Faculty of Resource Science and Technology

DEVELOPMENT OF A MULTIPLEX GENE AMPLIFICATION PROTOCOL FOR RIBOSOMAL PROTEIN GENES ASSOCIATED WITH NASOPHARYNGEAL CARCINOMA

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Development of a Multiplex Gene Amplification Protocol for Ribosomal Protein Genes associated with Nasopharyngeal Carcinoma

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Table of Contents

Acknowledgement		I
Declaration		II
Table of Contents		IV
List of Abbreviations		VI
List of Tables		VII
List of Figures		VIII
Abstract		1
1.0	Introduction	2
2.0	Literature Review	
2.1	Multiplex PCR	4
2.2	Nasopharyngeal Carcinoma	5
2.3	Ribosomal Proteins, RPS15 and RPL14	6
3.0	Materials and Methods	
3.1	Primer Designing and Synthesis	8
3.2	Total RNA Extraction	10
3.3	RNA Quantification	11
3.4	Reverse Transcription	11
3.5	Polymerase Chain Reaction and AGE	12
3.6	Restriction Digestion	14

4.0 Results

4.1 Total RNA Extraction 16

4.2 Quantification of RNA using UV Spectrophotometry 17

4.3 Polymerase Chain Reaction (PCR) and Optimization 18

4.4 Restriction Digestion 25

5.0 Discussion

5.1 Total RNA Extraction 26

5.2 Reverse Transcription 27

5.3 Polymerase Chain Reaction and Optimization 28

5.4 Restriction Digestion 30

5.5 Multiplex Polymerase Chain Reaction 31

6.0 Conclusion 33

References 34

List of Abbreviations

AGE	Agarose Gel Electrophoresis
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EBV	Epstein-Barr Virus
EtBr	Ethidium Bromide
MgCl2	Magnesium chloride
M-MLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NPC	Nasopharyngeal Carcinoma
RNA	Ribonucleic acid
RP	Ribosomal protein
RPL	Ribosomal protein large subunit
RPL14	Ribosomal Protein Large 14
RPS15	Ribosomal Protein Small 15
RT-PCR	Reverse transcriptase polymerase chain reaction
WHO	World Health Organization

List of Tables

		Page
Table 1	List of primers (NCBI, primer blast)	8
Table 2	PCR Mix (Promega, USA)	12
Table 3	Thermal Cycling Conditions for PCR (Promega, USA)	13
Table 4	Restriction Enzymes, Buffers, Enzyme Assay Temperature and Heat Inactivation used for Restriction Digestion of the Genes	14
Table 5	Product Length of genes and their respective fragment sizes (New England Bio Labs® Inc.)	15
Table 6	Quantification of total RNA extracted from NP69 from UV spectrophotometer (Shimadzu, USA)	17
Table 7	Initial optimized PCR parameters for gene expression of *RPL14, and RPS15* in NP69 cellLines	18
Table 8	Expected Product Length of the genes	18
Table 9	Range of values for factors	19
Table 10	Multiplex PCR optimized parameters	19

List of Figures

		Page
Figure 1	PCR Amplification of RPS15 to obtain optimal annealing temperature	20
Figure 2	PCR Amplification of RPL14 to obtain optimal annealing temperature	21
Figure 3	PCR Amplification of RPS15 to obtain optimal primer concentration	22
Figure 4	PCR Amplification of RPL14 to obtain optimal primer concentration	22
Figure 5	PCR Amplification of RPS15 to obtain optimal Magnesium Chloride concentration	23
Figure 6	PCR Amplification of RPL14 to obtain optimal Magnesium Chloride concentration	23
Figure 7	Multiplex PCR amplification of RPL14 and RPS15	24

Development of a Multiplex Gene Amplification Protocol for Human Ribosomal Protein Genes Associated with Nasopharyngeal Carcinoma

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ABSTRACT

The amplification of multiple genes in a single reaction is a fairly new and not fully understood aspect of the Polymerase Chain Reaction as the factors which affect its optimal reaction varies based on the genes used. The factors which usually play a major role in the optimization is the concentration of magnesium chloride and also the concentration of the primers.These two factors were tested in this study with the genes being *RPL14* and *RPS15* from the normal Nasopharyngeal Carcinoma cell line.Incorporated within this project was techniques such as Reverse Transcriptase PCR (RT-PCR) and Restriction Enzyme Digestion, which the end goal was to achieve a simultaneous amplification of both genes in a single reaction. The expected amplicon sizes of the genes being 115bp and 773bp for *RPS15* and *RPL14* respectively would be able to give a clear indication as to the capabilities of the genes being amplified together, and that was achieved at the end of this study.

Keywords: *RPL14, RPS15*, RT-PCR, Restriction Enzyme Digestion

ABSTRAK

Penguatan lebih daripada satu gene dalam satu reaksi adalah sesuata yang masih baru dalam aspek rantaian tindakbalas polymeras dan kurang pemahaman yang sepenuhnya mengenainya kerana terdapat pelbagai faktor yang member kesan kepada tindakbalas yang optima berdasarkan gen yang digunakan. Faktor yang sentiasa memainkan peranan yang besar dalam mengoptimakan tindakbalasnya adalah kepekatan magnesium klorida dan juga primer, dan dua factor inilah yang diuji dalam projek ini, dan gen yang diuji adalah *RPS14* dan *RPL15* daripada sel normal kanser nasofarinks dengan teknik seperti *Reverse Transcription PCR (RT-PCR)* dan *Restriction Enzyme Digestion,* dimana tujuan utamanya adalah untuk menguatkan kedua-dua gen tersebut dalam satu reaksi tindakbalas. Saiz amplikon gen bagi *RPS15* adalah 115bp dan bagi *RPL14* adalah 773bp mampu memberi indikasi yang jelas mengenai keupayaan gen tersebut untuk dikuatkan bersama, yang telah pun dicapai pada hujung kajian ini.

Kata kunci: *RPL14, RPS15*, RT-PCR, *Restriction Enzyme Digestion*

1.0 INTRODUCTION

Ribosomal proteins being the constituents of ribosomes are actively involved in ribosomal biogenesis. Besides this, they are found to have extra ribosomal functions and many of them are found to be related to diseases and cancers, especially carcinomas due to the aberrations in the growth of the cell when influenced by the ribosomal protein genes and also in cases like Diamond-Blackfan Anemia (Freed, Bleichert, Dutca, & Baserga, 2010). Due to the nature of certain diseases sometimes being caused by the aberrations of more than one gene, in this case, ribosomal protein genes. The usage of Multiplex PCR helps the most as it can amplify the copies of cDNA of several ribosomal protein genes and help us analyze the cDNA sequence of the genes that possibly plays a part in the cause of the disease

In conducting a Multiplex Polymerase Chain Reaction (PCR) there are many factors that has to be considered and needs through planning as to ensure the success of it. From the design of the primers to the optimization of the entire multiplexing process. The reason extensive optimization is needed is due to the fact that many nonspecific products and primer dimers might interfere with the amplification of the needed products. The reason for the increase in preference of conducting multiplex PCR is due to its ability to simultaneously amplify a few DNA fragments in a single reaction thus saving on money and time (Sint, Rosa, & Traugott, 2012). Due to this benefit, multiplex PCR is often used in examining population genetics (Guichoux, et al., 2011), forensic studies (Hill, Butler, & Vallone, 2009) and even food safety studies(Randhawa, Chhabra, & Singh, 2009).

Ever since its first recorded usage in 1988 (Chamberlain, Gibbs, Ranier, Nguyen, & Caskey, 1988), Multiplex PCR has progressed a long way, however, methods on further optimizing this PCR method is rarely described in journals covering the usage of Multiplex PCR,

hence, the need to develop a protocol that is able to optimize the Multiplex reaction. With many companies offering kits that can be "ready to use" which is partly true in terms of the salt concentrations, but at the end of the day, the most important aspects are the primer specificity and concentration and also the thermocycling conditions.

With the aim of this research being carried out to simply develop a multiplex reaction that is able to amplify multiple genes related to nasopharyngeal carcinoma in a single reaction, the main objective of this research was to develop and observe an efficient protocol in amplifying *RPS15* and *RPL14* in a single reaction, for at the end, we expect that the end results would be the two genes being successfully amplified within a single reaction.

Thus far in the project, the methodologies applied are the initial and technical protocols which are the designing of primers, extraction of RNA, and quality and integrity analysis of the extracted RNA which involves UV spectrophotometry and the running of Agarose Gel Electrophoresis (AGE).

The specific objectives of this study was as follows:

1. To develop an efficient protocol in amplifying *RPS15* and *RPL14* in a single reaction
2. To observe the amplification of *RPS15* and *RPL14* in a single reaction

2.0 LITERATURE REVIEW

2.1 Multiplex Polymerase Chain Reaction

Multiplex PCR is generally known as a variant of PCR whereby two or more DNA sequences can be amplified in a single reaction and has been widely used in the fields of DNA testing which includes analyses of deletions(Henegariu, et al., 1994), mutations (Shuber, Skoletsky, Stern, & Handelin, 1993), and polymorphisms(Mutirangura, et al., 1993), which makes it a perfect candidate as to amplify multiple ribosomal protein genes associated with nasopharyngeal carcinoma in a single reaction.

The success of any PCR reaction falls heavily on the design of primers used in that reaction, and it is the same case as in Multiplex PCR where there are certain parameters that need to be followed if we wish to obtain a successful multiplex reaction with a high yield from a specific amplification. Primer length, melting and annealing temperature, specificity and avoiding the formation of primer dimers are essential in obtaining the optimal result. However, the main success factor relies on the rate at which the primers anneal to their target and their specificity which can be marred by poorly designed primers, lackluster buffer constituents and annealing temperature that is not suited to the primer. For an ideal result, the primers with near identical annealing temperatures and with an ideal length average of 20 bp and GC content around the 50% range with no primer dimer formation would be the best (Cha & Thilly, 1993; Dieffenbach, Lowe, & Dveksler, 1993; Henegariu, Heerema, Dlouhy, Vance, & Vogt, 1997)

2.2 Nasopharyngeal Carcinoma

A form of cancer that arises from the epithelium of the nasopharynx region is commonly called as Nasopharyngeal Carcinoma (NPC) which can be categorized into 3 types that are squamous cell carcinoma, keratinizing undifferentiated carcinoma, and non-keratinizing undifferentiated carcinoma which is also known as lymphoepithelioma and is considered the most common.

There are many reasons that can lead to a person having a high risk of contracting NPC, amongst them are because of their ethnic background or hereditary, environmental influences or even due to them being exposed to the Epstein-Barr virus (Zhang & Zhang, 1999). Although the initial cause was believed to be due to the smoking of tobacco and even a high consumption rate of fermented or salted fish and other food products, it has also been discovered in recent studies that Epstein-Barr Virus (EBV) is the primary etiologic agent in the pathogenesis of NPC due to the expression of a specific subgroup of EBV latent proteins (Young & Rickinson, 2004).

2.3 Ribosomal Proteins, RPL14 and RPS15

Genes in the RPS family gives instructions for producing ribosomal proteins that makes up part of the cellular structure called ribosomes. Ribosomes catalyze protein synthesis. Ribosomes consist of 2 subunits, which are the small (40s) and large (60s) subunits. Each ribosomal protein is part of a huge ribonucleoprotein (Ishii, et al., 2006). Ribosomal subunit accumulation needs a deposition of many ribosomal proteins on ribosomal RNA (rRNA) to form two ribonucleoprotein particles, a small and a large subunit that assist in protein translation (Wild, et al., 2010).

These RNA-binding proteins which are available in every cell are complex cellular organelles and a vital catalyst that is responsible in the manufacturing of amino acids in a living organism (Brodersen&Nissen, 2005). The relationship between these ribosomal proteins and p53 allows it to exhibit one of its many extraribosomal functions which are as many as 30 (Wool. 1996) and amongst it is the ability to cause cell cycle arrest and even apoptosis (Warner J.R. 2006). In relation to its association with diseases and cancers, due to its unique properties of being novel regulators of the Mdm2-p53 activation pathway, ribosomal proteins with its aberrations were linked to Turner syndrome, Diamond-Blackfan anemia and Noonan syndrome (Mao-De & Jing, 2006) especially in terms of ribosomal stress.

Ribosomal protein L14, or widely addressed by *RPL14* gene belongs to the L14E family of ribosomal proteins. *RPL14* encodes for ribosomal proteins from subunit 60S. It contains a basic region-leucine zipper (bZIP)-like domain (Wool, Chun &Gluck, 1996). Besides that, Wool et al. (1996) reported that *RPL 14* contains a trinucleotide (GCT) repeat tract whose length is highly polymorphic whereby these triplet repeats result in a stretch of alanine residues in the encoded protein. Transcript variants utilizing alternative polyA signals and alternative 5'-terminal exons exist but all encode the same protein. There are multiple processed pseudogenes

of this gene dispersed through the genome. Human ribosomal protein gene (RPL14) is located on chromosome 3p21.

RPS15 gene, which is a protein-coding gene, is situated in the cytoplasm. This gene codes a ribosomal protein that is part of the 40S subunit and also sequence proteins that belong to the S19P family of the ribosomal proteins at 19p13.3. This gene seems to be functional in many different tumors, example insulinomas, colon cancer and esophageal cancers (Kitagawa, et al., 1991). *RPS15* has been tested for its relatedness and association with diseases and has been suggested to be involved in ribosome pathways and processes for ribosomal 40S transport out of the nucleus, 40S subunit biogenesis, rRNA processing, translational elongation and translation (Rouquette, Choesmel, & Gleizes, 2005). Proteins encoded by the *RPS15* normally has molecular function, like DNA, RNA and protein binding and structural component of ribosome, and to confine in different subdivisions, example nucleoplasm, cytosol, cytosolic 40S subunit, ribosomes, cytoplasm, intracellular and nucleus. As cited in Rouquette et al. (2005), *RPS15* is needed for the nuclear export of the pre-40S particles, and may directly or indirectly communicate with the nuclear transport machinery.

3.0 MATERIALS AND METHODS

3.1 Primer Design and Synthesis

Forward and reverse primers for *RPS15* and *RPL14* genes were designed using NCBI primer designing tool. The mRNA sequences of *RPS15* and RPL14 was retrieved from NCBI database. The primers were then checked for potential hairpin formation using Oligocalc (DANA-Farber Cancer Institute). Furthermore, the designed primers were also checked for the secondary structure calculations which are their lowest folding free energy (kcal/mole) andtheir strongest folding Tm by using IDT secondary structure analyzer in order to obtain the best result. And due to the nature of this research being a multiplex PCR, the primers were designed to have high specificity so that they did not interfere with the amplification of the other cDNA.

Table 1: List of primers (NCBI, primer blast)

Primer	Sequences (5'-3')	Length (bp)	GC Content (%)	Expected Product Length (bp)
RPS15 (F)	GAA GAC GCA CCT GCG GGA CA	20	65.00	115
RPS15 (R)	GTG GCC GAT CAT CTC GGG CTT G	22	63.64	
RPL14 (F)	TTC TTC CTT CTC GCC TAA CG	20	50.00	773
RPL14 (F)	CCT CCT AAC TTC AGC CTC AA	20	50.00	

The 4 major parameters that needed to be considered when designing a multiplex PCR are primer length, melting temperature, specificity, and to avoid primer dimer formation. In terms of the primer length, a recommended size of 19-20 base pairs is suitable as larger sizes would result in reactions that need to be carried out at higher temperatures to allow for annealing process and also it would yield unspecific results. In terms of the annealing temperature, the smaller the gap between all three sets of primers' annealing temperature the better as we would not need to vary the annealing temperature. Specificity is also another major contributing factor for a successful multiplex PCR as there are multiple targets in the reaction tube hence to avoid mismatch occurring. This can be achieved by designing the primers to be highly specific and running them through primer-BLAST to ensure specificity. And to avoid the formation of primer dimers, it can be observed via OligoAnalyzer (Intergrated DNA Technologies, USA).

The two primer sequenceswas based on primers which were available in the lab was used for the project with careful considerations on the primer properties. Also to be noted during the primer design, the primers was chosen such that their designhad a similar annealing temperature of about ± 3°C and yet concurrently targeting their specific genes in a single reaction.

3.2 Total RNA Extraction

In conducting total RNA extraction, the TRIzol method (Chomczynski, 1993) was utilised to extract the NP69 cell line via the Life Technologies method.

The obtained cell monolayer was made to undergo the standard procedures in extracting the RNA which was firstly by rinsing it with ice cold PBS followed by 1ml of TRIzol reagent added to the culture dish containing the cell line and further aided by mechanical lysing via the cell scrapper and pipetting up and down the cell line culture. 0.2 ml of chloroform per 1 ml of TRIzol reagent (Invitrogen™, USA) was added and the sample tubes was capped securely.

Samples were then vortexed vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. Centrifugation was carried out at 12, 000g for 15 minutes at 2-8°C. Upon centrifugation, the mixture was observed as three layers, where only the upper colorless aqueous layer was transferred without disturbing the interphase into a clean 1.5ml tube. 0.5ml of isopropanol was then added to the aqueous solution and mixed well.

The sample was then incubated at room temperature for 10 minutes and centrifugation was carried out at 10, 000g for 20 minutes at 4°C in order to pellet the RNA. The supernatant was completely removed from the tube. The RNA pellet was then washed with 1 ml of 75% ethanol per 1 ml of TRIzol reagent and then was vortexed briefly.

The sample was then centrifuged at 7, 500rpm for 5 minutes at 4°C. The ethanol was discarded while the RNA pellet was air dried for 5-10 minutes. The RNA pellet was resuspended in RNase-free water. The sample was then stored at -80°C for continuous use.

3.3 RNA Quantification

Concentration and purity of RNA isolated was determined by measuring absorbance via UV spectrophotometer (Shimadzu, USA). Volume of 1μL of RNA from the cell line was diluted separately in 999μL of nuclease free waterto make 1000X dilution factor in a glass cuvette. The prepared RNA sample was inserted intothe UV spectrophotometer to read the absorbance values. The readings were then recorded.

3.4 Reverse Transcription

For this process, cDNA was synthesized for the purified RNA from the cell line by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (Promega, USA). 1 μg of RNA samples was added with 0.5 μg of random primers and nuclease-free water to reach a final volume of 25 μl. The mixtures were then incubated at 70 °C for 5 minutes to melt the secondary structures within the template. Then, it was cooled immediately on ice and centrifuged briefly to collect the solution at the bottom of the tube.

Annealed primers was then added with 5μl of M-MLV RT 5X reaction buffer, 1.25 μl of 10 mM each dNTP, 0.63 μl of Recombinant RNasin® Ribonuclease Inhibitor, 1 μl of M-MLV RT and finally was filled up with nuclease-free water until a final volume of 25 μl. The mixtures were then incubated at 37 °C for 60 minutes for extension process. The mixtures were incubated again at 70 °C for 15 minutes for the denaturation process. The cDNA can be stored at -20 °C to be proceeded with PCR.

3.5 Polymerase Chain Reaction and AGE

Conventional RT-PCR was performed on cDNA samples with gene specific primers in the two samples with negative control. PCR was necessary to be carried out at an optimized melting point and a proposed 30 cycles of amplification which was started at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, followed by annealing at 51.4/53.5°C for 45 seconds, then extension at 72°C for 90 seconds and finally extension at 72°C for 10minutes based on the protocol using Taq DNA Polymerase from Fermentas.

Table 2: PCR Mix (Promega, USA)

Components	Final Volume (Total volume of 25µl)	Final Concentration
Taq DNA Polymerase	0.125µl	1.25u
Magnesium chloride solution, 25mM	1µl	1.0mM
dNTP mix, 10mM	0.5µl	1mM
Forward primer	2.5µl	1µM
Reverse primer	2.5µl	1µM
Taq DNA Polymerase buffer	2.5µl	1X
Template DNA	1µl	<0.5µg/25µl
Nuclease free water, to top up	14.875µl	-

Before carrying out the multiplex PCR, all sets of primers that were designed was tested through a standard PCR to ensure correct amplification and annealing temperature so that we are able to obtain the fragments that we desire. Upon obtaining clear and positive results, then only the Multiplex PCR is carried out by in 2 levels. The first level manipulates the primer and magnesium chloride concentration upon determining the annealing temperature to ensure that the primer pairs produced strong results and if there were weak results, the primer concentration was

changed. Finally, the second and final step of Multiplex PCR is carried out whereby all the targets produce a strong result when the template cDNA mix was used.

Table 3: Thermal Cycling Conditions for PCR (Promega, USA)

Step	Temperature	Time	Number of cylces
Initial denaturation	95°C	2 minutes	1
Denaturation	95°C	30 seconds	30
Annealing	51.4-53.5°C	30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	5 minutes	1
Soak	12°C	∞	1

The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. Since Taq DNA Polymerase was used, the reaction was loaded onto the gel after amplification with the use of loading dye.

3.6 Restriction Digestion

Restriction digestion was carried out to verify the expressed band on Agarose Gel Electrophoresis was the gene of interest, where a known specific restriction enzyme for each gene namely, *RPL14*and *RPS15* that were used to cut the gene of interest into two fragments of known sizes. First, one specific restriction enzyme for each of the genes were determined from NEB Cutter V2.0 (New England Bio Labs® Inc.) while the suitable Restriction enzyme activity in Promega 10X Buffers, Reaction Temperature and the heat inactivation for each of the restriction enzyme chosen were obtained from Promega (Table 4).

Table 4: Restriction Enzymes, Buffers, Enzyme Assay Temperature and Heat Inactivation used for RestrictionDigestion of the Genes (Promega, USA)

Genes	Restriction Enzyme	Promega 10x Buffer	Enzyme Assay Temperature	Heat Inactivation
RPL14	*Hinc II*	R Buffer	37°C	65°C
RPS15	*Mbo I*	Multicore Buffer	37°C	65°C

A typical restriction digestion protocol was adopted from Promega (USA), in which 5μL of the PCR product of each gene was mixed with 2μL of speeific Restriction Enzyme 10X buffer and 12.5μL of nuclease free water in a sterile microcentrifuge tube. The mixture was mixed by pipetting before adding 0.5μL of specific Restriction Enzyme. The reaction mixture was then mixed gently by pipetting and the closed tubes were allowed to centrifuge for a few seconds. Then, the mixture was incubated at the enzyme's optimum temperature for 2 hours, followed by inactivation for 20 minutes.

Restriction digestion was tested by running the reaction mixture on Agarose Gel Electrophoresis, in which 6X DNA loading dye was used to visualize the bands, along with the control for each gene, which is the uncut PCR product. The size of each band expression was