BINDURA UNIVERSITY OF SCIENCE EDUCATION

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DEPARTMENT OF CURRICULUM STUDIES

OPTIMISATION OF POLYMERASE CHAIN REACTION (PCR) CONDITIONS FOR THE AMPLIFICATION OF RUBISCO GENE *(RBCL)* IN *TRITICUM AESTIVUM (WHEAT)*.

BY

MANHIMANZI WASHINGTON

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SUPERVISOR: MS. MUYAMBO

APPROVAL FORM

Optimisation of Polymerase Chain Reaction (PCR)conditions for amplification of theRubisCO gene *(rbcL)*in*Triticumaestivum (Wheat)*

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This dissertation is suitable for submission on to the Faculty of Science Education and was checked for conformity with the faculty.

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I certify to the best of my knowledge that the required procedures have been followed and the preparation criteria have been met for this dissertation.

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DECLARATION

I Manhimanzi Washington, declare that this writing is solely my effort and that it has not been submitted anywhere for any degree in any other university. I certify that the information in this dissertation which is not my own has been acknowledged. This writing is being submitted in partial fulfilment of the requirements of the Bachelor of Science Education Honours Degree at Bindura University of Science Education.

Signature. _____ *washomanhi12* _____ Date: _____16/11/2020_____

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NAME OF STUDENT: Manhimanzi Washington

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CHAPTER 1: INTRODUCTION

1.0 BACKGROUND TO THE STUDY

Polymerase Chain Reaction (PCR) is a powerful tool for the amplification of specific gene sequences. Sometimes, even though using an established PCR protocol that has been optimized and successfully used for the amplification of a DNA segment, use of that same protocol on a different region can result in a less than desirable outcome. Poor amplification can result from one of several causes: non optimal temperatures for thermal cycling, poor quality template, or a reagent (or reagents) may be at suboptimal concentration(s)(Miller and Levine, 2002). When developing a protocol for PCR amplification of a new target, it may be important to optimize all parameters including reagent concentrations, cycling temperatures, time, and cycle numbers.

The optimal reagent concentrations and thermal cycling parameters for any specific target/primer set and the optimal thermal cycling parameters that will give the highest yield, more often than not, are determined by following a particular protocol whilst varying the concentrations of the reagents (Hauser *et al*., 2015). Taking particular care when optimizing PCR can provide rewards in several ways. An optimized PCR will improve both product yield and reproducibility between reactions, while reducing amplification of non-specific products. When electrophoresed on an agarose gel, an optimized reaction will give a brighter product band of expected size with minimal background (Miller and Levine, 2002).

1.1 STATEMENT OF THE PROBLEM

Most PCR reactions fail due to not optimised conditions resulting in waste of resources. Each gene segment has unique PCR amplification conditions, therefore no one protocol will fit all gene amplifications to be done in Molecular Biology.

1.2 RESEARCH QUESTIONS

What are the optimum conditions for PCR amplification of the RubisCO gene *rbcL* from *Triticum aestivum*(wheat)?

1.3 AIM OF THE STUDY

The aim of the study was to optimise PCR conditions for the amplification of the RubisCO gene *rbcL* from *Triticum aestivum* (wheat)

1.4 OBJECTIVES OF THE STUDY

The objective of the study was to determine the optimum conditions for PCR amplification of the RubisCO gene *rbcL* from *Triticum aestivum* (wheat)

1.5 SIGNIFICANCE OF THE STUDY

The Rubisco gene is responsible for the production of the enzyme Ribulose 1, 5-bisphosphate (RuBP) carboxylase/ oxygenase (RubisCO) which is found in all photoautotrophic and chemoautotrophic organisms. The enzyme initiates photosynthetic carbon metabolism by combining atmospheric carbon dioxide with RuBP to form 3-phosphoglyceric acid (1, 2) – an organic form of carbon (Miller and Levine, 2002). The study of the RubisCO gene can provide some useful information on the possibilities of some phylogenetic relationships between organisms from the three kingdoms:-(Archaea –the anaerobic archaeans, Protista – plant-like Protistians and plantae), which have photoautotrophic or chemoautotrophic organisms (Hauser *et al.,* 2015). Moreover, studying the RubisCO gene may also help explaining the endosymbiotic theorem that states that mitochondria and chloroplasts were once free-living and a nucleated cell engulfed these prokaryotes, in which they became organelles (Miller and Levine, 2002).

The presence of chloroplast gene *rbcL* which produces eight larger subunits from which the enzyme Ribulose 1,5-bisphosphate is formed may suggest that a photosynthetic prokaryote was engulfed by the nucleated cell so as to improve its carbon assimilation (Hauser *et al*., 2015). The chloroplast *rbcL* gene would combine with the nuclear *rbcS* gene to produce a more effective enzyme for carbon fixation while the chloroplast would gain shelter and other forms of nourishment – a symbiotic relationship(Hauser *et al*., 2015). The enzyme Ribulose 1, 5-bisphosphate is often the limiting factor for the process of photosynthesis in plants. It fixes just between three and ten Carbon dioxide molecules each second per enzyme molecule while other enzymes can catalyse up to thousands of chemical reactions each second (Pavlov *et al.*, 2006). Studying the RubisCO gene would give room for improving carbon fixation leading to improved productivity in crop fields.

Modifying the RubisCO genes through biotechnology may increase their rate of expression thus improving the efficiency of carbon fixation and photosynthesis in plants. This may be a major step towards increasing the availability of food and reducing the ever rising concentrations of carbon dioxide which can be a very important climate change strategy. PCR

techniques can be used to amplify the *rbcL* gene which is the first step in most genetic modification processes.

1.6. DELIMITATION OF THE STUDY

The study seeks to optimise a PCR for the RubisCO gene segment *rbcL* in *Triticum aestivum*.

1.7. LIMITATIONS OF THE STUDY

Out of the genes segments that code for the enzyme Ribulose 1, 5 -bisphosphate, the research only focused on the optimisation of the RubisCO gene segment *rbcL* and not the *rbcS*.

1.8. DEFINITION OF KEY TERMS

- **In-vivo**: Occurring or taking place in living cells of organisms.
- **In-vitro:** Occurring in a test tube, culture dish or somewhere outside living organisms.
- **PCR**: Acronym for polymerase chain reaction, an in-vitro method to amplify DNA segments rapidly in temperature-controlled cycles of DNA denaturation, oligonucleotide base pairing, and DNA synthesis
- **Gel electrophoresis**: A method for separation and analysis of macromolecules i.e. (DNA, RNA and proteins) and their fragments, based on their size and charge, achieved by placing them in a gel matrix and then exposing them to an electrical field.
- **Amplicon**: The specific sequence of nucleotides in a target molecule of nucleic acid which is to be copied (amplified) by a nucleic-acid-amplification technique such as PCR.
- **Annealing:** The binding of primers to template DNA.
- **Buffer:** Is a solution that resists changes in pH in a PCR reaction. It is used to ensure that the DNA polymerase is in an environment in which it will have optimum activity.
- **Exons**: are coding regions of the gene.
- **Intron:** are non coding regions of the gene.
- **Nanodrop Spectrophotometer:** A technology based on UV spectrophotometry that allows the measurement of nanogram concentrations of nucleic acids.
- **Primer**: A short oligonucleotide chain which flank the region of interest and indicate the point at which the polymerase enzyme should start complementary strand synthesis.

CHAPTER 2.0: LITERATURE REVIEW

2.1. POLYMERASE CHAIN REACTION OPTIMIZATION

Polymerase chain reactions (PCRs) is a commonly used molecular biology tool for amplifying small quantities of DNA samples. This in-vitro process is analogous to the in-vivo DNA replication in that the two processes use old DNA strands as templates to produce new daughter strands. However, the two are dissimilar in that:

a) The in-vivo DNA replication uses RNA primers synthesised by the enzyme *primase* unlike the synthetic oligonucleotide DNA primers used in PCR.
b) Okazaki fragments are only found on an in-vivo DNA replication folk and not present in the PCR folks.
c) In most cases there is no proofreading in a PCR as *Taq* polymerase does not have the proof reading ability which is possessed by the enzymes involved in in-vivo DNA replication, but however, due to biotechnological modifications recombinant *Taq* or high fidelity *Taq* now have the proof reading ability.
d) In-vivo DNA replication is carried out at a temperature of 37ºCfor humans unlike the in-vitro DNA replication processes which take place in temperature cycles for example 95˚C denaturation, 56˚C annealing and 72 ˚C extension.

Molecular biologists have developed various laboratory methods for PCR optimization to improve the performance of the PCR and minimize its failure. A PCR may fail due to several reasons some of which include contamination of PCR reagents, poor primer design, primer dimers, polymerase errors, magnesium ion concentrations, target DNA size, primer concentration and deoxyribonucleotide concentrations.

2.2. CONTAMINATION OF PCR REAGENTS.

DNA amplification by the PCR method is an extremely sensitive method in that it requires just a few DNA molecules, as little as just one copy, in a single reaction for amplification across several orders of magnitude. Because of this sensitivity it is imperative to take adequate measures to avoid contamination of the DNA sample from any form of DNA present in the lab environment (Karp., 2010). The major contaminant in a PCR is water as it may contain foreign DNA (Karp., 2010). Non-sterile water also contains microbes which are

sources of nucleases which may degrade the DNA resulting in the failure of the PCR (Karp., 2010).

Some PCR reactions may also fail because of contaminations from products formed from previous PCR amplifications (Pavlov *et al.*,2006). Molecular biologists try to optimise PCR reactions by implementing several procedures that reduce contamination some of which may involve dividing the laboratory into separate working areas for example : one laboratory area may be dedicated for the preparation and handling of pre-PCR reagents known as the clean room and the setup of the PCR reaction, while the other may be for post-PCR processing, such as gel electrophoresis or PCR product purification(Karp., 2010).

In the setup of PCR reactions, standard operating procedure (SOP) involve the use of sterile pipettes with filler tips and wearing fresh laboratory gloves each time, a laminar flow cabinet with a UV lamp maybe used as a working station so as to ensure that no foreign DNA contaminates the PCR reagents(Pavlov *et al.*, 2006). To check for contamination the PCR is routinely assessed against a sterility control which is performed alongside the experimental PCR (Pavlov *et al.,* 2006). The negative control is identical in every aspect to the experimental PCR, but without template DNA and, a band in the negative control signifies a contamination which automatically invalidates the results (Kramer and Coen.2006).

2.3. POOR PRIMER DESIGN

2.3.1 PRIMER SECONDARY STRUCTURE FORMATION

The formation of secondary structures in the primers can result in the folding or knotting of primers (Pavlov *et al*., 2002). The formation of these secondary structures leads to failure of the PCR or decreased product yield. Hairpins, which consist of internal folds caused by complementary base-pairing between nucleotides in inverted repeats within a single-stranded DNA molecule, are the commonest secondary structures and may result in failed PCRs (Pavlov *et al.* 2002). As such methods, in primer designs, that include a check for potential secondary structures in the primers, or addition of glycerol to the PCR to minimize secondary structures in the DNA template are important when designing primers for all PCRs (Pavlov *et al.*,2002).

2.3.2. PRIMER DIMERS

The annealing of the 3' end of one primer to itself or to the second primer may result primer extension, resulting in the formation of the so-called primer dimers which can be seen as low-molecular-weight bands on PCR gels(Pavlov *et al.*,2006). The formation of primer dimers often competes with amplification of the DNA fragment of interest for the building blocks i.e. the deoxyribonucleotide triphosphates(dNTPs). Therefore great care should be taken when designing primers so that they lack complementarity especially at the 3' end of itself or the 3' of the other primers used in the reaction(Pavlov *et al.*, 2006). However, if primer design is limited by other factors so that primer-dimers have a likelihood of occurring, methods to limit their formation may include the optimisation of the magnesium chloride ($MgCl_2$) concentration or to increase the annealing temperature in the PCR.

2.4. POLYMERASE ERRORS

Polymerase errors can also be sources of failure of a PCR reaction. The enzyme *Taq* polymerase which is used in most PCRs lacks a 3' to 5' exonuclease activity(Pavlov *et al.*, 2006). The error-proof-reading activity consists of excision of any newly misincorporated nucleotide base from the nascent (i.e., extending) DNA strand that does not match with its opposite base in the complementary DNA strand (Kramer and Coen, 2006). The lack of 3' to 5' proofreading ability of the *Taq* enzyme results in a high error rate (mutations per nucleotide per cycle) of approximately 1 in every 10,000 bases, which affects the fidelity of the PCR. The fidelity of the PCR is greatly affected in instances where errors occur early in the PCR when the starting materials are still in their very small amounts; such errors result in the accumulation of a large proportion of amplified DNA with incorrect sequence in the final product (Kramer and Coen, 2006).

To reduce these polymerase errors several "high-fidelity" DNA polymerases, with engineered 3' to 5' exonuclease activity, have become available. These “high fidelity” polymerases permit more accurate amplification Examples of polymerases with 3' to 5' exonuclease activity include: KOD DNA polymerase, a recombinant form of *Thermococcus kodakaraensis* KOD1; Vent, which is extracted from *Thermococcus litoralis, Pfu* DNA polymerase which is extracted from *Pyrococcus furiosus* and *Pwo* which is extracted from *Pyrococcus woesii* -a thermophylic bacteria (Pavlov *et al*., 2006).

2.5. MAGNESIUM CONCENTRATION

Magnesium ions are a co-factor for the thermostable DNA polymerase. *Taq* polymerase is a magnesium-dependent enzyme therefore determining the optimum magnesium concentration for use is critical for the success of a PCR reaction (Markoulatos *et al.,* 2002). Other components of the reaction mixture such as template concentration, dNTPs and the presence of chelating agents (EDTA) or proteins can reduce the amount of free magnesium present thereby reducing the activity of the enzyme.

Excessive magnesium concentrations increase non-specific binding i.e. primers which bind to incorrect template sites resulting in decreased specificity of the reaction. Excessive magnesium concentrations can also stabilize double stranded DNA preventing complete DNA denaturation during PCR reducing the product yield per cycle (Markoulatos *et al.*, 2002).

PCR reactions may also fail due to the inadequate thawing of magnesium chloride (Markoulatos *et al.*, 2002). Inadequate thawing may result in the formation of concentration gradients within the Magnesium Chloride solution supplied with the DNA polymerase thus contributing to too many failed experiments (Markoulatos *et al.*, 2002). To avoid such errors the magnesium chloride should be completely thawed and vortexed to ensure uniformity in the concentration of the solution.

2.6. SIZE AND OTHER LIMITATIONS

A PCR works readily with a DNA template of between 100bp to 300bp in length. However, when the size of the DNA template exceeds 3Kbp, the product yields often decrease, as with increasing length stochastic effects such as premature termination by the polymerase begin to affect the efficiency of the PCR (Park, 2004). In special PCR reactions it may be possible to amplify larger DNA pieces of up to 50Kbp; but however the reactions would require a slower heating cycle and special polymerases like *Topo* and *Pfu2* which are usually fused to a processivity-enhancing DNA-binding protein which enhance the adherence of the polymerase to the DNA(Park,2004).Other valuable properties of these polymerases include enhanced thermostability, specificity and resistance to contaminants and inhibitors(Park, 2004).

2.7. NON-SPECIFIC PRIMING

Non-specific binding of primers is a frequent occurrence which may result in the failure of a PCR reaction. There are several reasons why non-specific binding may occur and these include repeat sequences in the DNA template to be amplified, non-specific binding between primer and DNA template, high or low G-C content in the DNA template, or incomplete primer binding which leaves the 5' end of the primer unattached to the DNA template(Pavlov *et al.*,2006).Varying magnesium ion concentration and primer annealing temperatures may be used as strategies to increase the specificity of a PCR. For example, the use of lower magnesium concentrations or other cations may reduce or prevent non-specific primer interactions, thus enabling a successful PCR (Pavlov *et al.*,2006).

In some cases, "hot-start" polymerase enzymes whose activity is blocked unless it is heated to high temperature of between 90–98˚C during the denaturation step of the first cycle are favoured so as to prevent non-specific priming during reaction preparation when temperatures are still low(Clark, 2005).

Nested PCRs and Touchdown PCRs can also be the other methods used to increase specificity of a PCR.A Nested PCR is a variation of PCR in which two sets of primers are used in two successive reactions; the two sets of primers are used so as to increase the specificity of DNA amplification(Clark, 2005). In the first PCR, one pair of primers, i.e. forward and reverse primers, is used to generate DNA products, which will be the target segments for the second reaction. One type of primer can be used for the amplification process or two different sets of primers whose binding sites are located within the first set of primers can be used. A nested PCR is often more successful in specifically amplifying long DNA products than conventional PCR and can be used in the detection of pathogens that occur in few amount (Nelson and Cox, 2004).

In Touch down PCRs, the annealing temperature is gradually decreased in later cycles. The annealing temperature in the early cycles is usually 3-5 °C above the standard annealing temperature of the primers used, whereas in the later cycles it is a similar amount below the annealing temperature. The initial higher annealing temperature leads to greater specificity for primer binding, while the lower temperatures permit more efficient amplification at the end of the reaction (Nelson and Cox, 2004).

2.8. CONCENTRATIONS OF DEOXYRIBOXYNUCLEOTIDES

Deoxyribonucleotide triphosphates (dNTPs) may bind Mg^{2+} ions thus affecting the concentration of free magnesium ions in the reaction (Pavlov *et al.*, 2006). Excessive amounts of dNTPs can increase the error rate of DNA polymerase and may even inhibit the PCR reaction (Pavlov *et al.*, 2006). An imbalance in the proportion of the four dNTPs may result in the misincorporation into the newly formed DNA strand and contribute to a decrease in the fidelity of the enzyme DNA polymerase. Therefore, it is important to order a dNTP mix which has equal concentrations of the four dNTPs in the reaction mixture for the PCR to be successful (Park, 2004).

2.9. RUBISCO GENE ORGANIZATION

The RubisCO genes of higher plants are in the nucleus and in chloroplasts. The genes are only expressed in those parts of the plant which contain photosynthetic pigments and as such, the highest levels of mRNA encoding for the enzyme Ribulose 1,5-bisphosphate are found in the leaves, with the photosynthetic tissues of stems, petals, and pods also producing the enzyme but in smaller quantities (Pavlov *et al.*,2006).

The enzyme RubisCO is composed of eight large (51–58 kDa) and eight small (12–18 kDa) subunits encoded by a chloroplast gene *rbcL* and five nuclear *rbcS* genes (Hauser *et al.*,2015; Pavlov *et al.*, 2006). The *rbcS* genes are composed of a small multigene family ranging from between two to twelve closely linked members suggesting that they may have arisen from multiple gene duplications (Pavlov *et al.*, 2006). The nuclear *rbcS* genes contain one to three introns and encode mature proteins made up of 120 amino acids, and are more divergent than the chloroplast-encoded *rbcL* genes which with rare exceptions, contains no introns and encodes a mature protein of 475 amino acids (Pavlov *et al.*,2006).Phylogenetic analysis indicates that there are three similar forms of the RubisCO proteins i.e. forms I, II and III, all of which catalyse the same type of reaction(Hauser *et al.*, 2015). In addition to the three RubisCO proteins, there exists another form of RubisCO-like protein, form IV protein. Even though it does not catalyse RuBP carboxylation or oxygenation, the protein shows great similarities with the RubisCO forms I, II and III suggesting that they might have common origin.

CHAPTER 3.0: MATERIALS AND METHODS

3.1. DNA EXTRACTION

The plant DNA was extracted using a modified cetyl trimethylammonium bromide (CTAB) protocol (Nelson and Cox, 2004). The leaf specimen was frozen at -20˚C overnight, crushed in 500µL of CTAB buffer using sterile mortar and pestle and vortexed until a homogenous mixture was obtained. The CTAB/ plant extract mixture was transferred into a microfuge tube and incubated for about 15minutes at 55˚C in a water bath. After the incubation period the mixture was spun at 12000g for 5minutes to separate cell debris from the liquid part of the mixture. The supernatant was then transferred into a clean microfuge tube and 250µL of Chloroform: IsoAmyl Alcohol (24:1) v/v was added, and the solution mixed by inversion before being spun at 13000rpm for 1minuteso as to precipitate proteins.

The upper aqueous phase, which contained the DNA, was transferred into a clean microfuge tube and 50µl of 7.5M Ammonium Acetate followed by 500µl of ice cold absolute ethanol were added after of which the tubes were inverted slowly several times so as to precipitate the DNA. The DNA was pelleted by spinning the tube at 13000rpm for a minute. The supernatant was then removed, and the DNA pellet was washed by adding two changes of ice- cold 70%ethanol, the supernatant was then removed, and the DNA was air dried for about 15minutes. The DNA was resuspended in 200 µl of nuclease free water, incubated at 65˚C for 20minutes to destroy any DNases which might have been present and later stored at -20 ^{0}C for future use (Park, 2004).

3.2. DNA QUALITY CONFIRMATION

The quality of the DNA extracted was checked by running it on 1% agarose gel. 1% agarose solution was prepared by melting 2g of agarose in 200mL of 0.5xTBE buffer in a microwave

for approximately 2 minutes. The gel was allowed to cool to a temperature of about 55ºC then 2.5mL of ethidium bromide was added and the mixture stirred until a homogeneous solution was formed. Ethidium bromide intercalates into the major grooves of the DNA to increase resolution as it fluoresces under UV light allowing DNA bands to be seen on gel. The gel was run for 1 hour at 100volts using a 100 bp gene ruler (Thermofisher, California, USA). The gel was viewed under UV light and a photograph was taken using the gel viewer.

3.3. PRIMER DESIGN

The primer sequences of the *rbcL* were obtained from previously published work. The forward and reverse primers were blasted the National Centre for Biotechnology Information (NCBI) Primer blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to check for the specificity of the primers for use in the PCR. A good primer set should have a G-C content of 40-60%, their annealing temperatures should be within 5ºC of each other and generally should be 15-30 bps in their lengths.

3.4. PCR OPTIMIZATION

The optimisation process was done by varying the concentrations of the primers solutions (forward and reverse primer solutions), bivalent ions i.e. Mg^{2+} ions in ($MgCl_{2\ (aq)}$) solution, annealing temperatures and the concentration of dNTPs.

Five different combinations, between 0.1 µM and 0.5 µM, of the *rbcL* forward and reverse primer solutions, five different concentrations of dNTPs of between 50 µM and 250 µM, five concentrations of one *Taq* standard reaction buffer of between x1 and x5, and five different concentrations of Magnesium Chloride between 0.5 mM and 5mM, were tested to determine the optimal amplification signal. A negative control containing Nuclease-Free water instead of DNA was included in each run to detect any contamination. The following set of forward

and reverse primers were used in the amplification of rbcL gene: rbcL 1F_ ATG TCA CCA CAA ACA GAA AC and rbcL 724 R_ TCG CAT GTA CCT GCA GTA GC and rbcL 636F_ GCG TTG GAG AGA TCG TTT CT and rbcL 1426 R_ TCC TTT TAG TAA AAG ATT GGG CCG AC. A temperature gradient to determine optimum annealing temperatures for the primers was done from 50 to 55 ºC and 55 to 63 ºC. DNA was amplified in a Biorad T100 thermocycler (Biorad, California, USA) with the following conditions: initial denaturation at 95ºC for 2 minutes, cycle denaturation at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension of the target strand at 72ºC for 1 minutes per cycle, for a total of 35 cycles. The PCR products were run on 1.5 % agarose gel stained with ethidium bromide and ran for 1 hour at 100 Volts.

4.0. RESULTS

4.1 DNA QUALITY CONFIRMATION

The extracted DNA was of high quality as indicated by sharp distinct bands with no smears (Figure 1).

MWM S 1 S 1 S 1 S 2 S 2 S 2 S3 S3 S3 S4 S4 S4

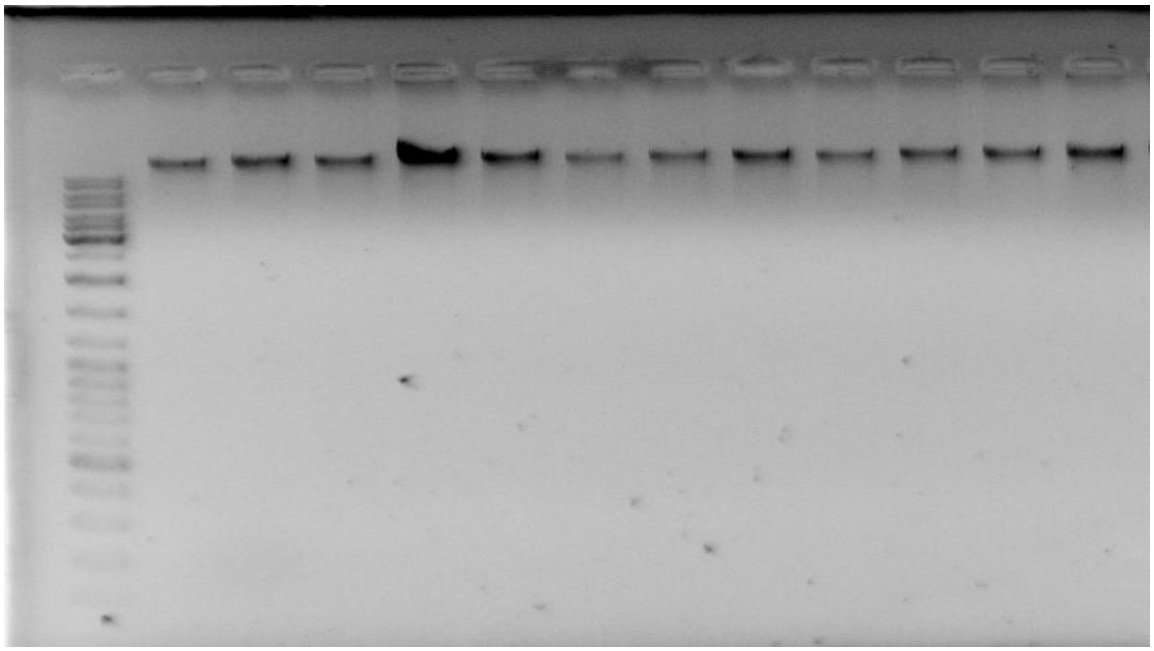
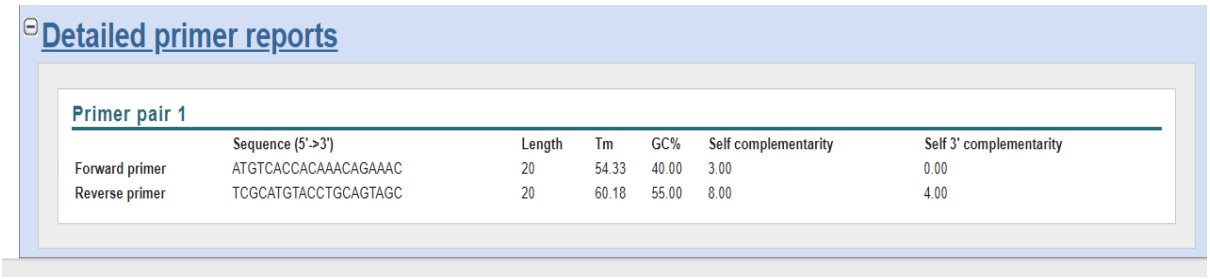


Fig 1: Integrity gel for extracted DNA from wheat leaves

Lane 1- Molecular weight marker (MWM), Lane 2-4 Sample 1 (S1), Lane 5-7 Sample 2 (S2), Lane 8-10 Sample 3 (S3), Lane 11-13 Sample 4 (S4)

4.3 PRIMER BLASTING

The forward and reverse primers were blasted on NCBI Primer blast and the results in Fig 2 generated. Both primer sets had GC % content difference greater than the stipulated 5 % (Fig 2). The self-complementarity and self- 3'complementarity for both primer sets were less than or equal to 8 except self-complementarity for primer set 2 which was 11. The melting temperature difference for primer set 1 was greater than 5 whereas for primer set 2 it was less than 5 (Figure 3).

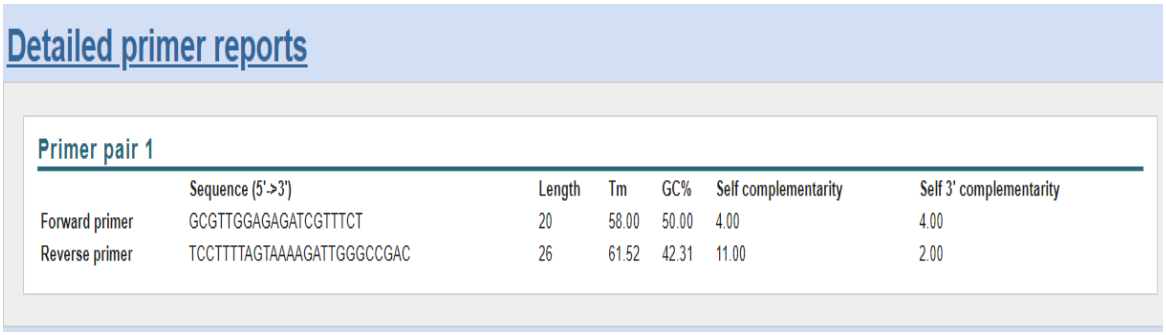


Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATGTCACCACAAACAGAAAC	20	54.33	40.00	3.00	0.00
Reverse primer	TCGCATGTACCTGCAGTAGC	20	60.18	55.00	8.00	4.00

Figure 2: Primer Blast Report for pair 1 (rbcl 1F and rbcl 724R): Amplicon size 723 bp



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCGTTGGAGAGATCGTTTCT	20	58.00	50.00	4.00	4.00
Reverse primer	TCCTTTTAGTAAAAGATTGGGCCGAC	26	61.52	42.31	11.00	2.00

Figure 3: Primer Blast Report for pair 2 (rbcl 636F and rbcl 1426R): Amplicon size 2621 bp

4.4. OPTIMISING ANNEALING TEMPERATURE

The annealing temperature for the first primer pair was optimised over a 50-55 ^{0}C range. Non- specific bands were observed in lane 3 (53.5 ^{0}C) to lane 9 (50 ^{0}C) and only lane 1 and 2 had the expected band (Figure 4).

Gene ruler**1000bp Plus** L1 L2 L3 L4 L5 L6 L7 L8 L9 L10

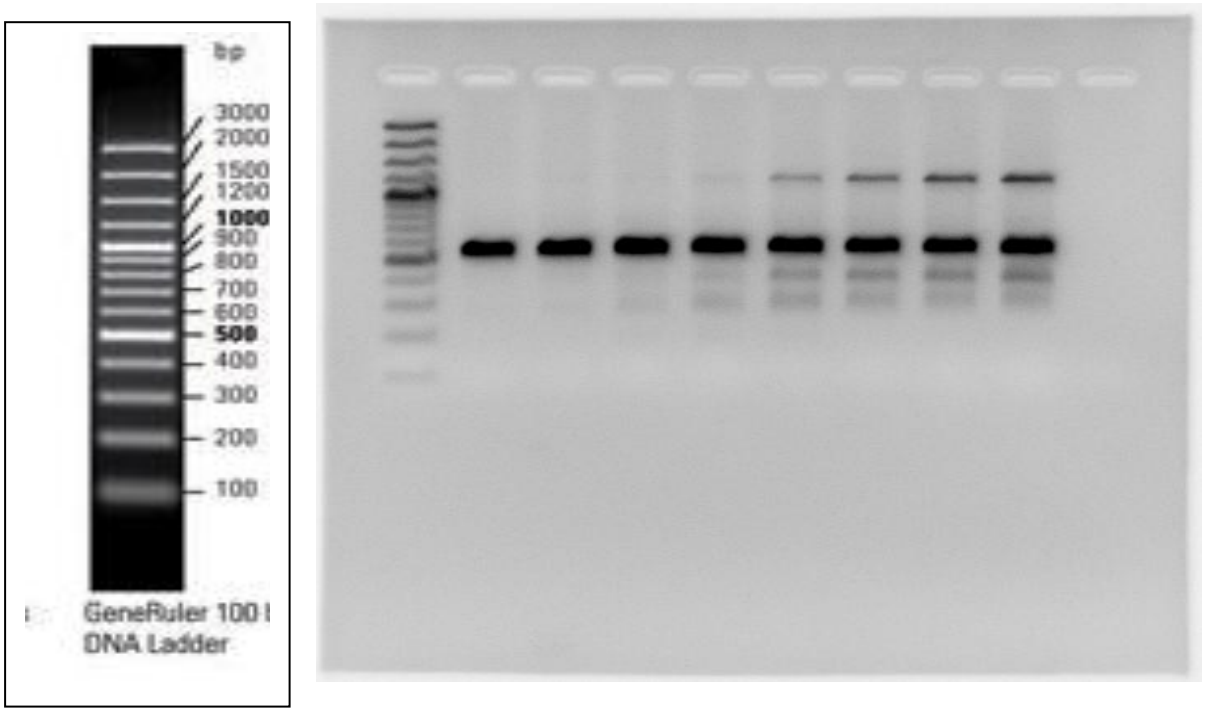


Figure 4: Annealing Temperature Optimisation. Lane 1 – Molecular Weight marker, Lane 2 – Lane 9 (Temperatures 55; 54.2; 53.5; 52.9; 52; 51.5; 50.7; 50) and Lane 10 --- negative template control

4.5. ANNEALING TEMPERATURE OPTIMISATION 2

A second primer annealing temperature optimisation was done from 55^{o}C to 63^{o}C. There was good amplification at all temperatures in this range indicating that the PCR could be done at any of these primer annealing temperatures. An annealing temperature of 59^{0}C was therefore chosen for all PCRs.

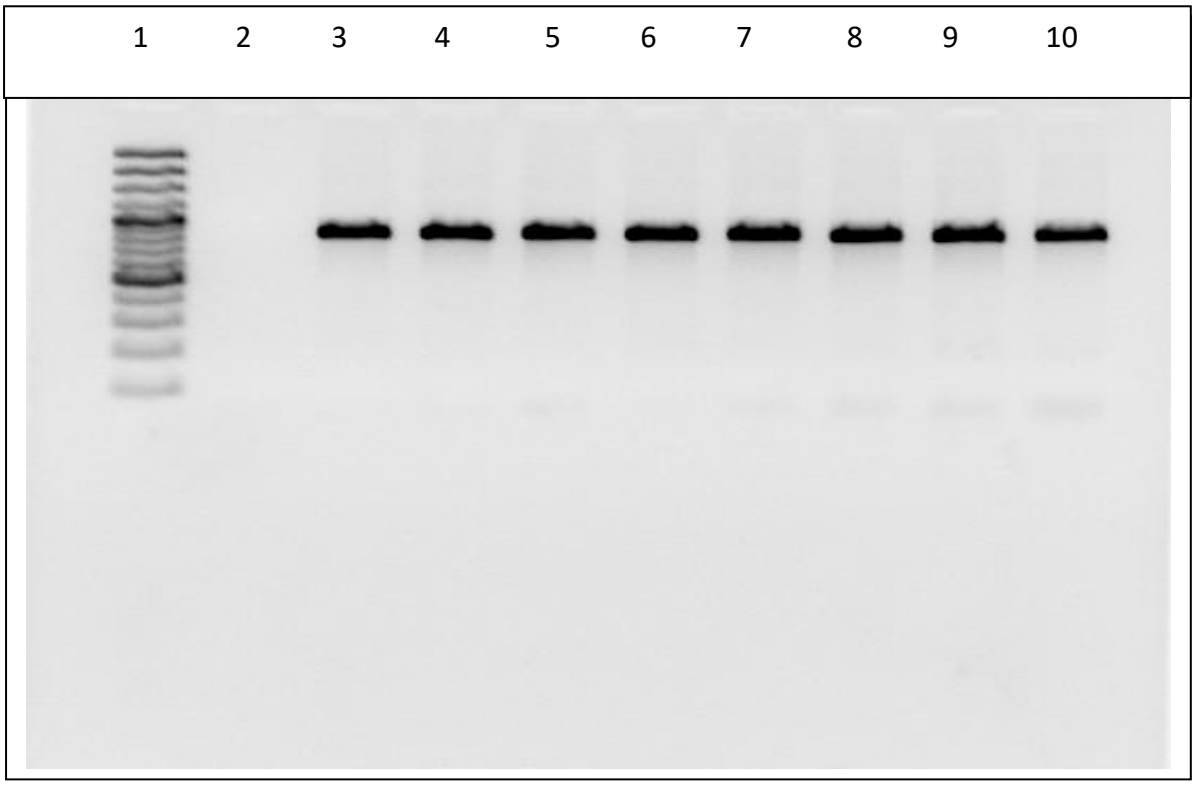


Figure 5: Second primer annealing temperature optimisation. Lane 1--- Molecular Weight marker, Lane 2- no template control, LANE 3- 10 (Temperatures (^{o}C): 55; 56.5; 57.6; 58.5; 59.2; 60; 61; 63)

4.6. OPTIMISED CONDITIONS FOR AMPLIFICATION OF THE RBCL GENE WITH PRIMER SET 1

The optimised PCR conditions for the rbcL gene amplification were: initial denaturation at 95ºC for 2 minutes, cycle denaturation at 95ºC for 30 seconds, annealing at 59ºC for 30 seconds and extension of the target strand at 72ºC for 1 minutes per cycle, for a total of 35 cycles. The reaction components concentrations were: 1X PCR buffer; 0.4mM deoxyribonucleotides; 0.4 µM of each of the primers (set 1), 3 mM Magnesium chloride and 0.13 units of go Taq polymerase enzyme. These optimised conditions were used to amplify rbcL gene and results in figure 6 obtained.

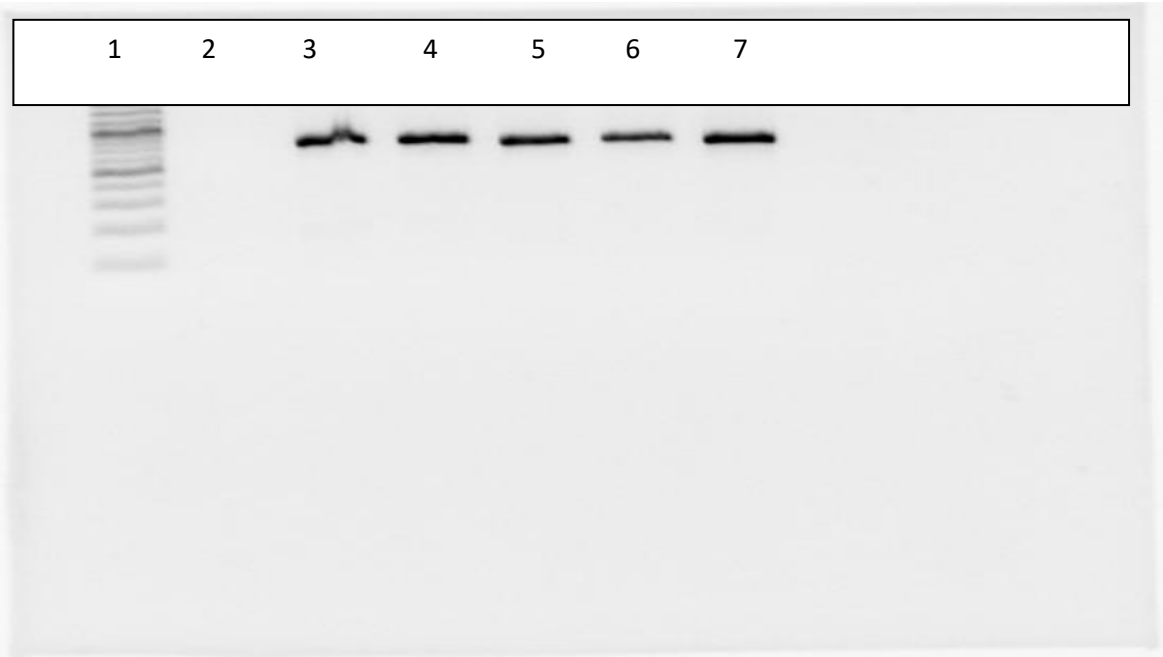


Figure 6: Amplification of the *rbc*L gene using the optimised conditions. Lane 1- Molecular weight marker, Lane 2- No template control, Lane 3- Wheat sample 1, Lane 4- Wheat sample 2, Lane 5- Wheat sample 3, Lane 6 – Wheat sample 4, Lane 7 – positive control (*rbc*L gene amplified from another lab).

5.0. DISCUSSION

5.1. DNA EXTRACTION AND QUALITY CONFIRMATION

The DNA extracted from the four *Triticum aestivum* samples was of very high integrity, this can be noted by sharp distinct bands seen when the samples were run on a 1% Agarose gel. Absence of smears in any of the lanes indicated that no sample was degraded. Unfortunately, the concentration and purity of DNA could not be determined as the Nanodrop Spectrophotometer was down. Hence, DNA concentrations were not optimised for the PCR reaction.

5.2. PRIMER DESIGN

The forward and reverse primers had GC % content difference greater than the stipulated 5 %. GC content is important as it affects the melting temperatures of the primers. Higher GC content difference might cause difficulties in optimising the primer annealing temperatures. The self-complementarity and self- 3'complementarity for both primer sets were less than or equal to 8 except self-complementarity for primer set 2. These self-complementarity parameters predict the likelihood of formation of primer dimers in the PCR reaction which might lead to failure of the PCR.

5.3. OPTIMISING PCR CONDITIONS

Low annealing temperatures of between (50°Cand 54.2°C) resulted in non- specific binding. Modifications to higher temperatures in the range 50°C to 63°C produced good amplification which was evidenced by correct size of amplicon at the annealing temperatures 55 to 63 °C.

Optimum primer concentrations are critical for success of a PCR reaction. The primers bind to the template DNA indicating the specific points at which the polymerase enzyme can start complementary strand synthesis. Higher concentration of primers will result in primer dimers which might affect downstream applications like sequencing. There will be need to clean up the amplicon thoroughly with exonuclease enzyme to ensure sequencing reaction is successful. Recommended primer concentrations for most PCRs range from 0.2-0.5 µM and using less results in no amplification so it becomes imperative to carefully optimise the

primer concentrations. The second set of primers was not optimised since it required a long PCR because of the big fragment size.

Divalent and monovalent cations ensure binding of primers and efficiency of Taq polymerase enzyme. A Magnesium ion concentration of 3mM used according to the previously published protocol and other concentrations were not tried.

PCR buffer maintains correct pH and ionic strength for Taq polymerase activity. Most PCR work best with a final buffer concentration of x1. There was no need to optimise buffer concentration as the x1 gave good amplification.

Finally, dNTP concentration of 200µM produced a very good amplification signal. Deoxyribonucleotides are the building blocks of DNA and there important in complimentary strand synthesis during PCR. A low concentration of dNTPs will result in thin bands where optimum concentration will lead to thick and distinct bands of DNA on gel. Deoxyribonucleotides should be optimised in conjunction with primer and template concentration to ensure good amplification takes place.

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APPENDIX: CALCULATIONS FOR CONCENTRATIONS AND VOLUMES OF DIFFERENT REAGENTS FOR THE PCR REACTION

Magnesium chloride ($MgCl_2$) will be added to a series of PCR tubes in concentrations of 1.5 mM, 2 mM, 2.5mM and 3mM. The stock solution of $MgCl_2$ has a concentration of 25 mM.

i. For a final concentration of 1.5 mM $MgCl_2$:

$$C_1 = 25 \text{ mM}$$

$$C_2 = 1.5 \text{ mM}$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50 \text{ µL}$$

$$C_1V_1 = C_2V_2$$

$$(25 \text{ µM})(\boldsymbol{x}) = (1.5\text{mM})(50 \text{ µL})$$

$$\boldsymbol{x} = \frac{(1.5\text{mM})(50 \text{ µL})}{(25\text{mM})}$$

$$\boldsymbol{x} = 3 \text{ µL}$$

3µL of the 25 mM $MgCl_2$ stock solution should be dispensed into a single 50 µL reaction to achieve a final concentration of 1.5 mM Magnesium Chloride.

ii) For a final concentration of 2mM $MgCl_2$

$$C_1 = 25 \text{ mM}$$

$$C_2 = 2\text{mM}$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50 \text{ µ L}$$

$$C_1V_1 = C_2V_2$$

$$\boldsymbol{x}\ (25\text{mM}) = (2\text{mM})(50 \text{ µL})$$

$$\boldsymbol{x} = \frac{(2\text{mM})(50 \text{ µL})}{(25\text{mM})}$$

$$\boldsymbol{x} = 4\text{µL}$$

Add 4 µL of the 25mM $MgCl_2$ stock solution into a single 50 µL reaction, to achieve a final concentration of 2mM magnesium chloride.

iii) For a final concentration of 2.5mM $MgCl_2$:

$$C_1 = 25 \text{ mM}$$

$$C_2 = 2.5\text{mM}$$

$$V_1 = \boldsymbol{x}\ \mu\text{L}$$

$$V_2 = 50\ \mu\text{L}$$

$$C_1V_1 = C_2V_2$$

$$(25\text{mM})\ (\boldsymbol{x}) = (2.5\text{mM})\ (50\ \mu\text{L})$$

$$\boldsymbol{x} = \frac{(2.5\text{mM})\ (50\ \mu\text{L})}{(25\ \mu\text{M})}$$

$$\boldsymbol{x} = 5\ \mu\text{L}$$

Into a single 50 µL reaction, add 5 µL of the 25mM $MgCl_2$ stock solution to yield a final concentration of 2.5mM magnesium.

iv) For a final concentration of 3.0mM $MgCl_2$:

$$C_1 = 25\text{mM}$$

$$C_2 = 3\text{mM}$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50\ \mu\text{L}$$

$$C_1V_1 = C_2V_2$$

$$(25\ \mu\text{M})\ (\boldsymbol{x}) = (3\text{mM})\ (50\ \mu\text{L})$$

$$\boldsymbol{x} = \frac{(3\text{mM})\ (50\ \mu\text{L})}{(25\ \mu\text{M})}$$

$$\boldsymbol{x} = 6\ \mu\text{L}$$

Add 6µL of the 25mM $MgCl_2$ stock solution to a single 50 µL reaction tube to get a final concentration of 3mM magnesium chloride.

VARYING THE CONCENTRATION OF THE FORWARD PRIMER *rbcL* 724*F*

The forward primer ***rbcL 724F***will be added to a series of PCR tubes in concentrations of 0.2µM, 0.3µM and 0.4µM. The stock solution of ***rbcL 724F*** has a concentration of 100µM.

i) For a final concentration of 0.2µM ***rbcL 724F***

$$C_1 = 100\mu M$$

$$C_2 = 0.2\mu M$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50\ \mu L$$

$$C_1V_1 = C_2V_2$$

$$(100\mu M)\ \boldsymbol{x} = (0.2\mu M)\ (50\ \mu L)$$

$$\boldsymbol{x} = \frac{(0.2\mu M)\ (50\ \mu L)}{(100\mu M)}$$

$$\boldsymbol{x} = 0.1\ \mu L$$

Add 0.1 µL of the100µM forward primer ***rbcL 724F*** stock solution into a single 50 µL reaction, to achieve a final concentration of 0.2µM ***rbcL 724F***.

iii) For a final concentration of 0.3µM forward primer ***rbcL 724F***:

$$C_1 = 100\mu M$$

$$C_2 = 0.3\mu M$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50\ \mu L$$

$$C_1V_1 = C_2V_2$$

$$(100\mu M)\ (\boldsymbol{x}) = (0.3\mu M)\ (50\ \mu L)$$

$$\boldsymbol{x} = \frac{(0.3\mu M)\ (50\ \mu L)}{(100\mu M)}$$

$$\boldsymbol{x} = 1.5\ \mu L$$

Into a single 50 µL reaction, add 0.15 µL of the 100µM ***rbcL 724F*** stock solution to yield a final concentration of 0.3µM ***rbcL 724F***.

iv For a final concentration of 0.4 µM ***rbcL 724F***

$$C_1 = 100\mu M$$

$$C_2 = 0.4\ \mu M$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50\ \mu L$$

$$C_1V_1 = C_2V_2$$

$$(100\mu M)\ (\boldsymbol{x}) = (0.4\ \mu M)\ (50\ \mu L)$$

$$\boldsymbol{x} = \frac{(0.4\ \mu M)\ (50\ \mu L)}{(100\mu M)}$$

$$\boldsymbol{x} = 0.2\ \mu L$$

Add 2 µL of the 10µM ***rbcL 724R*** stock solution to a single 50 µL reaction tube to get a final concentration of 0.4µM forward primer ***rbcL 724F***.

VARYING THE CONCENTRATION OF THE REVERSE PRIMER *rbcL* 724R

The reverse primer ***rbcL 724R***will be added to a series of PCR tubes in concentrations of 0.2µM, 0.3µM and 0.4µM. The stock solution of ***rbcL 724R*** has a concentration of 100µM.

i) For a final concentration of 0.2µM ***rbcL 724R***

$$C_1 = 100\mu M$$

$$C_2 = 0.2\mu M$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50\ \mu L$$

$$C_1V_1 = C_2V_2$$

$$(100\mu M)\ \boldsymbol{x} = (0.2\mu M)\ (50\ \mu L)$$

$$\boldsymbol{x} = \frac{(0.2\mu M)\ (50\ \mu L)}{(100\mu M)}$$

$$\boldsymbol{x} = 0.1\ \mu L$$

Add 0.1 µL of the100µM reverse primer ***rbcL 724R*** stock solution into a single 50 µL reaction, to achieve a final concentration of 0.2µM ***rbcL 724R***.

iii) For a final concentration of 0.3µM reverse primer ***rbcL 724R***:

$$C_1 = 100\mu M$$

$$C_2 = 0.3\mu M$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50\ \mu L$$

$$C_1V_1 = C_2V_2$$

$$(100\mu M)(\boldsymbol{x}) = (0.3\mu M)\ (50\ \mu L)$$

$$\boldsymbol{x} = \frac{(0.3\mu M)\ (50\ \mu L)}{(100\mu M)}$$

$$\boldsymbol{x} = 1.5\ \mu L$$

Into a single 50 µL reaction, add 0.15 µL of the 100µM***rbcL 724R*** stock solution to yield a final concentration of 0.3µM***rbcL 724R***.

iv For a final concentration of 0.4 µM ***rbcL 724R***

$$C_1 = 100\mu M$$

$$C_2 = 0.4\ \mu M$$

$$V_1 = \boldsymbol{x}$$

$$V_2 = 50\ \mu L$$

$$C_1V_1 = C_2V_2$$

$$(100\mu M)(\boldsymbol{x}) = (0.4\ \mu M)\ (50\ \mu L)$$

$$\boldsymbol{x} = \frac{(0.4\ \mu M)\ (50\ \mu L)}{(100\mu M)}$$

$$\boldsymbol{x} = 0.2\ \mu L$$

Add 2 µL of the 10µM ***rbcL 724R*** stock solution to a single 50 µL reaction tube to get a final concentration of 0.4µM reverse primer ***rbcL 724R***.