MOLECULAR CLONING AND EXPRESSION STUDIES OF GROWTH FACTOR RECEPTOR BOUND PROTEIN 14 (Grb14) GENE IN ZEBRAFISH DEVELOPMENT

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Molecular Cloning and Expression Studies of Growth Factor Receptor Bound Protein 14 (Grb14) gene in Zebrafish Development

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This thesis project was submitted in partial fulfilment of the requirement for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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2011
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DECLARATION

I hereby declare this thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was done under the guidance of Dr. Lee Kui Soon, of Department of Molecular Biology, Faculty of Science and Technology, University Malaysia Sarawak.

Signature: ..................................

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LIST OF ABBREVIATIONS

BPS (Between PHand SH2)
EGFR (Epidermal growth factor receptor)
FGFR (Fibroblast growth factor receptor)
Grb14 (Growth factor receptor bound protein 14)
IR (Insulin receptor)
PIR (Phosphorylated insulin receptor interacting region)
IGF-IR (Insulin-like Growth Factor Receptor)
PDGFR (Platelet-derived growth factor receptor)
RTK (Receptor tyrosine kinase)
SH2 (Src homology 2)
GM (Grb/Mig region)
PH (Pleckstrin homology domain)
CORT (Cloning of receptor target)
ZIP (Zeta interacting protein)
PCR (Polymerase chain reaction)
RNA (Ribonucleic acid)
cDNA (Complementary deoxyribonucleic acid)
AGE (Agarose gel electrophoresis)
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Molecular Cloning and Expression Studies of Growth Factor Receptor Bound Protein 14 (Grb14) gene in Zebrafish Development

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ABSTRACT

Grb14 was the most recently identified members of Grb7 superfamily of adaptor protein, which participate in the functionality of multiple signal transduction pathways under the control of a variety of activated tyrosine kinase receptors (RTK). To date, the functional roles of Grb14 adaptors is not fully understood. At the present time, a role for this Grb14-RTK interaction was only established in the regulatory effect on RTK signaling especially in insulin receptor signaling inhibition as well as cell migration and tumor progression. In respect that most of the current knowledge and understanding of Grb14 expression come from murine studies, therefore, in this project the expression studies of Grb14 will be determined from 10 different developmental stages in Zebrafish (Danio rerio) which comprise of 4-cells, 3hpf, 5hpf, 11hpf, 18hpf, 24hpf, 36hpf, 48hpf, 3dpf and 5dpf. From Reverse Transcription (RT-PCR), the expression degree from each respective stage will allowed us to comprehend when or which developmental stage that Grb14 started to be expressed and how strong the expression degree in distinct developmental stage. Based on the RT-PCR semi-quantitative analysis result of gene expression in this study, Grb14 was expressed in all the embryonic developmental stages of zebrafish with dissimilitude in expression degree. Beside that, direct sequencing result from BLAST showed that the nucleotide sequences exhibit 99% of homology similarity from Grb14 NCBI database. Later on, Grb14 which had been successfully cloned can be used in assisting in future functional studies of Grb14 using in situ hybridisation followed by gene known down approach.

Keywords: Grb14, Zebrafish (Danio rerio), molecular cloning and gene expression.
CHAPTER 1 INTRODUCTION

The growth factor receptor-bound protein 14, Grb14 was the latest described member of the Grb7 superfamily of adaptors protein that comprises of Grb7, Grb10 and Grb14. It was cloned initially using the CORT (cloning of receptor target) by interacting with EGFR. The study of Daly, Sanderson, and Sytherland (1996) proposed that Grb14 was identified in a screen of a breast epithelial cDNA library. Structurally, Grb14 contain a C-terminal Src homology 2 (SH2) domain, a central region Pleckstrin homology (PH) domain, a putative RA (Ras associating) domain, a BPS (between PH and SH2) domain and lastly a N-terminal (Kasus-Jacobi et al., 2000).

The studies of Baker, Sutherland and Daly (1996) discovered that human Grb14 gene is localized on chromosome 2q22-24 close to ErB4 family by fluorescent in situ hybridization. According to the newly adopted nomenclature, there are three variants of Grb14 which differ from their species of origin including hGrb14, rGrb14 and mGrb14 (Daly, 1998). Peptide sequence alignment showed that human and rat or mouse partake 83% sequence identity as most conserved region is found in the C terminus of the protein.

Currently, Grb14 has been described in several vertebrate species including human (Holt and Daly, 2005), rat (Kasus-Jacobi et al., 1998) and mouse (Reilly et al., 2000). In rat, Northern and Western blot analysis revealed that Grb14 is specifically expressed in insulin sensitive tissue such as skeletal muscle, liver, pancreas and kidney (Kasus-Jacobi et al., 1998). Expression of Grb14 can be detected in human in the same organ mentioned above, however with some degree of difference. It is noteworthy to notice that Grb14 seems to be preferentially expressed in insulin target tissues such as liver, skeletal muscle, white adipose tissue and insulin sensitive organs like pancreas and brain. Grb14 also personate its roles in tumor progression as it is found to be expressed in human cell lines including those derived
from normal breast epithelial strains breast cancer and prostate cancer (Cariou et al., 2004; Daly et al., 1996). Expression of Grb14 in these human cell lines might underscore its potential implication in cancer biology. However, the functional role of this protein still remains to be elucidated.

Grb14 binds to various receptor tyrosine kinases (RTKs) under ligand induction, for instance insulin receptor (IR) and fibroblast growth factor receptor (FGFR). Several studies validate that Grb14 play a role in regulating insulin signaling. Conspicuous studies by Kasus-Jacobi et al., 2000 showed that binding of Grb14 to the Insulin tyrosine kinase loop may alter the catalytic activity of the receptor thus resulting in inhibition of insulin signaling. Inhibitory action on the IR kinase activity is mainly mediated by BPS domain of Grb14. Numerous researches proved that Grb14 plays role in insulin signalling. Besides that, there were research reported that Grb14 may plays role in cell migration as well as tumor progression but the functional role of this gene remains to be clarified.

The main objectives of this study are:

(i) To extract total RNA of 10 different embryonic developmental stages from zebrafish for cDNA synthesis to perform Reverse Transcription (RT)-PCR.

(ii) To establish, detect and study the semi-quantitative expression analysis of Grb14 at different developmental stage using RT-PCR.

(iii) To clone the Grb14 gene for the used for in situ hybridization on whole mount zebrafish embryo in the coming future.

Consequently, expression studies in this project is promising to provide some hint to the biological function of the Grb14 which is crucial in assisting in future functional studies of Grb14 using in situ hybridisation followed by gene known down approach.
2.1 Growth factor receptor-bound protein (Grb14)

2.1 (a) Structure of Grb14 and its tissue distribution

Grb14 is the latest described member of Grb7 family of adaptors that include Grb7, Grb10, and Grb14. Adaptors protein generally lack enzymatic activity, but play an important role in multiple signal transduction pathways in the cells. They function by bringing together in proper conformation manner, different components of given signaling pathways to achieve their correct functionality in transmitting signals along defined routes. These proteins shared a series of conserved domain involved in protein-protein and protein lipid interactions: an amino terminal proline rich region, a C terminal SH2 domain, a central Gm region containing a RA, a PH domain, and a newly described PIR region (Kasus-Jacobi et al., 1998).

Figure 1: Schematic representation of Grb14 primary structure. (GM: Grb and Mig region, PP: proline rich conserved motif, RA: Ras homology domain, PH: Plecktrin homology domain, SH2: Src-homology domain, PIR/BPS: phosphorylated insulin receptor interacting region, also known as between PH and SH2 domain, IR: insulin receptor, FGFR: fibroblast growth factor receptor, EGFR: epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptor; ZIP: protein kinase C zeta interacting protein.) (Adapted from Frontiers in Bioscience 9)

Using fluorescent in-situ hybridization, the Grb14 gene was localized in the long arm of human chromosome 2, more precisely in 2q22-q24 locus. The human Grb14 mRNA is highly expressed in liver, kidney, pancreas, ovary, testis, heart and skeletal muscles and it is also
very abundantly present in kidney embryonic cells, some prostate cancer cell lines and breast cancer cells. On the other hand, to date there is no in situ hybridization data available for the Grb14 expression in either mouse or rat, however Northern and Western analysis revealed that Grb14 was expressed in the liver, skeletal muscle, heart, white adipose tissue pancrease and brain (Kasus-Jacobi et al., 1998).

2.1 (b) Potential roles of Grb14 in insulin receptor catalytic activity

Grb14 binds to various receptor tyrosine kinases (RTKs) under ligand induction. Interaction that involved BPS/PIR domain is likely the determinant in the specificity action of Grb14 as BPS/PIR as the main interacting region. Coincidentally, PIR is also the responsible domain that allowed the binding of insulin like growth factor-1 receptor (IGF-IR) to Grb14 (Bereziat et al., 2002). On the other hand, research conducted by Kasus-Jacobi et al., (1998) pointed out that SH2 domain may involved but do not play a crucial role to bind with insulin receptor.

According to Bereziat et al., (2002) and Reilly et al. (2000), Grb14 exerts its functional roles upon its association with the insulin and thereby inhibiting insulin receptor catalytic activity. Uniquely, Grb14 binds to the phosphorylated tyrosine kinase loop that is responsible in the transmission of insulin signal by maintaining it in an inactive conformation that directly decreases the substrate phosphorylation. Nevertheless, Grb7 and Grb10 inhibition on the IR catalytic activity is less effective as compared to Grb14 (Cariou et al., 2004).

The most compelling evidence on the role of Grb14 in insulin signaling was provided by the Grb14 gene knockdown experiment performed on mice. Interestingly, the Grb14 deficient mice whose Grb14 gene had been knockdown exhibit improved glucose tolerance and a better insulin signaling glucose homeostasis. In support of this statement, in ex vivo studies,
insulin–induced 2-deoxyglucose uptake was enhanced in soleus muscle, yet not in adipose tissue (Cooney et al., 2004). This metabolic effect highlights that Grb14 is an important tissue-specific inhibitor of insulin signaling. In future prospect, Grb14 may appear as a new target for anti-diabetic drugs subsequently to Type 2 diabetic as could disrupt the association of Grb14 with IR.

Moreover, another area of future interest will be to identify new downstream partners of Grb14 beside tankyrase 2 and PKC-zeta interaction protein. As an adaptor protein, it is likely that Grb14 will interact with its other conserved domains such as the PH domain or the proline rich region. Through this, the identification of these Grb14 binding partner will be of considerable interest to fully understand the function of this protein.

2.1 (c) Potential roles of Grb14 in cell migration and tumor metastasis

The central region of similarity, shared with *Caenorhabditis elegans* gene product, Mig10 which participate in migration of neuronal cells is almost 60% identical between Grb7, Grb10 and Grb14. This provides a hint that Grb7 families may play a role in cell migration such as tumor progression (Han et al., 2001). With this inhibition effect of Grb14 on fibroblast growth factor receptor (FGFR) which associated in estrogen-independent breast cancer cell growth, MDA-MB-231 cell line had been performed on Xenopus oocyte in an anti-cancer research. The result of this experiment denotes that Grb14 applied its inhibition effect by blocking the PLCγ activation with it bind to Y766 FGFR site (Cailliau et al., 2005). Forasmuch, research in Grb14 is avail in pharmacological for the inhibition of FGFR receptors which are associated in the estrogen-independent breast cancer cell growth, MDA-MB-231 cell line.
2.2 Zebrafish (*Danio rerio*)

Zebrafish (*Danio rerio*) belong to the family of *Cyprinidae* of order Teleostri (bony fish) are mostly found in the tropical fresh water between 20-28°C. Physically, males fish prone to be slimmer build than females and richer in colour with a slight yellow or red undertone in the anal fin where interestingly adult female could lay up to 300 transparent eggs as larvae hatch at approximately 2 days post fertilization. Zebra fish become a popular animal model for scientific studies by playing its significant role in toxicology, developmental and genetics studies. It is widely used because the fertilization process and all subsequent developmental events occur outside the mother making all of these events easily accessible for study. Besides that, the transparent embryos provide the possibility to visualize cells directly in the living embryo as well as for *in situ* mRNA hybridization analysis in the whole embryos.

Besides that, embryonic development is rapid as well. Because of their relatively short reproductive cycle, the large number of progeny that can be produced, and the relatively small space needed to maintain large numbers of offspring, the zebrafish is an efficient vertebrate model system for genetic analysis. Besides that, it is easy to get and easy to rear. They also have far lesser cells in their organ to fulfill the equivalent function compared to other higher vertebrates. Rapid embryonic development in zebrafish provides the opportunity
to follow the entire course of development of the same cell from birth through to the expression of its differentiated phenotypes.

Zebrafish are also useful in genetic studies. Although its generation time is about 2-4 months which are quite long, but the large number of progeny greatly facilitates genetic analyses (Eisen and Weston, 1993). Zebrafish and humans share much of the same genetic material. So, by studying the development of zebrafish from its egg to its adult stage, it can give light on human development, especially during human’s initial developmental stages. In addition, the embryo has a few cells that are relative to the embryo of many classes of vertebrate, causing it possible to recognize individual cells as well as to study the same identified cell in many different individual.

In addition, there was experiment been done on the transparent zebrafish embryos that screened for defects in overall embryonic pattern, morphogenesis or organ formation, these screens have identified a substantial number of mutations that affect the formation of organ systems, including defects in the nervous system, skeletal muscle, craniofacial region, kidney and endocrine organs, cardiovascular and gastrointestinal systems. It is likely that many of these mutations affect genes which are also relevant and relational to human development and disease processes such as cancer and neurodegenerative diseases. The zebrafish offers the opportunity of using classical genetics to define gene functions.

Last but not least, gene knockdown using reagent such as morpholinos (modified anti-sense oligonucleotide) has also widely used to study the function of individual genes of interest in expression study (Froese and Pauly, 1996).
CHAPTER 3 MATERIAL AND METHODS

3.1 Primer design and sequences

All the primer in this project was designed according to Lee (2008) as dominating guideline by using two different programmes. Firstly, suitable primer pairs for PCR amplification were suggested by Primer3 (http://waldo.wi.mit.edu/cgi-bin/primer3.cgi/primer3www.cgi). Then the primer pairs were construing for hairpin, palindromes, dimmers and melting temperature (Tm) using NetPrimer as second programme(http://www.premierbiosoft.com/netprimer/netprimer.html). Primers with a rating of at least 85(out of 100, Netprimer) were selected.

3.2 Zebrafish crossing, breeding and developmental stages observing.

Zebrafish will mate and deposit fertilized eggs on the bottom of the tank in the morning when light turn on. The evening before that, several egg bearing females and male zebrafish were selected and separated solely using two plastic bottle and labeled with male and female.

Male and female fish were then mix together on the next morning in the ratio male to female (1:1) or male to female (1:2). The bottles should contain a layer of clean marbles or a divider to prevent the adult fish eating they own eggs. In succession to that, the fishes were exposed to light and after an hour or two, the female zebrafishelayed its precious eggs. The eggs were
collected using a tea stainer and washed with distilled water before putting them into an embryo medium.

Eggs were collected under the divider

The embryo eggs were then examined and observed under a dissecting microscope at low magnification. Healthy embryos were transfer into a clean Petri dish with embryo medium using a Pasteur pipette. The eggs with cloudy dark appearance or ruptured were discarded.Embryos were maintained at 28°C in an incubator.

3.2 Sample preparation

Zebrafish embryos were obtained from the fish facility in Animal Biotechnology Laboratory, Department of Science and Technology, Univeristy Malaysia Sarawak. A total number of 10 disparate developmental stages were selected for total RNA isolation. The developmental stages include 8-cells, 3hpf, 11hpf, 16hpf, 24hpf, 48hpf, 54hpf, 3dpf, 4dpf and 5dpf (Lee, 2008). The embryos were excavate and finely minced using gauge and syringe. Promptly, the homogenate were placed separately into eppendorf tubes with each tube containing Tri Reagent (Chomczynski, 1993; Chomczynski and Mackey, 1995) for extraction purposes.
3.3 Isolation of total RNA using Tri Reagent

The homogenate in eppendorf tubes were centrifuged at 12,000rpm for 10 minutes at 4°C. Supernatant are transferred to a new tube before 200μl of chloroform added. After that, the samples were centrifuged again at 12000rpm for 5 minutes and the upper phase was transferred to new tubes. 500μl of isopropanol was added and the samples were incubated at room temperature for 10 minutes to precipitate the RNA. To pellet the RNA, the samples were centrifuged at 12,000rpm for 10 minutes. The Supernatant was discarded and RNA pellet was washed with 70% ethanol and re-centrifuged at 7,500rpm for 5 minutes. Supernatant was discarded for last and the RNA pellet was air dried for 5 minutes before 10μl of Nuclease-Free water was added into the tubes.

3.4 Agarose gel electrophoresis on RNA isolated

For a 1% agarose gel, 1.0 g of agarose was added into 100ml of 1x TAE buffer. The mixture was heated up in a microwave for approximately 2 minutes until all the agarose powder was dissolved. The solution was then cooled to approximately 60°C and 2 μl of ethidium bromide was added. The solution was slowly poured into the gel tray. It was allowed to cool for 5-10 minutes before the gel was formed. 1μl of loading dye (6x) and 4μl of distilled water was added to 1μl of RNA sample to load the gel. The gel was run at 120V for approximately 30 minutes.

3.5 Quantification of RNA

For spectrophotometric determination of RNA purity and concentration, 2 μl of RNA aliquots for each sample 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 therefore measured and recorded.
The purity of the samples was determined by calculating the ratio of $A_{260-320}/A_{280-320}$ and the concentration of RNA was calculated as shown below:

\[
RNA (\mu g/\mu l) = \frac{A_{260} \times (40 \mu g \text{ RNA/ml})/(1 \text{ A}_{260} \text{ unit}) \times (\text{dilution factor})}{1000}
\]

3.6 cDNAsynthesis

cDNA synthesis for each respective stage RNA was carried out using SuperScript™ III First Strand Synthesis System (Invitrogen) according to manufacturer’s instruction. On ice, 1 µl of RNA and 1 µl of oligo(dT) were mixed in 3 µl of Nuclease-Free Water to a final volume of 5µl per RT reaction. The mixtures were incubated at 65 °C for 5 minutes using the Supercycler SC-200 KyratecPCR machine. After incubation, the mixtures were then chilled on ice immediately for at least 5 minutes. The tubes were kept on ice until the reverse transcription reaction mix was prepared. 15 µl of reverse transcription reaction aliquots were prepared as below:

Table 1: cDNA synthesis reaction components and volume of experiment samples

<table>
<thead>
<tr>
<th>Components</th>
<th>1X Reaction</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT buffer</td>
<td>2.0 µl</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>4.0 µl</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2.0 µl</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>RNaseOUT&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>1.0 µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Superscript&lt;sup&gt;TM&lt;/sup&gt; III RT</td>
<td>1.0 µl</td>
<td>—</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>5.0 µl</td>
<td>6.0 µl</td>
</tr>
<tr>
<td><strong>Final Volume</strong></td>
<td><strong>15.00 µl</strong></td>
<td><strong>15.00 µl</strong></td>
</tr>
</tbody>
</table>

15 µl of reverse transcription reaction aliquots prepared was then mixed with 5 µl of primer mix for a final reaction volume of 20 µl per tube. After that, the tubes were incubated for 50 minutes at 50 °C for extension. Next, the tubes were incubated at 85 °C for 5 minutes for
inactivation of reverse transcriptase. Finally, 1 μl of RNase H were added and let incubated for 20 minutes at 37°C. On the other hand, the 25μl of negative control were done for each respective stage by not adding SuperScript RT yet replaced by 1μl of nuclease free water. The cDNA synthesis reaction were stored at -20°C or used for PCR immediately.

3.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

PCR was carried out in order to amplify Grb14 cDNA fragment. A final volume of 25 μl of PCR mixture per tube was prepared by master mix. The below components the ingredients for PCR master mix except for the template DNA (cDNA). The components were combined in the order listed and were vortexed gently to mix. Grb14 primers (reverse and forward) were used for the master mix while beta actin (β-actin) control primer was used for the positive control.

Table 2: RT-PCR reaction conditions and volume for 1X reaction and master mix

<table>
<thead>
<tr>
<th>Components</th>
<th>1X reaction (Volume per 25 μl reaction)</th>
<th>Master Mix (X 24) (including negative RT control)</th>
</tr>
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<tbody>
<tr>
<td>10X PCR Buffer minus Mg</td>
<td>2.50 μl</td>
<td>60.00 μl</td>
</tr>
<tr>
<td>10 mM dNTP mixture</td>
<td>0.50 μl</td>
<td>12.00 μl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>0.75 μl</td>
<td>18.00 μl</td>
</tr>
<tr>
<td>1 μM Forward Primer</td>
<td>1.25 μl</td>
<td>30.00 μl</td>
</tr>
<tr>
<td>1 μM Reverse Primer</td>
<td>1.25 μl</td>
<td>30.00 μl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>0.50 μl</td>
<td>12.00 μl</td>
</tr>
<tr>
<td>MiliQ water</td>
<td>16.75 μl</td>
<td>402.00 μl</td>
</tr>
<tr>
<td>Template DNA (added separately for each tubes)</td>
<td>1.50 μl</td>
<td>36.00 μl</td>
</tr>
<tr>
<td><strong>Final Volume</strong></td>
<td><strong>25.00 μl</strong></td>
<td><strong>600.00 μl</strong></td>
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</table>
Amplification was carried out at 95 °C for 2 minutes and followed by 35 cycles of amplification at 94 °C for 30 seconds, 59 °C for 1 minute and 72 °C consecutively for 1 minute, ended by a 5 minutes final extension at 72 °C. Then, the RT-PCR products were separated on a 1% agarose gel electrophoresis using 5μl of each sample and 1μl of loading dye, followed by visualization of the products under UV transilluminator and photographed using detection system.

3.8 Recovery of PCR products from Agarose Gels

The PCR products DNA band of interest from 10 respective zebrafish embryonic development stages was cut from the gel and DNA were isolated using a Promega Wizard® SV Gel and PCR Clean-Up System kit according to the supplier instructions. Firstly, the gel was loaded and run using an established protocol. Then, a 1.5ml microcentrifuge tube for each DNA fragment to be isolated was weighted and the weight was recorded. The DNA was visualized and photographed using a long-wavelength UV lamp. Following that, the DNA fragment of interest was excised using a clean scalpel or razor blade. The gel slide was transferred to the weighted microcentrifuge tube and the weight was recorded. The weight of the empty tube was subtracted from the total weight to obtain the weight of the gel slice. Membrane binding solution was added at a ratio of 10μl of solution per 10mg of agarose gel slides. The mixtures were vortex and incubated at 65°C for 10 minutes or until the gel slide is completely dissolved. The dissolved gel mixture was transferred to the SV Minicolumn assembly and incubated for 1 minute at room temperature. The liquid in the collection tubes were discarded. The column was washed by adding 700μl of membrane wash solution to the minicolumn. Minicolumn was centrifuged for 1 minute. The liquid in collection tubes were emptied and wash was repeated by adding another 500μl of membrane wash solution and centrifuged at 14000rpm for 5 minutes.