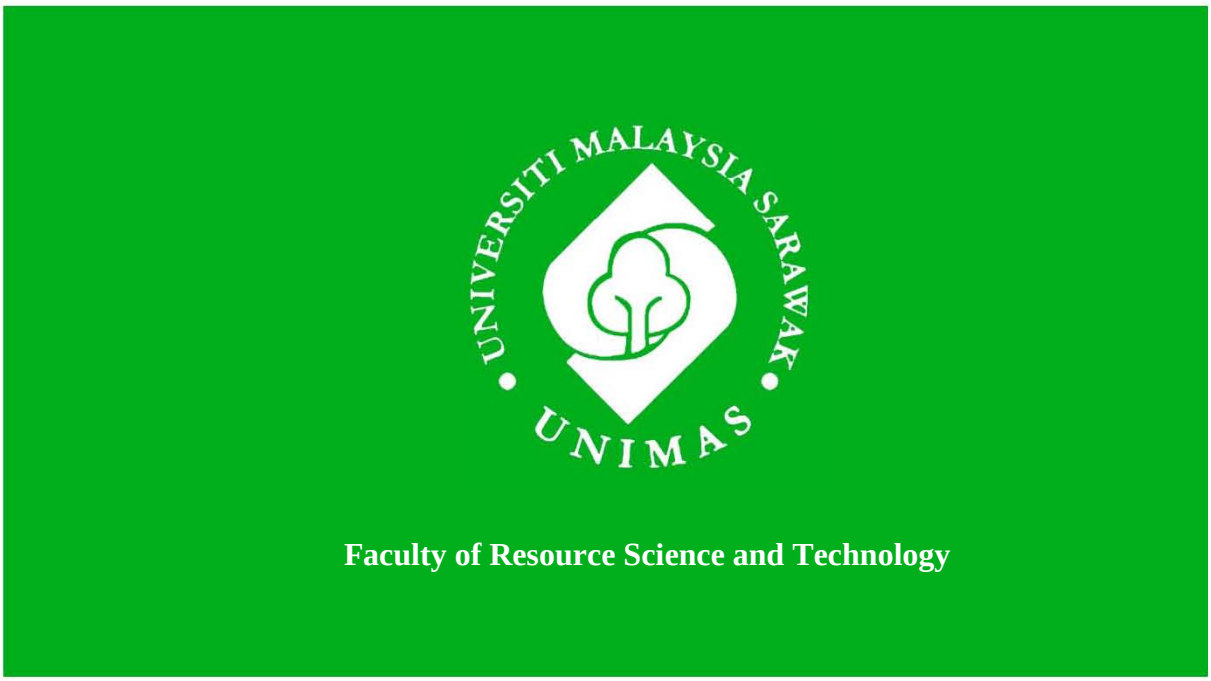

Faculty of Resource Science and Technology

COMPARATIVE ANALYSIS OF EXPRESSION PATTERNS AMONG THE RIBOSOMAL PROTEIN GENES OF RPL27, RPL37a, AND RPL41 IN HUMAN CELL LINES

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Bachelor of Science with Honours
(Resource Biotechnology)
2013

Comparative analysis of expression patterns among the ribosomal protein genes of RPL27, RPL37a, and RPL41 in human cell lines.

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A final year project submitted in partial fulfillment for the degree of Bachelor of Science with Honours (Resources Biotechnology)

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ACKNOWLEDGEMENT

First and foremost, I would like to thank God for the strength and wisdom from Him throughout the entire project. Without His abundant grace and blessings, I would not be able to accomplish this task successfully.

Next, I would like to express my gratitude to my supervisor, Assoc. Prof. Dr. Edmund Sim Ui Hang for giving me the chance to conduct this project. A million thanks for his tremendous support and guidance throughout the process of completing this project.

Besides, a standing ovation and salute to the postgraduate student of Human Molecular Genetics Laboratory (HMGL), UNIMAS, Miss Ma Xiang Ru for providing informative suggestions and her precious advices. Not forgetting, laboratory assistants and research assistants of HMGL for their generous assistance in the laboratory.

In addition, I would like to express my appreciation to my laboratory mate, Ting Chee Enn. A thousand thanks for his generous to share some useful information and his effort to tackle the problems we faced during the experimentation. Moreover, I would like to express my deepest gratitude to my family members. It was their unconditional love and strong faith that I am able to complete this project.

Last but not least, I would like to thank the universiti and Faculty of Resources Science and Technology for providing sophisticated instruments and other facilities that enable the completion of this project as well as everyone who has directly or indirectly helped me in this project.

DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions of higher learning.

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LIST OF ABBREVIATIONS

cDNA	Complementary Deoxyribonucleic Acid
NPC	Nasopharyngeal Carcinoma
RP	Ribosomal Protein
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase chain reaction

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Comparative analysis of expression patterns among the ribosomal protein genes of RPL27, RPL37a, and RPL41 in human cell lines.

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ABSTRACT

Ribosomal proteins are essential in protein biosynthesis process and they also have extraribosomal functions. These proteins have been found to be closely associated with various inherited diseases and cancers. Previous studies revealed that the ribosomal protein large subunit (RPL) gene expressed differently among cell line derived from human nasopharyngeal carcinoma (NPC). However, *RPL27*, *RPL37a*, and *RPL41* are found to be significantly downregulated in all cell lines derived from NPC tumours when compared to normal control. The main objective of this study is to evaluate quantitatively the expressed transcript level among the RP genes of *RPL27*, *RPL37a*, and *RPL41* in the human cell line system. The method of Reverse Transcription Polymerase Chain Reaction (RT-PCR) coupled with band intensity analysis will be used to meet this objective. Result from this finding show that there are no significant different in expressions *of RPL27*, *RPL37a*, and *RPL41* in NPC tumours and normal control. Besides that, *RPL27* and *RPL37a* show significant correlation when increase in sample size.

Keywords: *RPL27, RPL37a, RPL41*, nasopharyngeal carcinoma (NPC) tumours, RT-PCR and band intensity analysis.

ABSTRAK

Protein ribosom adalah penting dalam proses biosintesis protein dan mereka juga mengandungi fungsi extraribosomal. Protein ini telah didapati berkait rapat dengan pelbagai penyakit yang diwarisi dan kanser. Kajian terdahulu menunjukkan bahawa gen protein ribosom unit besar (RPL) dinyatakan berbeza dalam kalangan barisan sel berasal dari karsinoma nasofarinks manusia (NPC). Walau bagaimanapun, RPL27, RPL37a, dan RPL41 didapati ketara penurunan pengekspresan dalam semua barisan sel berasal dari tumor NPC apabila dibandingkan dengan kawalan normal. Objektif utama kajian ini adalah untuk menilai secara kuantitatif tahap transkrip antara gen RPL27, RPL37a, dan RPL41 dalam sistem sel manusia. RT-PCR and analisis pancaragam intensiti digunakan untuk memenuhi matlamat ini. Keputusan yang didapati dalam kajian ini menunjukan bahawa tiada berbezaan signifikan pada ekspresi RPL27, RPL37a , dan RPL41 dalam tumor NPC dan kawalan normal. Selain itu, RPL27 dan RPL37a menunjukkan kolerasi signifikan apabila meningkatkan saiz sampel.

Kata-kata kunci: RPL27, RPL37a, RPL41, tumor karsinoma nasofarinks (NPC), RT-PCR dan analisis pancaragam intensity.

1.0 INTRODUCTION

Ribosome is the site of translation which plays an important role in protein synthesis. It is highly conserved in all cells and this signified the important of ribosome protein in all organisms. Ribosome is the association of rRNA and ribosomal protein which eventually form the small and large ribosomal subunits.

Due to the essential function of ribosomal protein in transcription process, studies have related ribosomal protein with human congenital disorder and cancers (Ruggero & Pandolfi, 2003; Anupama & Benjamin, 2012**).** For example the expression of *RPL27* is found to be inhibited in the NPC-derived cell line, CNE2/DDP that is resistant to anticancer drug (Jiang *et al*., 2003). Moreover, the putative zinc finger in *RPL37a* protein can bind to DNA and is suspected to take part in the regulation of tumour suppressor, oncogenes or cell-cycle genes (Saha *et al*., 1993). Besides that, deletion in RPL41 is found in 59% of tumour cell lines (Wang et al., 2010). Studies by Sim *et al.* (2010) also identified that *RPL27, RPL37a,* and *RPL41* are essentially downregulated in cell lines derived from NPC tumours when compare to the normal control. However, the co-expression among these genes in the human cell line systems is yet to be evaluated.

The objectives of this research project are:

1. To quantify the expressed transcript level among the RP gene of *RPL27, RPL37a,* and *RPL41* in a human cell line system.
2. To identify the relationship among *RPL27, RPL37a,* and *RPL41* based on their expression level in a human cell line system.

2.0 LITERATURE REVIEW

2.1 Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal Carcinoma (NPC) is a head and neck cancer with malignancy originating at the nasopharynx region. The occurrence of NPC is due to a combination of genetic and environment factors (Chou *et al*., 2008). NPC is only common in certain geographic areas, such as Southern Asia, and is more prevalent among the Chinese ethnic group (Chou *et al*., 2008). Environmental factors of this disease may be the exposure to nitrosamines in salted and pickled food (Chou *et al*., 2008).

Histologically, NPC is due to the malignancy of squamous epithelial cell around the ostium of the Eustachian tube in the lateral wall of the nasopharynx. Squamous cells are flat-type epithelial cell which can be found in many part of the body. According to the World Health Organization, NPC is categorized into three type; Type 1, keratinizing squamous carcinoma; Type 2, nonkeratinizing squamous carcinoma; Type 3, nonkeratinizing squamous carcinoma (Chou *et al*., 2008). Type 2 and 3 are associated with Epstein-Barr Virus (EBV) but not Type 1. Since almost all NPC tumours are predisposed by EBV infections, it is believed that the EBV infection could be one of the main etiology of (Chou *et al*., 2008).

2.2 Ribosome

Ribosome is made up of four ribosomal RNA species and 79 ribosomal proteins in mammals . It consists of 2/3 of ribonucleic acid and 1/3 of protein (Gerard *et al*., 2007). The ribosomes found in eukaryotes are composed of two subunit; large subunit (60s) and small subunit (40s). Ribosome plays an important role in the biosynthesis of protein. Protein is synthesized through the translation process whereby with the aid of ribosome,

the tRNA will code for the genetic instruction within the mRNA. The translation product of ribosome which is located in different place will have different destination (Gerard *et al.*, 2007). Ribosome normally can be found in two places; in the cytoplasm or bounded to the endoplasmic reticulum and nuclear. Ribosomes found in the cytoplasm are called 'free ribosome' (Gerard *et al.*, 2007). The main functions of these ribosomes are to synthesize protein used inside the cell. Ribosomes which are bounded to the nuclear or on the endoplasmic reticulum are mainly predestined to synthesize proteins for insertion into the plasma membrane and to be exported out of the cell (Gerard *et al.*, 2007).

2.3 Ribosomal Protein

Ribosomal protein is the protein that is associated with rRNA to make up the ribosomal subunits involved in the translation process. Apart from protein synthesis, ribosomal protein is also involved in extraribosomal functions, such as DNA repair, apoptosis, transcription regulation, and translation regulation (Wang *et al*., 2010).

Studies have related ribosome protein with human congenital disorder and cancers (Ruggero *et al.*, 2003; Anupama *et al.*, 2012**)**. The example of ribosomal protein genes disorder which can causes diseases are Turner's syndrome and Diamond-Blackfan anaemia syndrome (Draptchinskala *et al*., 1999). Thus, this is believed that the ribosomal protein play a role in causing diseases. There are up to 33 RP genes are found to be overexpressed in tumours of colorectal carcinoma relative to their normal controls (Sim *et al*., 2006). In a recent study by Sim *et al.* (2010) *RPL27, RPL37a,* and *RPL41* are downregulated in cell lines derived NPC tumours when compare to a normal control. This finding supports the existence of NPC-associated RP genes and shows their essential role in human nasopharyngeal development.

2.4 RPL27

RPL27 gene is located on loci 17q21 of Chromosome 21 which encode for the 60s ribosomal protein *L27* in human. The human ribosomal protein *L27* gene is found to be developmentally regulated in the kidney (Gallagher & Malik, 1994). Besides that, *RPL27* is also one of the candidate housekeeping genes that exhibit stability in expression (Jonge *et al*., 2007). *RPL27* is inhibited in the NPC-derived cell line, CNE2/DDP that is resistant to anticancer drug, hence suggesting its role in the chemo-response of malignant cells (Jiang *et al*., 2003). *RPL27* is one of the genes, which is found to be significantly downregulated in malignant nasopharyngeal cell line relative to normal counterpart cells (Sim *et al*., 2010). Moreover, it also has an increased copy numbers and expression levels in gastric carcinoma (Varis *et al*., 2002)

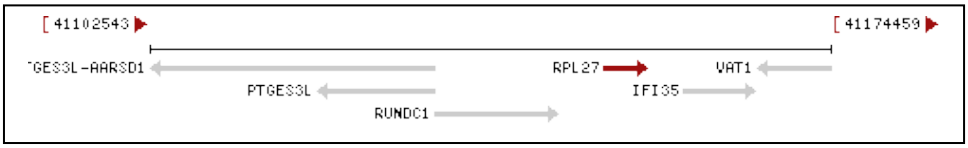

Figure 1: Location of *RPL 27* on Chromosome 17 (Taken from National Center for Biotechnology Information, 2012).

2.5 *RPL37a*

RPL37a gene is located at loci 2q35 on the Chromosome 2 (Hillier *at al*., 2005) which is responsible to encode for 60s ribosomal protein *L37a* in human. The gene belongs to the *L37AE* gene family. The main function of ribosomal large subunit is to transfer peptidyl (Liljas, 2004). The full cDNA length of *RPL37a* included the polyA tail is 366 nucleotides (Saha *at al*., 1993) whereas the gene itself is 2624 base pair long (Hillier *at al*., 2005).

Ribosomal protein *L37* functions as a housekeeping gene has been used as an optimal reference gene for normalization of gene expression in tumour studies (Pfister *at al*., 2011). It is one of the ribosomal protein genes that are found to be significantly downregulated in NPC-derived cell (Sim *et al.*, 2010). Moreover, *RPL37a* is highly expressed in skeletal muscle cells (Liljas, 2004).

RPL37a also has a single zinc finger-like motif of the C2-C2. The length of the structural protein encoded by *RPL37a* is predicted to be 92 amino acids long with the zinc finger located in between the central region of the gene (Saha *at al*., 1993). Besides that, the structural protein is ribredoxin-like and this enables it to bind to zinc ions, magnesium and monovalent metals even though having putative zinc fingers (Liljas, 2004). The putative zinc finger in *RPL37a* protein can bind to DNA and is suspected to take part in the regulation of tumour suppressor, oncogenes or cell-cycle genes (Saha *et al*., 1993).

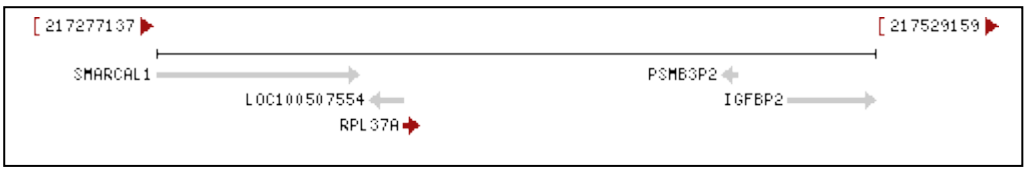

Figure 2: Location of *RPL37a* on Chromosome 2 (Taken from National Center for Biotechnology Information, 2012).

2.6 *RPL41*

RPL41 gene is located at loci 12q13 on Chromosome 12 (Wang *et al.*, 2010) which encodes for 60s ribosomal protein *L41* in human. The *RPL41* gene is a basic (positively charged) peptide which code for the shortest coding sequence size among the other

ribosomal proteins. It only consists of 25 amino acids but is highly specified and highly conserved in eukaryotes.

Deletion in *RPL41* is found in 59% of tumour cell lines and its down regulation is found in 75% of primary breast cancers (Wang *et al*., 2010). These have suggested that there is a tumour suppression role for *RPL41*.

RPL41 is associated with several cytoskeleton components and is bounded directly to polymerized tubulins. It is believed that *RPL41* play an important role in stabilization on microtubule (Wang *et al*., 2010). Since microtubule spindles are important in the chromosome segregation during mitosis, the knockdown of *RPL41* will show abnormal spindles, frequent failure of cytokinesis and the formation of polynuclear cells. *RPL41* is important in mitosis as it has been proved to be a microtubule associated protein which is important for functional spindles and the integrity of centromere (Wang *et al.*, 2010). In other words, the abnormal mitosis and disrupted centromere are linked to the *RPL41* down-regulation and this might lead to malignant transformation of cell (Wang *et al*., 2010).

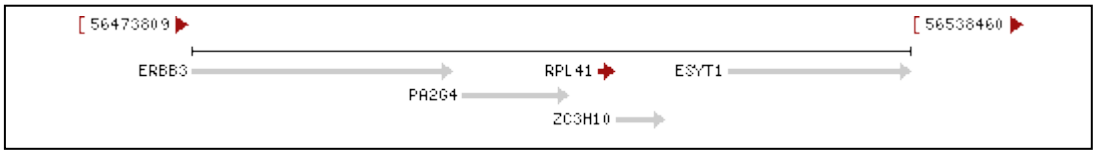

Figure 3: Location of *RPL41* on Chromosome 12 (Taken from National Center for Biotechnology Information, 2012).

2.7 Reverse Transcriptase-PCR

Reverse Transcriptase Polymerase (RT-PCR) is modified version of PCR. It uses the enzyme Reverse Transcriptase to reverse transcribed the RNA strands into complementary

DNA (cDNA). The cDNA will be amplified using PCR. RT-PCR is a sensitive approach and theoretically it can detect very low number of RNA copies (Fátima *at al*., 2001). Other than that, RT-PCR is preferably more suitable for a rapid analysis of a large number of samples when compare to the other technique (Fátima *et al*., 2001). It is normally used to detect genetic disease and to study the genome of RNA viruses.

3.0 METHODS AND MATERIALS

3.1 Cell line samples

In this study, two kinds of human epithelium cell lines were used; they are normal and tumour cell lines. Normal cell line used was NP69 (Tsao *et al,*. 2002), whereas for tumour cell lines were HONE1, SUNE1 and HK1.

3.2 Total RNA isolation

Total RNA isolation from cell lines was performed by using Trizol RNA Isolation Protocol Trizol reagent consists of a mono-plastic solution of phenol and Guanidine isothiocyanate, which helps to maintain the integrity of the RNA, while disrupting cell and dissolving cell components, during sample homogenization or lysis. The materials used were Trizol reagent, nucleus-free water, PBS (phosphate-buffered saline), chloroform, isopropyl alcohol and 70% ethanol.

The cells were grown in the flask till a monolayer of 70% confluent was achieved. Then cells media were discharged and the attached cells were rinsed with PBS solution. The cells were dislodged from the culture flask by adding 1ml of TRIZOL Reagent per 3.5cm diameter dish. These cells were scraped off with a cell scrapper and the lysed cells were passed through a pipette and were vortexed. The sample was incubated in the TRIZOL Reagent for 5 minutes at room temperature in the culture dish and then transfers it to a centrifuge tube.

After that, 0.2ml of chloroform per 1 ml of TRIZOL Reagent was added into the tube. The sample was mixed and vortexed vigorously for 15 seconds and then incubated at room temperature for 5 minutes. Then, the sample was centrifuged at 10,000rpm for 10 minutes at 4 ℃. After centrifugation, the mixture was separated into a few layers. The RNA

was in the upper aqueous phase. The upper aqueous phase was transferred carefully into a new centrifuge tube.

Then, the RNA from aqueous phase was precipitated by mixing it with 0.5 ml of Isopropyl alcohol per 1 ml of TRIZOL Reagent into the tube and incubated at 10 minutes at room temperature. Then, the tube was centrifuged at 10,000 rpm for 10 minutes at 4℃. Before the centrifugation, the RNA precipitate is often invisible, which forms a gel-like pellet on the side and bottom of the tube. After centrifugation, the supernatant was removed completely. After that, 1 ml of 70% ethanol per 1 ml of TRIZOL reagent was added into the tube to wash the pellet. The sample was mixed by vortexing for 15 seconds and centrifuge at 10,000rpm for 5 minutes at 4℃. Next, the leftover ethanol was removed and the RNA pellet will be air-dried for 5 minutes. Subsequently, the RNA was dissolved in 30 ml of nucleus-free water and the RNA was stored at -80℃ until use.

3.3 Agarose Gel Electrophoresis

The isolated RNA was checked using Agarose Gel Elecrophoresis (AGE). The gel was prepared by mixing 0.4 g of agarose powder and 40 ml of 1× TAE Buffer. The gel mixture was heated in a microwave oven for 70 seconds and subsequently cooled for 5 minutes. The gel mixture was then be poured onto the gel electrophoresis tank with combs inserted before the gel mixture was solidified. Then, the solidified gel was submerged in 1 × TAE buffer. The RNA was loaded into the well of the gel by using a pipette. Two µl of RNA samples was mixed with 1 µl of gel loading dye prior to being loaded into the wells. Electrophoresis was run for 24 minutes at 90 Volts. Then, the gel was post-stained with ethidium bromide by submerging the gel in an enclosed box of ethidium bromide solution. The gel was then be visualized in a UV transilluminator and image was taken.

3.4 RNA quantification

Two µl of RNA were diluted with 68 µl of nuclease free water to make a ratio of 1:35. The nuclease free water will be used as a standard for measurement of absorbance reading via a UV spectrophotometer.

3.5 Reverse Transcription

The RNA do not serve as a template for PCR (polymerase chain reaction), thus it must be synthesised into cDNA by a Reverse Transcriptase. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega Corporation, USA) was used for the reverse transcription of RNA into cDNA. One µg of RNA samples and 1 µl of random primers were added into a sterile RNase-free microcentrifuge tube. RNase free water was then add up to get a final volume of 25 µl.

The tube was heated at 70℃ for 5 minutes to melt the secondary structure within the template. Then, it was cooled down immediately on ice to prevent secondary structure from reforming. It was centrifuged briefly to collect the solution at the bottom of the tube. After centrifugation 5 µl of M-MLV 5X reaction buffer, 1.25 µl of 10 mM dNTP, 1.6 µl of Ribonuclease inhibitor (RNasin), and 1 µl of M-MLV RT were added. The mixture was mixed gently by flicking the tube and it was incubated at 37℃ for 60 minutes. Then, the mixture was incubated again at 70℃ for another 15 minutes. After that, it was stored at -20℃ for future used.

3.6 Primer Used

The characteristic of the primer used in the experiment for *RPL27, RPL37a, RPL 41* and *GAPDH* was shown below (Table 1, 2, 3and 4).

Table 1: Primer sequences for *RPL 27*

Primer sequences	Length	T_m	GC%	Product Size
Forward Primer 5'CAAGTTCATGAAACCTGGGAAG3'	22	60.87	45.45	398
Reverse Primer 5'GCAGTTTCTGGAACAACCACTT3'	22	59.79	45.45	398

Table 2: Primer sequences for *RPL 37a*

Primer sequences	Length	T_m	GC%	Product Size
Forward Primer 5'CTTTCTGGGCTCGGACCTA3'	19	60.34	57.89	343
Reverse Primer 5'AGGCCAGTGATGTCTCAAAGA3'	21	59.86	47.62	343

Table 3: Primer sequences for *RPL 41*

Primer sequences	Length	T_m	GC%	Product Size
Forward Primer 5'TAGCCGTAGACGGAACTTCG3'	20	59.27	55.00	227
Reverse Primer 5'TCCCACAACTTGTACCAGCA3'	20	59.16	50.00	227

Table 4: Primer sequences for *GAPDH*

Primer sequences	Length	T_m	GC%	Product Size
Forward Primer 5'TGCACCACCAACTGCTTAGC3'	20	61.17	55.00	87
Reverse Primer 5'GGCATGGACTGTGGTCATGAG3'	21	61.02	57.14	87

3.7 Polymerase Chain Reaction (PCR) Amplification

The PCR Mastermix was prepared by mixing 5 µl of 5X Green Go Taq® Flexi Buffer, 2.5 µl of $MgCl_2$ Solution at $25mM^{-1}$, 0.5 µl of dNTP, 0.125 µl of GoTaq® DNA Polymerase (5 µ/µl), 1 µl of cDNA template, forward primer and reverse primer. Nuclease free water was then been added to bring the final volume of mixture to 25 µl.

The PCR parameter was set for 95℃ for 2 minutes (initial denaturation), then 25~35 cycles of 95℃ for 30 seconds (denaturation), 55℃~65℃ for 30 seconds (annealing) and 72℃ for 90 seconds (extension) and a final extension of a final extension of 72℃ for 5 minutes. The PCR products were co-separate with GeneRuler™ 100 bp DNA ladder (Fermentas, USA) as marker by using 1.7% AGE system. The gel was then be visualized in a UV transilluminator and image was taken.

3.8 Expression Analysis

The gel image of PCR products obtained from the amplification of *GAPDH, RPL27, RPL37a* and *RPL41* genes was analysed using TotalLab Quant (TotalLab, USA). The software was used to measure the intensity of bands of PCR products. Each of the band intensity values for *RPL27, RPL37a* and *RPL41* gene will be divided by the band intensity values for *GAPDH* genes on the corresponding samples in order to obtain ratio of band intensity values. Then, the ratio of band intensity values will be further analysed by student t-test and One-way ANOVA to determine the variation of gene expression level within different cell line and also between normal and NPC (HK, HONE1 and SUNE1) cell line.

4.0 RESULTS

4.1 Total RNA Isolation

The RNA ladder and RNAs samples (NP69, HK, HONE1 and SUNE1) were analysed via electrophoresis and showed a clear ribosomal RNAs band of 28s and 18s (Figure 4). This signified the total RNAs was successfully isolated from the cell lines.

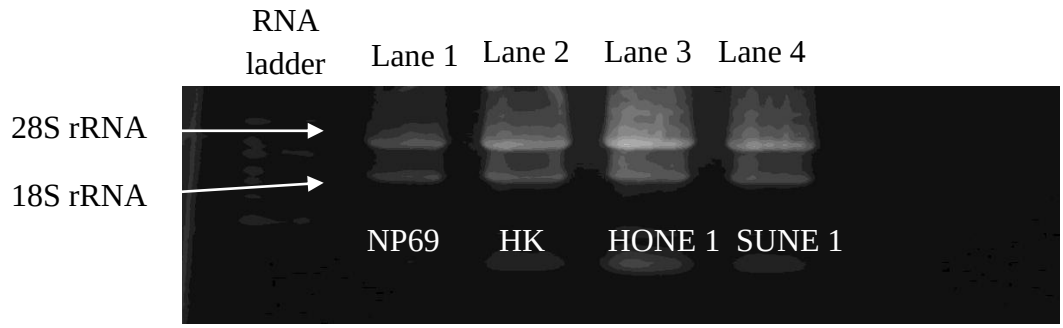

Figure 4: Total RNA bands for four different isolated cell lines. **Lane 1:** NP69**, Lane 2:** HK, **Lane 3:** HONE1 and **Lane 4:** SUNE1.

4.2 RNA Quantification

Spectrometric analysis was carried out to determine the quantity and quality of isolated RNAs.

Table 5: RNA quantification of NP69, HK, HONE1 and SUNE1

	NP69	**HK**	**HONE1**	**SUNE1**
230nm	0.663A	0.881A	1.544A	1.031A
260nm	0.483A	0.944A	1.839A	1.675A
280nm	0.296A	0.580A	1.031A	0.944A
320nm	0.093A	0.181A	0.159A	0.031A
A_{260}/A_{280} ratio (µg/µl)	1.925	1.915	1.928	1.800
A_{260}/A_{230} ratio (µg/µl)	0.684	1.091	1.213	1.644
Concentration (µg/µl)	0.546	1.069	2.353	2.302

In Table 5, it shows the RNA quantification value of NP69, HK, HONE1 and SUNE1. To indentify a good quality RNA, the concentration (µg/µl) of RNA should be around 1 µg/µl, whereas the A_{260}/A_{280} ratio which above 1.6 was considers having high purity (Ausubel *et al*., 2002). According to the absorbance ratio of A_{260}/A_{280} of isolated total RNA, the RNA which contains the highest purity was HONE1, NP69, HK and SUNE1 accordingly. All of the isolated RNA contain a concentration of RNA which above 1 µg/µl except for NP69. The concentrations obtained were used to calculate the volume of RNA needed from different cell line for cDNA conversion.