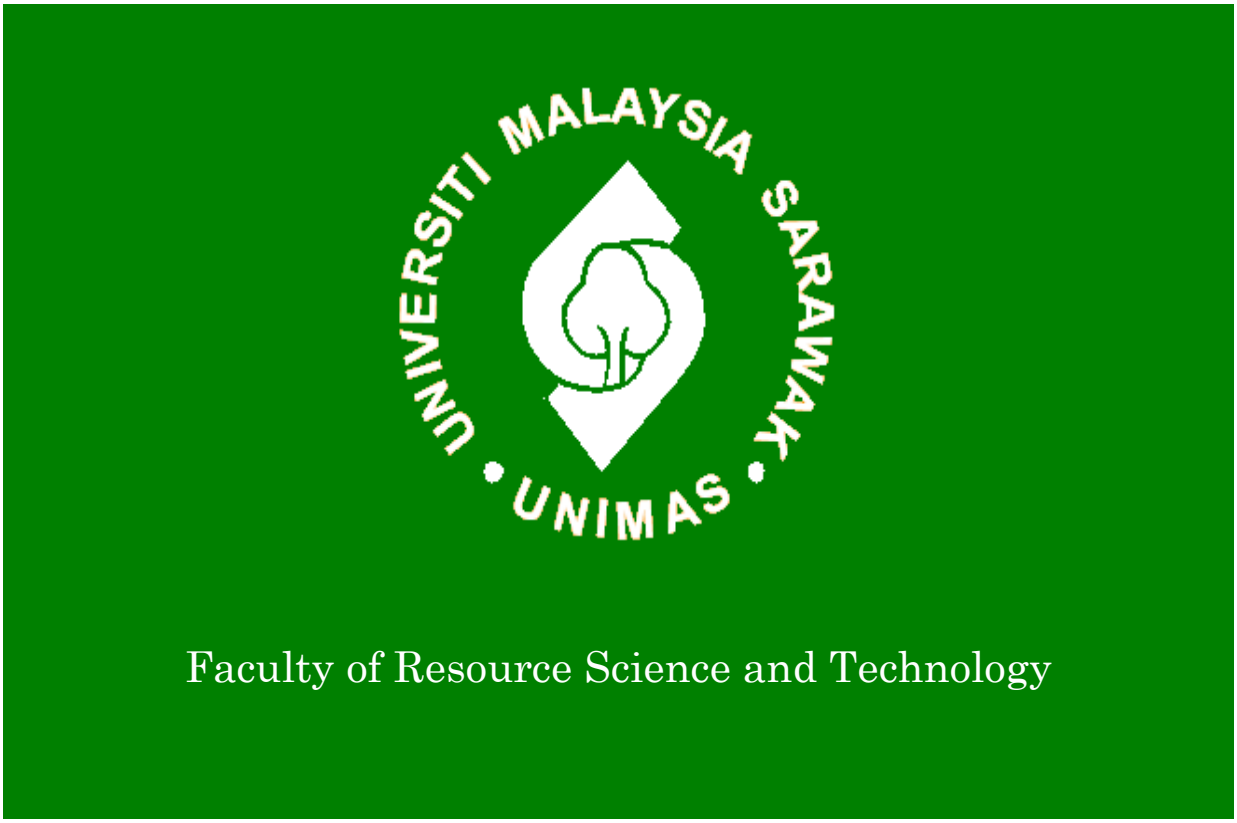

Characterization and Expression Studies of Grb10a Genes in Zebrafish

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Bachelor of Science with Honours
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LIST OF ABBREVIATIONS

Grb10	Growth factor receptor bound protein
PH	Pleckstrin Homology
BPS	Between PH and SH2
SH2	Src homology 2
IGF-IR	Insulin-like growth factor 1 receptor
EGF	Epidermal Growth Factor
PDGF	Platelet-Derived Growth Factor
EphB2/ELK	Ephrin Receptor
IR	Insulin Receptor
SRS	Silver-Russell syndrome
IRK	Tyrosine Kinase Domain of the IR
PI3-K	Phosphatidylinositol 3-kinase
Jak 2	Janus Kinase 2
Bcr-AbI	Breakpoint Cluster Region-Ab
RT-PCR	Reverse Transcription Polymerase Chain Reaction

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Characterization and Expression Studies of Grb10a Gene in Zebrafish

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ABSTRACT

Grb10 is the member of super family of adaptor proteins which is characterized by the presence of proline rich region, PH domain, BPS region and SH2 domain. Several studies reported a role of Grb10 in regulating the metabolic and mitogenic responses to insulin and IGF-I. Two Grb10 genes namely Grb10a and Grb10b were found in the zebrafish due to duplication. Total RNA was extracted from 10 different organs (eyes, brain, gills, heart, liver, stomach, intestine, muscle, skin and bone) of zebrafish. The extracted RNA is then used to synthesize cDNA template to perform RT-PCR. Interestingly, Grb10a is expressed in all the isolated organs of zebrafish and this expression patterns were similar to the mammalian expression based on the expression profiles.

Key words: Grb10, Grb10a, expression, evolutionary conserved.

ABSTRAK

Grb10 merupakan ahli keluarga bagi super adaptor protein yang dicirikan dengan adanya prolin domain yang banyak, PH domain, BPS domain dan SH2 domain. Beberapa laporan telah menunjukkan peranan Grb10 dalam pengaturan metabolisme dan mitogenik bertindak balas terhadap insulin dan IGF-I. Dua gen Grb10 iaitu Grb10a dan Grb10b telah dijumpai dalam ikan zebra yang dipercayai berasal daripada duplikasi. RNA diekstrak daripada 10 organ yang berbeza (mata, otak, insang, jantung, hati, perut, usus, otot, kulit dan tulang) daripada ikan zebra. RNA yang diekstrakkan kemudian digunakan untuk mensintesis cDNA untuk melakukan RT-PCR. Menariknya, Grb10a mengekspres dalam semua organ yang diasingkan dari zebra ikan dan pola-pola ekspresi ini sama dengan mammalian ekspresi yang dilaporkan.

Kata kunci: Grb10, Grb10a, , ekspresi, evolusi dikekalkan.

CHAPTER I

INTRODUCTION

The growth factor receptor-bound protein 10 (Grb10) belongs to a small family adaptor proteins including Grb7, Grb14, and Mig10 (a protein of *Caenorhabditis elegans*). This family of proteins are adaptor proteins which contain an NH_2-terminal proline-rich region, a Ras-associated-like domain, a pleckstrin homology (PH) domain in the central region, a BPS (between PH and SH2) domain, and a C-terminal Src homology 2 (SH2) domain (except Mig10) (Stein *et al*., 2001).

According to Hitchin *et al*. (2001), the human and murine Grb10 gene is highly conserved, with 88 and 99% amino acid similarity in the PH and SH2 domains respectively. The remaining segments of the two proteins showed 70-99% similarity. Four Grb10 isoforms have been identified in human which denoted as hGrb10α, hGrb10β (or Grb-IRSV1), hGrb10γ, and hGrb10δ (Figure 1.1). hGRB10α has an incomplete PH domain with a 46-amino acid deletion due to exclusion of exon 7A. hGRB10β has a short amino terminus and complete PH domain. hGRB10γ has the same amino terminus as hGRB10 α, but with a full-length PH domain while the hGRB10δ cDNA encodes a unique amino terminus and full PH domain.

All of these isoforms are different at the amino-terminus and the PH domain due to alternative splicing events (Hitchin *et al*., 2001). In murine, Grb10 gene is located on the proximal arm of chromosome 11 with two major isoforms which are mGrb10α and mGrb10δ (Lim *et al*., 2004). The difference between mGrb10α and mGrb10δ is mGrb10δ

lacks exon 5 and resulting in the absence of 25 amino acids located between the proline-rich region and the PH domain (Figure 1.2).

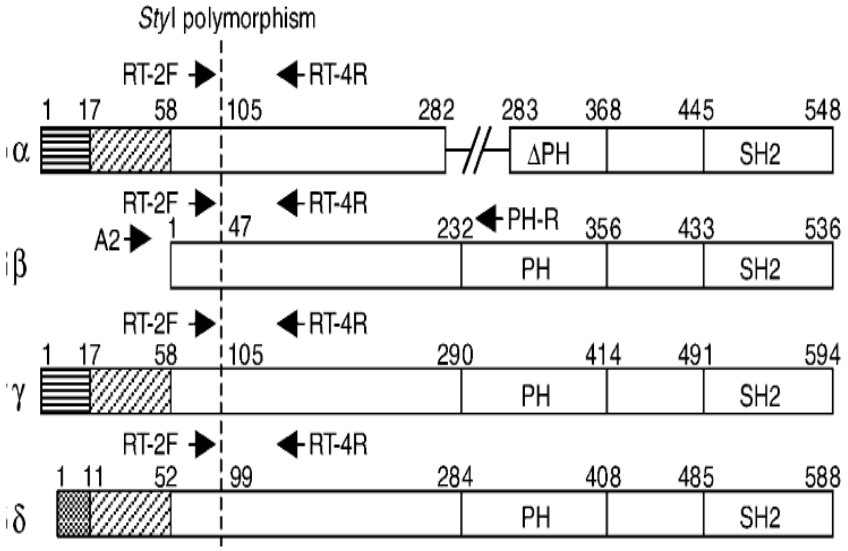

Figure 1.1 Grb10 protein isoforms in human. Schematic of the four known hGrb10 isoforms aligned according to regions of similarity (Adopted from Hitchin *et al*., 2001).

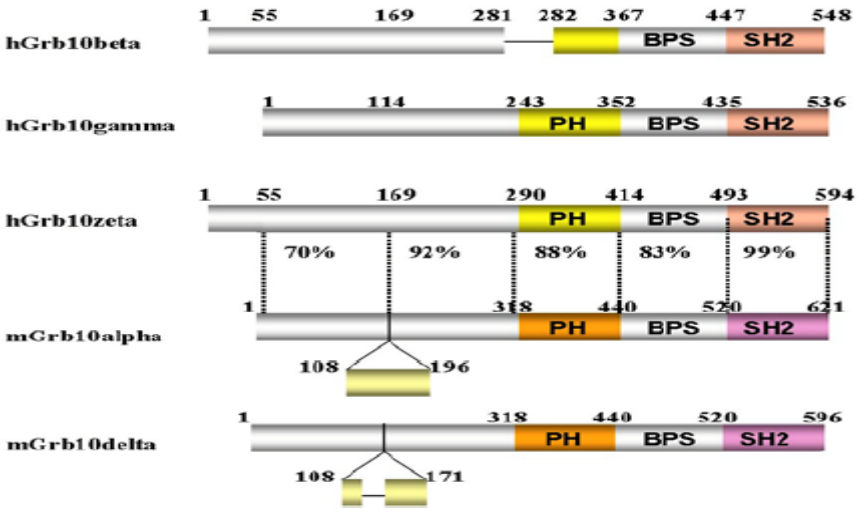

Figure 1.2 Schematic representation of the different Grb10 isoforms. The scale is different for each protein in order to facilitate homology comparisons of the different domains between the human and mouse splice variants (Adopted from Lim *et al*., 2004).

Although many studies have been done on the Grb10 gene, the role or function of Grb10 remains controversial. Interestingly, a role of Grb10 in regulating the metabolic and mitogenic responses to insulin and IGF-I is mostly discussed in various reports. Grb10 gene has been shown to play positive and negative role in insulin and IGF-I signaling (Lim *et al*., 2004). Several researches have also indicated that Grb10 is an imprinting gene which controls the embryonic growth and development or post-natal development as disruption of maternally transmission of Grb10 cause overgrowth of embryo and placenta in mice (Charalambous *et al*., 2003). Besides, Grb10 imprinting and SRS disease in human also has been predicted to be closely related (Hitchins *et al*, 2001).

There are two copies of Grb10 gene in zebrafish which denotes as Grb10a and Grb10b (Lee, 2008). These genes might be originated from a gene duplication event during the evolution either through subfunctionalization or neofuncionalization process which can be explained by the duplication-degeneration-complementation hypothesis (DDC). In subfunctionalization process, it is argued that the ancestral Grb10 gene is partitioned between duplicate Grb10a and Grb10b. Thus, both of the Grb10a and Grb10b might be complement each other to establish the gene expression throughout evolution. However, in the neofunctionalization hypothesis, it is believed that either one of the duplicated Grb10 retains the ancestral function of Grb10 while another copy acquires a new functions (Force *et al*., 1999).

The objectives of this study are to extract and purify total RNA of 10 different organs from zebrafish for cDNA synthesis to perform RT-PCR. Besides, it is also aimed to study and compare the expression patterns of Grb10a gene in zebrafish with mammals.

CHAPTER II

LITERATURE REVIEW

2.1 Growth Factor Receptor-Bound Protein 10 (Grb10)

Grb10 was firstly discovered as a binding partner of the EGF receptor; however the function of Grb10 in EGF has not been established. It was followed with detection of cDNA encoding mouse Grb10 by using EGF receptor as a probe. Further research had identified human Grb10 as a cellular partner of the insulin receptor in a yeast-2-hybrid screen of a HeLa cells cDNA library by using the cytoplasmic domain of the insulin receptor as bait (Lim *et al*., 2004). Since then, the 4 isoforms of human Grb10 and 2 isoforms of mouse Grb10 were discovered and described the roles and functions of gene in different experiment strategies (Lim *et al*., 2004).

The Grb10 domains consist of proline-rich region, a Ras-associated-like (RA) domain, a PH domain, a BPS domain, and a SH2 domain. Each of these domains has specific function and thus determines the role of Grb10 gene. RA domain which locates between the proline-rich region and PH domain is identified through the sequence comparison analysis. Typically, RA domain has shown to mediate the association with members of the Ras superfamily GTPases. However, it is still unknown whether the RA domain in Grb10 promotes the binding to Ras superfamily GTpases (Poting & Benjamin, 1996). Grb10 family members contain either one or two additional proline-rich motifs (Lim *et al*., 2004). This proline-rich region has been shown to provide a binding site for SH3 and WW domain (Nantel *et al*., 1999; Wojcik *et al*., 1999). The WW domain is commonly found in protein with signaling or regulatory functions while SH3 domain is

associated with signal transduction and cytoskeletal proteins (Manser *et al*., 1997). Proline-rich domain also requires for the actions of Grb10 on receptor tyrosine kinase signaling which may involve the formation of a protein complex. The importance of proline-rich had been determined by using 3T3-L1 adipocyes overexpressiong full-length (FL-Grb10) and N-terminally truncated Grb10 (BPS-SH2). Overexpression of FL-Grb10 inhibited insulin-stimulated receptor autophosphorylation and glucose uptake while BPS-SH2 fragment of Grb10 does not show any effect. It also has been suggested that Grb10 involves in metabolic actions of insulin through the proline-rich domain (Mori *et al*, 2005). Moreover, yeast-2-hybrid screen had identified that proline-rich sequences provide interface for two Grb10 interacting proteins, GIGYF1 (Grb10 Interacting GYF Protein 1) and GIGYF2 (Reidel, 2004). It was predicted that GIGYF1 may cooperatively enhance the association between Grb10 and IGF-IR.

The PH domain is a structural protein module of approximately 100 amino acid which originally identified in the cytoskeletol protein pleckstrin (Lim *et al*., 2004). It has been reported to play important roles in protein-protein and protein-lipid interaction (Dong *et al*., 1997 & 1998). Research study showed that an intact PH domain enhances the association of Grb10 with the insulin and IGF-1 receptors, which is mediated by the BPS and SH2 domains (Lim *et al*., 2004). hGrb10α which lacks intact PH domain inhibited insulin-stimulated substrate tyrosine phophorylation and PI 3-kinase activation in cells (Dong *et al*. 1997). Although PH domain is reported to mediate protein-protein interaction, however according to Maffucci & Falasca (2001), the major role of this domain lies in the binding of phosphoinositides which are typically produced in response to activation of cell surface receptors, resulting in the recruitment of PH domain proteins to the plasma

membrane. Furthermore, PH domain is involved in hGrb10 dimerization by providing additional specificity for the interaction (Dong *et al*., 1998).

The crystal structure of Grb10 SH2 domain is dimeric in solution and consists of approximately 100 amino acids. It is represented by a core anti-parallel beta sheet flanked on both sides by an alpha helix and broadly resembles other SH2 domains. It has been shown to have an interaction with the autophosphorylated insulin receptor and insulin-like growth factor-1 receptor (Dong *et al*, 1998; Hansen *et al*., 1996). It also had been proven that full-length Grb10γ with an inactive SH2 domain is unable to co-immunoprecipitate detectable levels of the IR (Wick *et al*., 2003). However, this interaction is specific as Grb10 SH2 does not bind to an insulin receptor which lacks 43 amino acids at the carboxyl terminus (Hansen *et al*., 1996). Some studies have indicated that SH2 domain may act cooperatively with BPS domain to facilitate the interaction of Grb10 with the IR.

BPS region was termed as it is located between the PH and SH2 domain which is identified through mapping studies by using the yeast-2-hybrid system and in vitro protein interaction assays (He et al., 1998). BPS domain which has represented a major interface with the IGF-IR as it shares high sequence homology to the IR but not with the EGF receptor (Lim *et al*., 2004). The study demonstrated that binding of the BPS domain to IRK directly inhibits substrate phophorylation. Moreover, it also inhibited the catalytic activity of autophosphorylated IGF1R kinase (Stein *et al*., 2001). According to Wick *et al*. (2003), both of the BPS and SH2 domains together are necessary for disruption of interactions between IRS-1/2 and IR. BPS domain may mediate the interaction with the IR and the SH2 domain facilitates a physical disruption of IR/IRS interaction. Thus, it is strongly suggested

that the Grb10 involves in insulin signal transduction which is greatly influence the growth of the human and mouse.

In the mouse, Grb10 is highly expressed in a variety of muscle tissue including the face and trunk, the intercostals muscles, the diaphragm and cardiac muscle, and the tongue and the limb. In the brain, Grb10 expression is most abundant in the subependymal layers, in the meninges, and in the choroid, and in the choroid plexus. The liver, bronchioles, and cartilage of the atlas, ribs, and long bones all express high levels of Grb10. Grb10 expression is limited to the developing tubules and the mesenchyme in the kidney. Grb10 expression could also be detected in the adrenal gland and the pancreatic bud (Charalambous *et al*., 2003). On the other hands, Grb10 expression also has been found to occur in liver, lung, kidney, skeletal muscle, heart, spleen, skin, and brain in both human fetal and postnatal tissues (McCaan *et al*., 2001).

Besides that, studies also indicated that Grb10 played a positive or negative role in regulating insulin and IGF-I signaling. IR signaling primarily regulates metabolic functions including protein synthesis, glucose uptake and glycogen synthesis, whereas the IGF-I receptor signaling mediates growth and differentiation. Suppression of Grb10 expression in the cell increases the insulin receptor (IR) protein level. However, overexpression of Grb10 gene causes the reduction of the insulin receptor protein levels. It is a mechanism of negative regulation as the Grb10 inhibits the insulin receptor kinase activity by binding directly to the IR (Ramos et al., 2006). On the other hands, the study of Wang *et al*. (1999) showed that Grb10 function as a positive, stimulatory, mitogenic signaling adapter in insulin and IGF-I action.

Grb10 is an imprinted gene where the expression gene is determined by the parent of origin. The Grb10 gene has been identified as a maternally inherited and imprinted gene in mice that located on proximal chromosome 11. It has been proven by Charalambous *et al.* (2003) by designing *Grb10*Δ*2-4* with deletion of exon 2-4 which ablated the major adaptor-encoding transcript. Maternal transmission of *Grb10*Δ*2-4* enhances the growth of placental and embryo while paternal transmission of *Grb10*Δ*2-4* did not result in significant deviation from wild type weight. It demonstrated that maternally inherited of Grb10 plays its role as a growth suppressor.

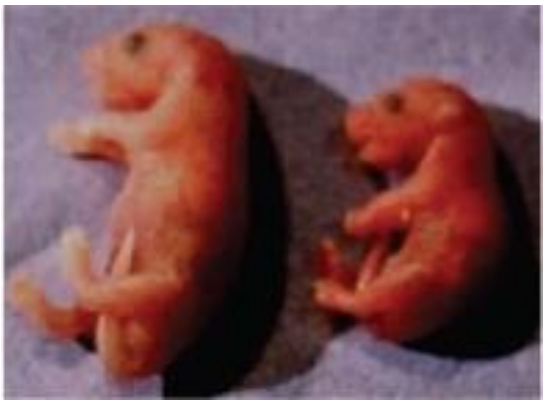

Figure 2.1 Crown-rump length. *Grb10*Δ*2-4*/+ (left) and wild-type (+/+, right) litter-mates on the day of birth (Adopted from Charalambous *et al*., 2003).

Grb10 has been associated with Silver-Russell syndrome (SRS). SRS is a malformation syndrome characterized by pre- and postnatal growth retardation with a relative sparing of cranial growth, a large prominent forehead and triangular-shaped face with down-turned corners of the mouth, clinodactyly and asymmetry of the head, trunk and limbs. SRS seems to be a genetically heterogeneous disorder as it has been shown both autosomal dominant and autosomal recessive patterns of inheritance (Wakeling *et al.*, 1998). As Grb10 maps to proximal 11p in mouse and maternal duplications of this region cause growth retardation, the localization of human Grb10 to chromosome 7 may be an excellent candidate for at least some of the features of SRS. This has been proven by

Hitchins *et al.* (2001), as maternal duplication of Grb10 in human chromosome 7p11.2-p13 causes pre- and postnatal growth restriction. However, McCaan *et al*. (2001) proposed that Grb10 is unlikely involved in SRS disease as Grb10 does not imprinting in growth cartilage which control the height of human. Therefore, the role of Grb10 in SRS remained to be investigated.

However, there is no evidence of imprinting have been shown in non-mammalian including zebrafish and this can be explained by the 'conflict theory' (Moore, 2001 & Moore & Haig, 1991). Moore & Haig (1991) proposed that imprinting only developed gradually in mammals and flowering plants because their offspring are nourished directly from maternal tissues. Zebrafish is external fertilization and does not nourished their offspring, thus no imprinting is involved.

2.2 Zebrafish (*Danio rerio*)

Zebrafish is a teleost fish of the cyprinidal family which belong to the class of ray-finned fishes (*Actinopterygii*) (Christiane *et al*., 2002). The binomial name of zebrafish is *Danio rerio* (Figure 2.2). The zebrafish is found to live in rivers of northern India, northen Pakistan, Nepal, and Bhutan in South Asia (Bopp *et al*., 2006). The zebrafish can grow to 3-5 centimetres in length and the generation time is approximately 3-4 months. It is omnivorous and the primary food is zooplankton, insects, and phytoplankton. The zebrafish genome is 1.7 gigabases in size and divided up into 25 linkage group (Christiane *et al*., 2002). However, the absolute number of genes in zebrafish still unknown. Recently, zebrafish has become a common model species for vertebrate development biology research. It is because the zebrafish embryo has many characteristics that make it suitable for development studyings.

Zebrafish is chosen for laboratory experiment due to the low maintenance cost, a robust reproduction cycle, a rapid life cycle and a large number of offspring can be produced. It is also easy to maintain a large number of zebrafish in a small area which is important for large-scale genetic and behavioral studies (Elkhayat, 2009). The development of zebrafish is very similar to the embryogenesis in higher veterbrates as it develops externally from transparent egg to adult. (Wixon, 2000). The embryos are completely transparent during the first 24 hours of development. Thus, the developing organ can be observed even deep inside living embryos. It also facilitated the characterization and expression study of specific gene in the embryos. Moreover, the rapid embryonic development of zebrafish also allows the common vertebrate specific body can be seen after 2 days (Christiane *et al*., 2002).

Due to the advantages such as its versatility, amenability to genetic and experimental manipulation, and because it possess a large number of duplicated genes, zebrafish can be used to provide insight into the process of functional gene evolution (Kamei *et al*., 2008).

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Figure 2.2 Adult wild type zebrafish (*Danio Rerio*) (Adopted from Lopez, 2008).

2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR (Reverse Transcription Polymerase Chain Reaction) is a very important test in the field of gene expression and expression diagnostics because it gives researchers a mechanism to test whether any specific gene is active or inactive (Rudra, 2002). It is a rapid and sensitive method for analyzing gene expression to determine the presence or absence of transcripts and for producing cDNA for cloning. RT-PCR also is a laboratory technique used in molecular biology for amplifying a defined piece of a RNA molecule. It is the most sensitive technique for mRNA detection and semi-quantitation which is currently available as even a very low copy number of RNA molecules also can be detected through the exponential amplification.

The first step in RT-PCR is to use reverse transcriptase and primers to anneal and extend a desired RNA. RNA is first reverse transcribed into cDNA by using a reverse transcriptase. This step is very important as the DNA polymerase only can act on DNA templates. The next step involves the denaturation of the double strands DNA at 95ºC so that the double strands DNA are separated and the primers can bind again at lower temperature to begin a new chain reaction. After denaturation is the annealing process. The annealing temperature can be varying depend on the set of primers used. The optimal annealing temperature is the melting temperature of the primers which is depends directly on length and composition of the primers. The final step of RT-PCR is DNA extension from the primers by using thermostable Taq DNA polymerase at 72ºC. The numbers of cycles are controlled by a programmable thermal cycler. Finally, the PCR product is detected by using agarose gel electrophoresis for further analysis.

CHAPTER III

MATERIALS AND METHODS

3.1 Primer design and sequences

The primers used in the project were designed by using two different programmes. Suitable primer pairs for PCR amplification were suggested by Primer3 (http://waldo.wi.mit.edu/cgi-bin/primer3.cgi/primer3www.cgi). All suitable primer pairs suggested by Primer3 were then analysed for hairpin, palindromes, dimmers and melting temperature (Tm) with a second programme, NetPrimer (http://www.premierbiosoft.com/netprimer/netprimer.html). Primers with a rating of at least 85 (out of 100; Netprimer) were selected.

3.2 Isolation of Total RNA using Tri Reagent

Total RNA from 10 isolated organs (eyes, brain, gills, heart, liver, stomach, intestine, muscle, bone and skin) were extracted using Tri Reagent. The organs were placed in a 2 ml eppendoff tube and followed by 1 ml of Tri Reagent. The organs were minced by using the blade and transferred to the tubes which contain Tri Reagent. The homogenate were stored for 10 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, the homogenate were centrifuged at 12,000 rpm for 10 minutes at 4ºC. After centrifugation, 200 µl of chloroform was added. The samples were then shaken for 15 minutes and left at room temperature for approximately 5 minutes. The samples were then centrifuged at 12,000 rpm for 15 minutes and the upper phase was transferred to a new tube. 500 µl of total isopropanol was nixed with each sample and stored at room temperature for 10 minutes to precipitate RNA. To precipitate the RNA, the samples were

centrifuged at 12,000 rpm for 10 minutes. The RNA pellet was then washed with 1ml of 70% ethanol and centrifuged again at 7,500 rpm for 5 minutes. The supernatant was discarded and the RNA pellet was air-dried for 3-5 minutes before addition of 10 µl of Nuclease-Free Water. To visualize the RNA, 2 µl of the solution was run on a 1% agarose gel at 120V for 20-30 minutes.

3.3 Agarose gel electrophoresis

A 1% agarose gel was prepared by adding 0.5g of agarose powder into 50 ml 1 × TAE buffer and heated up in a microwave at full power for 2 minutes to dissolve the agarose. The gel solution was then cooled to approximately 60ºC and 2.5 µl of ethidium bromide (10 mg/ml was added and the gel was poured into to a gel mould. 2 µl of loading dye (6×) and 6 µl of distilled water were added for every 2 µl of sample. The gel was loaded by using a pipette. After that, the gels were run at 120V for approximately 30 minutes in 1 × TAE buffer, and examined using an uv illuminator.

3.4 Purification of RNA using Phenol/Chloroform/Isoamyl alcohol Extraction

492 µl of autoclaved water was added to the 8 µl of RNA solution followed by 500 µl of phenol/chloroform/isoamyl alcohol. The mixture was mixed well by vortexing and then centrifuged at 12,000 rpm for 5 minutes. The upper aqueous supernatant was transferred to a new tube and mixed with 400 µl of chloroform followed by centrifugation at 12,000 rpm for 5 minutes. After centrifugation, the upper aqueous layer was transferred to a new tube again. RNA samples were precipitated by adding 30 µl of 3M sodium acetate and 60 µl of cold absolute ethanol. Then, the samples were left at -80ºC for an hour. The RNA was then recovered by centrifugation at 12,000 rpm for 15 minutes at 4ºC. The RNA pellet was washed with 1 ml of cold 70% ethanol and centrifuged again at 12,000 rpm for 5 minutes.

The supernatant was removed and the pellets were left to dry at room temperature and resuspend in 10 µl of distilled Nuclease-Free Water.

3.5 Quantitation of RNA

For spectrophotometric measurement, 2 µl of DNA aliquots was diluted with 68 µl of distilled water in the cuvette and quantified using spectrophotometer. The absorbance values of 260 nm, 280 nm and 320 nm were recorded. The purity of the samples was determined by calculating the ratio of $A_{260\text{-}320/A280\text{-}320}$ and the concentration of RNA was calculated as show below:

$$\textbf{RNA (µg/µl)} = [A_{260} \times (40\ \text{µg RNA/ml})/(1\ A_{260}\ \text{unit}) \times (\text{dilution factor})] / 1000$$

3.6 cDNA Synthesis

To synthesis first-strand cDNA for RT-PCR, ImProm-II™ Reverse Transcription System (Promega) was used according to the manufacturer instructions. 2 µl RNA and 1 µl random primer (0.5 µg/reaction) were mixed with Nuclease-Free Water to a final volume of 5µl per RT reaction. 1.2kb Kanamycin Positive Control RNA (1 µl) and oligo$(dT)_{15}$ Primer (0.5 µg/reaction) were used as positive control. The mixtures were incubated in ESCO Swift MiniPro PCR machine at 70 ºC for 5 minutes and immediately chill on ice for at least 5 minutes. After incubation, 15 µl of reverse transcription reaction aliquots were prepared and mixed with 5 µl of primer mix to a final volume of 20 µl per tube. The reverse transcription reaction mix were prepared and the tubes were incubated for 5 minutes at 25 ºC for annealing, followed by extension at 42 ºC for 1 hour. Inactivation of reverse transcriptase was carried out at 70 ºC for 15 minutes.

Table 3.1. cDNA synthesis reaction conditions

Experiment Reaction	**×1**	**Master Mix (×12)**
Nuclease-Free Water (to a final volume of 15 µl)	6.35 µl	76.20 µl
ImProm-II™ 5X Reaction Buffer	4.00 µl	48.00 µl
$MgCl_2$ (final concentration 4 mM)	2.40 µl	28.80 µl
dNTP Mix (final concentration 0.5 mM)	0.75 µl	9.00 µl
Recombinant RNasin® Ribonuclease Inhibitor	0.50 µl	6.00 µl
ImProm-II™ Reverve Transcriptase	1.00 µl	12.00 µl
Final volume	15.00 µl	180.00 µl

Table 3.2. Positive control for cDNA synthesis reaction conditions

Positive-Control	**×1**
Nuclease-Free Water (to a final volume of 15 µl)	5.15 µl
ImProm-II™ 5X Reaction Buffer	4.00 µl
$MgCl_2$ (final concentration 6 mM)	3.60 µl
dNTP Mix (final concentration 0.5 mM)	0.75 µl
Recombinant RNasin® Ribonuclease Inhibitor	0.50 µl
ImProm-II™ Reverve Transcriptase	1.00 µl
Final volume	15.00 µl