisms, particularly those that are less dominant in a sample, could be lost whilst ensuring the exclusion of PCR inhibitory substances during extraction. This makes DNA extraction prior to performing PCR very crucial and imperative (Khan, et al., 1991; Monteiro, et al., 1997; Watson and Blackwell, 2000).

Wholesomeness of DNA template: Various methods and approaches are employed by different workers to extract and isolate DNA from environmental or tissue samples. Essentially, DNA extraction fulfills three basic conditions. These include cell lysing, removal of protein/cell debris and potential PCR inhibitors, and precipitation of DNA. The size or wholesomeness of extracted DNA would depend mostly on the manner in which cells were lysed to release their DNA. Usually cells are lysed by mechanical, chemical or enzymatic means, or a combination of means (Kauffmann, et al., 2004; Miller, et al., 1999). Different soil DNA extraction methods have been shown to significantly affect the yield of DNA, as well as results of soil microbial diversity obtained from PCR-DGGE community fingerprinting studies (İnceoğlu et al., 2010). A greatly sheared DNA would portend a low quality PCR product or simply no amplification, especially if the region of interest in the DNA is a long sequence. Having sheared nucleic acids (DNA), after extraction, are likely to pose less challenge if the region of interest is a short DNA sequence. This notwithstanding, intact bulk genomic nucleic acids (DNA) sequences would be the preferred choice. It is therefore advisable to minimize shearing of DNA during extraction, as much as practicable.

Contamination: Apart from certain PCR inhibitory substances that are sometimes co-extracted along with genomic DNA, contamination of reagents could equally frustrate the performance of PCR. In undertaking a PCR, two controls (positive and negative) are vital (Kwok and Higuchi, 1989; Pellett et al. 1999; Scherczinger et al. 1999). Amplification of the positive control proves the functionality and reliability of the entire process. This control would have all the reagents except DNA obtained from sample(s) being investigated. In the place of DNA being studied this control would have DNA known to possess the sequence of interest under investigation. This control would therefore be expected to have positive amplification. A negative result with this control would cast doubt on, and nullify the entire PCR process. On the other hand, the negative control, often called the No Template Control (NTC) is to avoid accepting a false positive amplification. With this control all reagents are added except template DNA. In the place of DNA, nuclease free sterile water is added prior to PCR. This control would therefore not be expected to have a positive amplification. A positive amplification would mean only one thing, contamination, and that would also nullify the entire process. Having amplification with the NTC could be very frustrating because one would have to determine the source of the contamination. In most cases the entire reagents would be discarded and the process repeated with a new set of reagents. This could be expensive. To avoid wasting funds through such occurrences, it is advisable to aliquot amplification buffers and other reagents as soon as they are received or prepared and stored away from the work bench. Additionally, Gordon and Haseltine, (1982) advises that Eppendorf tubes, racks, micropipettes, pipette tubes etc be sterilized with a UV light before they are used for PCR.

Choice of polymerase: At the onset of PCR, thermostable DNA polymerase extracted from thermophilic bacterium *Thermus aquaticus*, a microorganism that dwells in hot springs at about 70°C was the choice of polymerase in PCR. However, several other sources of Polymerase enzymes have been identified over the years. The usefulness of these several other polymerases differs depending on efficiency, fidelity and cost (Bergseid, *et al.,* 1991). While some simply extend DNA through the use of deoxynucleoside-5'-triphosphate (dNTP) molecules available in the reaction matrix to build an oligonucleotide chain complementary to the template DNA, forming a double stranded DNA molecule again (Saiki *et al*., 1988), some others are, in addition, also able to proofread the extended sequence and rectifies errors of mismatch between the parent DNA strand and the newly formed strand (Sutton and Walker 2001).

Magnessium concentration: Magnessium is usually included in a typical PCR master mix as Magnessium chloride. A standard Mg^{2+} concentration of about 1.5mM is typically recommended in a PCR (Hill and Stewart, 1992; Etebu, 2008; Etebu and Osborn, 2009) Concentration of Magnessium chloride greatly affects the quality of yield of PCR product. Raising Magnessium concentration in a PCR mix reduces quality of product in terms of specificity, and generally increase yield; comparable to lowering the annealing temperature, and vice versa. Excessive Mg^{2+} reduces enzyme fidelity and increase non-specific amplifications which are usually observed multiple bands in gel electrophoresis (Innis and Gelfand 1990; Hill and Stewart, 1992; Ellsworth *et al*. 1993). Tackling complexities of most PCR are more easily accomplished by playing with other variables.

Number of PCR cycles: This also affects the quality and or yield of PCR products. Generally, increasing the number of cycles in a PCR, result to greater yield of product. However, this would often also lead to increase in nonspecific amplification (Hill and Stewart, 1992; Saiki, *et al*., 1988), evidenced by smeared bands upon gel

electrophoresis; affecting quality of product. Also, the efficiency of Taq polymerase generally reduces as the cyclic reactions progresses. Although, the number of cycles to set for a PCR would depend on the purpose of the investigation, a maximum number of 30 cycles has been said to be advisable (Hill and Stewart, 1992). Theoretically a single DNA molecule containing the region of interest would result to 2^{30} (1.07 billion) copies of DNA molecules with the sequence of interest after 30 cycles of a PCR. Depending on the time specified for each of the three stages (Denaturation, annealing and extension), a PCR of 30 cycles would be done with, in about 2 hours (Etebu and Osborn, 2009).

PCR Enhancers: Performing a PCR could be as frustrating, as it is exciting. A meticulous observance to the processes of a PCR may not necessarily guarantee a successful result. Certain reagents or organic chemicals, collectively termed enhancers in PCR investigations, are sometimes included in the master mix to enhance the process. They enhance PCR by aiding the yield increase of the desired product or decreasing the yield of undesired products. Some of the reagents used in this regard include Bovine serum, glycerol, polyethylene glycol (5–15%) and tetramethyl ammonium chloride (60mM), dimethyl sulfoxide (DMSO; 1-10%), betaine (1M), formamide (1–10%), gelatin (0.1–1.0%) and nonionic detergents such as Tween®-20, and Triton® X-100 (Cheng *et al*. 1995; Schwarzenbach, et al., 2007; Varadaraj and Skinner, 1994; Giambernardi et al. 1998; Gelfand and White, 1990; Chakrabarti and Schutt, 2001)

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Table 1: PCR Reaction Components for 1 Reaction mix

Components	Concentration/volume required
Forward primer (18-30bp)	20pmol (1-2µl)
Reverse primer (18-30bp)	20pmol (1-2µl)
Amplification buffer	One-tenth of final volume
dNTP	20-200µM each of dATP, dCTP, dGTP & dTTP
$MgCl_2$	50mM (typically 1.25µl)
Taq polymerase	1µl (1U)
Template DNA	10-100ng
Sterile distilled H_2O	Make up to 50µl

(Adapted from: Etebu, 2008; Etebu and Osborn, 2009)