Chapter 2 Materials and Methods

2.1. Construction of human *adiponectin* by overlap-extension PCR.

The coding region of the human *adiponectin* was divided into two exons. Three PCR runs were performed to construct the full length sequence of *adiponectin* with (*ADPs*) and without native signal peptide (*ADPws*). The components of each PCR reaction were 200μM of each dNTPs, 10μM of each forward and reveres primer, 150ng of DNA templates, 1X of polymerase specific buffer, 1.5mM of $Mg2^{+}$ and 1U of *Pfu* DNA polymerase (Fermentas, USA) or *Taq* polymerase (EuRX, Poland) according to the purpose of the PCR reaction .

2.1.1. Primers design

2.1.1.1. Primer design for expression of *adiponectin* in *P. pastoris*

The sequence of full length *adiponectin* was obtained from GenBank under the reference number NC_000003.11 (Sherer *et al.*, 1995). In order to construct the coding sequence for *ADPs* and *ADPws*, two sets of primers were designed to amplify exon 1. Forward primers included the endonuclease *Eco*RI restriction enzyme (GAATTC) to facilitate ligation in subsequent steps. The reverse primer of exon 1 contained six nucleotides that overlapped with the forward primer of exon 2. The reverse primer of exon 2 contains cleavage site of Factor Xa (TAACTTCCGGCG) to remove the poly histidine tag that use for purification by affinity chromatography. This primer also contains specific sequences (CGCCGGCG) that can recognize by endonuclease *Not*I restriction. The two restriction enzyme sites were inserted in primers to generate two sticky ends of *adiponectin* fragment that facilitate ligation with *P. pastoris* specific vector (Table 2.1).

2.1.1.2 Primer design for expression *adiponectin* in *E. coli*

In order to clone in *E. coli* specific vector, the *ADPws* sequence was used as a DNA template. Therefore, different primers were designed to amplify *adiponectin* fragments suitable to be cloned into *E. coli* specific vector. The forward primer started from the 5' end of the first codon of *ADPws*. However, the reverse primer started from the 3' end of *ADPws* that contains the sequence recognized by *Hind* III restriction enzyme (AAGCTT) (Table 2.2). All of these primers were synthesized commercially by Next Gene, Malaysia.

Table 2.1: Sequences of primers were used to express *adiponectin* in *P. pastoris.*

Primers	**Sequences**	**Remarks**
Exon1 forward with signal peptide	5'- AACGAGAATTCATGCTGTTGCTGGGAGCTGTTCT-3'	EcoRI site Tm°C= 68.0
Exon1 forward without signal peptide	5'-ATCGAGAATTCGGTCATGACCAGGAAACCACG-3'	EcoRI site Tm°C= 68.5
Exon1 reveres	5'-TAAGACCTGGATCTCCTTTCTCACCCT-3'	Tm°C=65.3
Exon2 forward	5'-ATCCAGGTCTTATTGGTCCTAAGGGAG-3'	Tm°C=64.0
Exon2 reveres	5'-TAATTGCGGCCGCGCGGCCTTCAATGTTGGTGTC-3'	NotI site Tm°C=73.6

Table 2.2: Sequences of primers were used to express *adiponectin* in *E. coli.*

Primer	**Sequence**	**Remarks**
Exon 1 forward	5'-ATGCTGTTGCTGGGAGCTGTTCTACTG-3'	Tm°C=65.3
Exon 2 revers	5'-TGCCGAAGCTTAATGTTGGTGTCATGG-3'	Hind III site Tm°C=64.4

2.1.2. PCR reactions

Normal PCR reaction was performed to construct exon one and exon two using human genomic DNA as template and the specific forward and reverse primers for each exon. The PCR conditions through 32 cycles were 95°C for 45s as denaturing step, 60°C for 45s as an annealing step and 72°C for 1min as an elongation step. The product of this reaction was exon 1 with the sequence of signal peptide, exon 1 without the sequence of signal peptide and exon 2. This product was purified by QIAquick Gel Extraction kit (Qiagen, USA) to eliminate any unspecific products.

The purified exon one and exon two were used in the next PCR reaction (overlap PCR) to construct the full length *adiponectin* gene. Each type of exon one was overlapped with exon 2 to get *ADPs* and *ADPws* sequences. The reaction conditions of an overlap-extention PCR through 10 cycles were denaturing step at 95°C for 45s, reannealing step at 60°C for 45s and elongating step at 72°C for 1min. In addition, the components of this reaction were dNTPs, *Pfu* polymerase and its buffer and exon 1 and exon 2. This reaction was carried out without primers and DNA template.

The product of the overlap PCR was used as a template in a new PCR reaction to amplify the full length of *ADPs* and *ADPws*. This reaction was carried out by forward primer of exon one and reverse primer for exon two and the polymerase enzyme was *Taq* polymerase. The PCR conditions through 32 cycles were 95°C for 45s as denaturing step, 60°C for 45s as an annealing step and 72°C for 1min as an elongation step.

Similar PCR reaction was performed by forward and reverse primers which were designed to express in *E. coli*. The polymerase enzyme was *Taq* polymerase and

PCR conditions through 32 cycles were 95°C for 45s as denaturing step, 60°C for 45s as an annealing step and 72°C for 1min as an elongation step.

2.2. Agarose gel electrophoresis

The expected size and approximate quantity for each of the previous PCR product were determined by carrying out agarose gel electrophoresis. The electrophoresis buffer consisted of 445mM Tris base, 445mM borate and 10mM EDTA called Tris-Borate EDTA (TBE). In order to prepare 1% agarose gel (Promega, USA), the TBE buffer was used to dissolve gel powder with heating in rotary oven. One microlitre of ethidium bromide was added after cooling and then the molten gel was poured into a tray. The wells in the top of the gel were made by fixing a suitable comb and the gel was allowed to solidify at room temperature for 20min. Samples were loaded into the wells after mixing with 6X loading dye prepared by mixing 0.25% bromophenol blue, 0.25% xylene cyanole FF and 30% glycerol. One of these wells was loaded with DNA marker (Promega, USA) to determine the expected size of DNA samples. Finally, the electrophoresis apparatus was installed and setup on 120 V for 30min. Alpha Imager system (Alpha Innotech Corp, USA) was used to document the gel and save gel data.

2.3. DNA extraction by gel extraction kit

QIAquick Gel Extraction Kit was used to extract DNA from agarose gel according to manufacturer's protocol (Qiagen, USA). Before using this kit, DNA samples were loaded into 1% agarose gel and the electrophoresis was performed as mentioned above (Section 2.2). Ethidium bromide was used to detect the specific bands

and after short exposure to ultraviolet (UV) illumination the specific bands were cut using sterile and sharp scalp. The gel slices were weighed and put into 1.5 ml microcentrifuge tube. The gel extraction buffer (QG buffer supplied with the kit) was added 3 v/w. After 10 min incubation at 50°C, one v/w isopropanol was added to the liquefied gel and the mixture was transferred to the spin column to spin at 13,000 rpm for 1min. The flow through was discarded and one v/w of QG buffer was added and centrifuged again at 13,000rpm for 1min. Next washing step was done by adding 750µl of 1:4 v/v of PE buffer (provided with kit) and ethanol and incubating for 2-5min followed by centrifugation at 13,000rpm for 1min. The flow through in the collection tube was discarded. Finally, 30µl distilled water was added into the spin column and incubated for 1min. The spin column was centrifuged for 1min to elute DNA into microcentrifuge tube. In order to confirm DNA extraction, another agarose gel electrophoresis was carried out using DNA marker to detect the molecular weight of the extracted DNA sample.

2.4. Adiponectin expression by *E. coli*

The pMAL™ Protein Fusion and Purification System (BioLabs, New England) was used to express human *adiponectin* (*ADPs* and *ADPws*) by *E. coli*. The protocols of transformation, expression and protein purification were based on instruction manual of this kit with some modification according to available materials and instruments.

2.4.1 Preparation of Luria-Bertani (LB) broth and agar media

Standard LB (Conda, Spain) was used to prepare LB broth (3.5% LB) which was poured in 10ml glass tubes and autoclaved on liquid cycle at 15lbs/sq.in., 121°C

for 20min. For preparation of agar medium, standard LB-agar (Conda, Spain) was used to prepare LB-agar plates (3.5% LB). After autoclave, the medium was cooled to 50°C and divided in two flasks, ampicilin (100μg/ml) was added to first flak and ampicilin (100μg/ml), X-Gal (80μg/ml) and IPTG (0.1mM) were added to the second flak. The two agar media was poured into Petri dishes and stored at 4°C until use.

2.4.2. Competent cells preparation of TB1 *E. coli* strain

The *E. coli* TB1 strain was provided with the kit (BioLabs, New England) and used for cloning. Other *E. coli* strains can be used for cloning which contain the *lacZΔM15* allele for α-complementation. A single colony of *E. coli* TB1 strain was inoculated into 10ml LB broth and grown overnight at 37°C, 250rpm. A subculture was carried out by inoculating 10ml of new LB broth with 100 μl of overnight culture and incubation at 37°C, with continuous shaking (220rpm) until OD_{600} was approximately in the range of 0.4 to 0.5. The early log phase bacteria was chilled in ice for 20min. and then centrifuged at 4°C, 2500*xg* for 20min. The cells was resuspended in 5ml of 0.1M $CaCl_2$ and incubated on ice for 20min. The resuspended cells were again centrifuged at 4°C, 2500*xg* for 20min and the supernatant was discarded. The bacterial cells were resuspended gently in 0.5 ml of 0.1M $CaCl_2$. The competent cells can either be used directly for transformation or stored at -80°C after adding 30% glycerol and a few seconds of cold shock treatment in liquid nitrogen or ice cold methanol.

2.4.3. Cloning of *adiponectin* into pMAL™ vector

The pMAL™-p4 vector was used to express *adiponectin* fragments (*ADPs* and *ADPws*) in *E. coli* periplasm. This vector (0.5μg) was digested with 10units of *Xmn* I

and 10 units of *Hind* III restriction enzymes (Promega, USA) at 37°C for 1 hour. At the same time, *adiponectin* PCR fragment (0.5µg) which was prepared for expression by *E. coli* (see Section 2.1.2) was digested with 10 units of *Hind* III restriction enzyme. Heat inactivation of the enzymes was applied by incubating reaction mixture at 65°C for 10min.

Agarose gel electrophoresis was used to check for complete digestion of the vector and *adiponectin* fragments. In addition, gel extraction kit (Qiagen, USA) was used to purify digested *adiponectin* fragments as describe previously (see Section 2.3). To purify the digested vector, first, EDTA was added to a final concentration of 20mM. In addition, an equal volume of a 1:1 phenol/chloroform mixture was added to the restriction digest mixed and removed the aqueous phase and placed in a fresh tube. This step was repeated with chloroform alone. The glycogen was added as carrier to digest, then 1/9th volume 3M sodium acetate was added, mixed and an equal volume isopropanol was added as well. Then, the mixture was incubated at room temperature for 10min, centrifuged and the pellet was rinsed and allowed to dry with 70% ethanol. Finally, each sample was resuspend in Tris-HCl/ EDTA buffer. The ligation mixture was prepared by adding digested vector and digested *adiponectin* fragment with DNA ligase and its suitable ligation buffer (New England Biolabs, UK). The ligation reaction (Table 2.3) was incubated overnight at 4°C.

Table 2.3: The components of the ligation mix.

Component	**Amount**
2X ligase buffer	5µl
DNA fragment	3µl
pMAL-p4 vector	1µl
Ligase enzyme	1µl
Total volume	**10µl**

2.4.4. Transformation into *E. coli*

Deep frozen competent cells were thawed in ice bath. A volume of 5µl of ligation mixture was added to 100µl of TB1competent cells and mixed gently. The mixture was incubated on ice for 30min and then transferred directly to water bath adjusted at 42°C to apply heat shock for 2 minutes. After heat shock, 900µl of LB broth was added and the cells were incubated at 37°C for 90min. Finally, 100µl of the cells were spread on an LB agar plate containing 100µg/ml ampicillin. The rest of cells culture was centrifuged and the medium discarded. The cell pellet was resuspended with 100µl of LB broth then, spread on an LB agar plate. After an overnight incubation at 37°C, normal PCR was used to detect the existence of *adiponectin* fragments in ampicillin resistant colonies.

2.4.5. Colony screening PCR

In order to select for recombinant transformants, overnight growing colonies were transferred by sterile loop to a gridded new LB-agar-ampicillin plate as a master library. Another library plate contains 100µg/ml ampicillin, 80µg/ml x-gal and 0.1mM IPTG was used to determine the *Lac* phenotype. After transferring each colony to the master library plate and the second library, the two libraries were incubated overnight and then the white colonies were determined in the second library and were used for colony screen PCR. The inoculation loop was rinsed into 0.5ml tube containing 50µl distilled water. The mixture was boiled at 100°C for 10min. and used as template (2µl) for PCR reaction. The reverse *adiponectin* primer and the forward primer of pMAL-p4 vector were used to detect the expected size and determine the correct orientation of the gene of interest.

2.4.6. Small scale expression of *adiponetin* by *E. coli*

Single colony of cells containing recombinant plasmid was used to inoculate 10ml of LB broth and grown overnight at 37°C. The expression medium consists of 100μg/ml ampicillin, 10g/liter tryptone, 5g/liter yeast extract, 5g/liter NaCl and 2g/liter glucose. An overnight culture (0.8ml) was used to inoculate 80 ml of an expression medium. The subculture was grown at 37°C with good aeration (250rpm shaking) until OD_{600} was approximately 0.5. A sample of 1 ml was taken as non induced cells and centrifuged for 2min. the cell pellet was then resuspended in 50μl of SDS-PAGE sample buffer and frozen at -20°C. For induction, isopropylthiogalactoside (IPTG) was added to the remaining culture to a final concentration of 0.3mM and the culture was incubated at 37°C with shaking at 250rpm for 4 hours. At each hour after induction a sample of 1ml was taken and prepared for SDS-PAGE analysis.

2.4.7. Native sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE without denaturing condition.

SDS-PAGE was carried out to detect the expression of recombinant protein and the efficiency of protein purification. First the apparatus was set up according to manufacturer's protocol (Mini-Protean®3 Cell system, Bio-Rad, USA). The separating gel solution was prepared by mixing 12.5% of 30% acrylamide, 10% SDS, TEMED, 10% APS and 1.5M Tris-HCl in distilled water. The mixture was immediately transferred to the sandwich glass plates which were fixed in specific holders to form gel cassette. Two gel cassettes were formed, one of them was used for staining with silver or coomassie stain and another was used for western blot. The gel was covered with butanol and left to solidify for 30 min after drying excess butanol with towel paper, a stacking gel was prepared by mixing 4.5% of 30% acrylamide, 10% SDS, TEMED,

10% APS and 0.5 M of Tris-HCl in distilled water. The stacking solution was poured into the sandwich glass plates on top of the separating gel. The comb was then fixed immediately; and the gel was left to solidify for 30 min. Then, the two gel cassettes were placed in its specific location on the SDS-PAGE apparatus and 1X electrophoresis buffer (25mM Tris, 200mM glycine and 3.5mM SDS) was poured to the top between these two cassettes and up to half of the tank height outside the cassettes.

For native SDS -PAGE, the sample was mixed with 2X gel loading buffer (125mM Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, and 0.25% bromophenol blue and 5% β-mercabtoethanol). The loading dye contained β-mercabtoethanol as a reducing agent and the sample was heated for 4min at 100°C to completely denature the protein. For the SDS- PAGE without denaturing condition the reducing agent and heating step were excluded. After loading samples, the Precision Plus Protein^{TM} Standards (Bio-Rad, USA) was used as a protein molecular weight marker and the complete apparatus was connected to 180V for one hour. When the run had been completed, one of the thin layer gels was used for western blot processing, and another one was used for staining with Silver stain or Coomassie Blue stain.

Silver staining was used to detect the level of expression and the expected size of the recombinant adiponectin. After careful collection of the thin layer gel, fixative solution (50% ethanol and 10% acetic acid) was added to the gel and incubated over slow shaker for 30min. The fixative solution was replaced by infiltrating solution (30% ethanol, 500mM sodium acetate, 0.125% glutaldehyde and 0.2% sodium thiosulfate) for another 30min. This was followed by three times washing with dH_2O (5min. for each time). The next step was staining with silver solution (0.1% silver nitrate and formaldehyde) for 20min and protein bands appearing with staining solution (2.5% sodium carbonate and formaldehyde). In order to stop further staining, the staining solution was replaced by stopping solution (1.5% EDTA) or distilled water.

Coomassie Blue stain was also used to detect the level of expression and the expected size of the recombinant adiponectin. The thin layer gel was incubated in fixating solution (40% methanol and 10% acetic acid) for 30 min. with slow shaking. For one and half hour the gel was incubated with Coomassie Blue solution (0.05% Coomassie Brilliant Blue R250, 50% methanol and 10% acetic acids) and the destaining step was carried out with destaining solution (20% ethanol and 7% acetic acid). In order to see the protein bands, the destaining solution was changed for several times to remove any stain remaining. The Alpha Imager system (Alpha Innotech Corp, USA) was used to document the gel and save gel data.

2.4.8. Western blot

The thin layer gel which was collected from the SDS-PAGE electrophoresis apparatus was used for western blot. The following materials were arranged respectively on the black side of the cassette holder, the first fiber pad was laid, the filter paper on top, the gel on top of the filter paper, the nitrocellulose membrane (Amersham Biosciences, USA), the filter paper and the next fiber pad. It was very important to remove any bubbles between the gel layer and nitrocellulose paper. The cassette holder was fixed in the western blot apparatus (Bio-Rad, USA) with ice block and the transferring buffer was added to the top of the tank. The apparatus was connected with electricity (100 V) for one hour. The nitrocellulose membrane was incubated overnight at 4°C with blocking solution. Then, the membrane was transferred into probing solution with monoclonal anti-human adiponectin antibody and incubated at room temperature for 60min with slow shaking. After the first antibody incubation, the membrane was rinsed with TBS solution and washed three times (15min each) with TBST solution then rinsed again with TBS solution. The second antibody (mouse IGg

antibody, Sigma, USA) was added after covering the membrane with probing solution. The membrane was incubated for one hour and the same step of washing with TBS and TBST solutions was repeated. Finally, the membrane was covered with Western Blue®stabilized substrate (Promega, USA) to catalyse by the alkaline phosphatase which was conjugated with the second antibody. Protein bands of interest were detected according to the protein maker using Alpha Imager System.

2.4.9. Scale up expression of adiponectin by *E. coli*

One litre rich medium (1% peptone, 0.5% yeast extract, 0.5% NaCl and 0.2% glucose) was used to scale up the expression of adiponectin by *E. coli.* After inoculation by 10 ml of an overnight culture (37°C and 250rpm), the one litre culture was grown at same condition until OD_{600} was around 0.5. Then, IPTG was added to a final concentration of 0.3mM for induction. The culture was grown for two hours after induction at the same condition. The cells were harvested by centrifugation at 4000x*g* for 20min. After discarding the supernatant, the cells were resuspended in 400ml of 30mM Tris-HCl, 20% sucrose (pH 8.0) and EDTA was added to final concentration of 1mM. The mixture was stirring for 10min. at room temperature. After centrifugation at 8000x*g* for 20min, the cells were resuspended in 30ml of ice-cold 5mM $MgSo_4$ and kept in -20°C overnight. The sample was placed in ice- water bath and after thawing 20g of glass beads were added. For seven times, a vortex step for 1min followed by incubation on ice for 1 min was applied. The mixture was centrifuged at 4°C for 10min. at 10,000rpm. The supernatant (crude extract) was transferred to a clean 50ml tube and 8ml of 1M Tris-HCl, pH 7.4 was added. Later, the crude extract was purified by affinity chromatography.

2.4.10. Protein purification by amylose resin column

The amylose resin was poured into a disposable polypropylene column of 25ml volume (BioRad Econo-Pac™, U.S.A.). The bed volume was 5ml and the column volume was 20ml. The column was washed with 8 column volume of column buffer (20mM Tris-HCl, 200mM NaCl and 1mM EDTA). Then, the crude extract was loaded into the column at a flow rate about 1 ml/minute. The column was shut off for 15min as this was important to enable optimal binding between the fusion protein and the amylose resin. Later, the column was washed with 12 column volume of column buffer. In order to elute the bound protein, one column volume of elution buffer (column buffer with 10mM maltose) was added and the fractions (3ml) were collected and kept in -80°C.

2.4.11. Cleavage, denaturing and re-purification of recombinant protein

First, a pilot experiment was carried out using small portion of protein sample to optimize the suitable time for Factor Xa (Biolabs, UK) cleavage. Factor Xa final concentration of 1% was added to the protein sample, and the positive control was a protein sample before purification (crude protein) incubated with same concentration of Factor X. All samples were incubated for 2, 4 and 8 hours. From each reaction 5µl sample were added to 5µl of 2X SDS-PAGE sample buffers and kept in 4°C. SDS-PAGE was performed (see Section 2.4.6) to determine the suitable time for cleavage. Then, the pilot experiment was scaled up for the portion of the fusion protein to be cleaved. Guanidine hydrochloride was added to the sample to a final concentration of 6M. Later, the sample was dialysed against 100 sample volumes column buffer (20mM Tris-HCl, 200mM NaCl and 1mM EDTA) three times for 2 hours each. In order to remove the rest of maltose binding protein, the fusion protein cleavage mixture was

loaded onto the hydroxyapatite column (BioRad Econo-Pac™, U.S.A.). The column was washed with 80ml of 20mM sodium phosphate, 200mM NaCl (pH 7.2). The protein mixture was eluted with 0.5M Na phosphate (pH 7.2). The collected fractions were loaded onto amylose column (as described in Section 2.4.10) and the flow through was collected should be free of maltose binding protein (MBP). Protein concentration was assessed by Bradford assay.

2.4.12. Bradford assay

The purified protein concentration was measured by the Bradford method. One volume of Bio-Rad Protein Dye (Bio-Red, U.S.A) was diluted with four volumes of dH_2O and then filtered through the filter paper. Six serial dilutions of bovine serum albumin (BSA) were prepared as a protein standard starting from 0.05 to 0.50mg/ml. The purified adiponectin and BSA protein (10µl) were transferred into the micro-titer plate wells in duplicate. Later, the diluted dye reagent (200µl) was added to each well and the mixture was mixed using the pipette. In order to let colour development the micro-titer plate was incubated at room temperature for 5min. The wave length of 595nm was applied to measure the colour intensity by ELISA reader.

2.5. Adiponectin expression by *Pichia pastoris*

The Easy Select ™ pichia Expression Kit (Invitrogen, Netherlands) was used to clone *adiponectin* fragments (*ADPs* and *ADPws*) into *P. pastoris*. Some modifications on instruction manual of this kit had been made according to available materials and instruments. *P. pastoris* strain that was used in this work was the wild type X-33 strain.

2.5.1. Cloning of *adiponectin* into pGEM-T vector

The purified PCR producs of *ADPs* and *ADPws* were cloned in pGEM-T vector and transferred in to JM109 *E. coli* strain. LB broth and LB-agar with ampicillin, X-Gal and IPTG were prepared as mentioned previously (see Section 2.4.1). In addition, the competent cells of JM109 *E. coli* were prepared using $CaCl_2$ method as mentioned in section 2.4.2.

2.5.1.1. Ligation reaction

The PCR products of *ADPs* and *ADPws* were amplified using forward primers containing *EcoR*I restriction site and reverse primer contains *Not*I restriction site was purified by QIAquick Gel Extraction Kit (Qiagen, USA) as mentioned in section 2.3. The purified PCR product (3μl) was mixed with 1 μl of pGEMT-T vector and 5μl of 2X buffer and 1μl of T4 ligase (all from Promega, U.S.A.). The reaction mixture was incubated in 4°C overnight.

2.5.1.2. Transformation

The JM109 *E. coli* strain competent cells were taken from -80°C and thawed on ice. Ligation mixture (5μl) was added to 100μl of competent cells and another 100μl of competent cells were used as control without ligation mixture. The transformation procedure was carried out as mentioned in section 2.4.4. Finally, the transformation mixture (100μl) was plated on LB-agar containing ampicillin, X-Gal and IPTG. The rest of the transformation mixture was briefly centrifuged and the supernatant was discarded. The cells were resuspended by 100μl LB broth and mixed by pipette then

plated on another LB-agar plate as a concentrated transformation mixture. The cultures were incubated overnight at 37°C.

2.5.1.3. Colony selection

An overnight plate cultures contained two types of *E. coli* colonies according to the *Lac* phenotype. The white colonies were transferred to a new gridded LB-agar plate. After transferring, each white colony was picked and the loop was rinsed into 0.5 ml tube contain 50µl dH_2O. DNA template for each colony was prepared by heating this tube at 100°C for 10min. Then 2µl was used as a template for PCR reaction. The forward and reverse M13 primers were used to detect the expected size of the gene of interest using gel electrophoresis.

2.5.1.4. Plasmid isolation

A positive bacteria single colony contains *adiponectin* inserts were inoculated in 10ml LB-broth contain 5µl of 100mg/ml ampicillin and left to grow overnight at 37ºC, 250rpm in the shaking incubator. A glycerol stock of recombinant *E. coli* was prepared by adding 150µl of glycerol into 850µl of bacteria culture and kept at -80°C for long term storage.

Overnight culture was transferred to 15ml plastic tube and centrifuged at 10,000rpm for 5min at room temperature. The supernatant was discarded and the tube was left standing upside down on absorbent paper to draw off the remainder of the supernatant. The cell pellet was resuspended by 200µl of solution I (50mM glucose, 10mM EDTA and 25mM Tris-Cl, pH 8) and vortexed until the entire pellet was dissolved. Then resuspended pellet was transferred to 1.5ml tubes and 200µl of freshly

prepared solution II (0.2N NaOH and 1% SDS) was added. The suspension was mixed gently for 4min at room temperature, and 200µl of solution III (3M KOAc and 10% acetic acid) was added, mixed gently and then left for 15min on ice. The suspension was then centrifuged at 13,000rpm for 10min at room temperature. The supernatant was transferred into a new 1.5ml tube. In order to remove any RNA contamination, RNase A (0.25µg/µl) was added and the mixture was incubated for 3 hours at 37°C.

To clean up plasmid DNA from protein contamination, phenol- chloroform extraction steps was applied. First, 600µl of phenol were added into the suspension and mixed well then centrifugated at 13,000rpm for 3min. The upper phase was carefully transferred into new 1.5ml tube. Second, the phenol step was repeated with same amount of chloroform. The precipitation steps included adding 5M NaCl (0.1 volume) and isopropanol (2.5 volume) to the suspension and the mixture was incubated on ice for 20min. The mixture was centrifuged at 13,000rpm for 15min and supernatant was discarded. As a washing step, 1ml of 70% ethanol was added to the pellet and vortex pPICZαA then centrifuged at 13,000rpm for 5min. Finally, ethanol was discarded and the tubes were placed in a Speed Vac Concentrator for 5min to dry the pellet. The pellet was resuspended with 30µl of distilled water and kept in 4°C for overnight to let the pellet dissolve completely. Gel electrophoresis was applied to check plasmid isolation process and plasmid concentration was determined by spectrophotometer.

2.5.1.5. Restriction enzyme digestion of pGEMT-*adiponectin* recombinant plasmid

Isolated pGEMT-*ADPs* and pGEMT-*ADPws* recombinant plasmids were digested by *Eco*RI and *Not*I (Promega, USA) to generate cohesive ends at both ends of the *adiponectin* fragments. The digestion reaction was consisted of 1µl of *Eco*RI (10 U/ µl) and 1X reaction buffer and 0.1 mg/ml of bovine serum albumin to digest 1µg of the recombinant plasmid. The reaction mixture was incubated for 4 hrs at 37 °C. The control sample contained all reaction materials except enzyme. After digestion period, gel electrophoresis run was performed to check the digestion. For 15min. at 65°C *Eco*RI enzyme had been inactivated and NotI enzyme (1µl of 12U/µl) with its specific buffer was added. The reaction mixture was incubated again for 4 hrs at 37°C. After heat inactivation, gel electrophoresis run was carried out to detect the expected size of *adiponectin* fragments. The *adiponectin* fragments flanked at both ends of *Eco*RI and *Not*I were purified by QIAquick Gel Extraction Kit (Qiagen, USA) as mentioned in section 2.3.

2.5.2. Clone of *adiponectin* in pPICZαA vector

The vector used to transfer *adiponectin* fragments into *Pichia pastoris* was pPICZαA (Invitrogen, Netherlands). pPICZαA plasmid was cloned previously into Top 10F' *E.coli* and stored as glycerol stock. This plasmid was isolated as mentioned above (Section 2.5.1) and the concentration was determined by spectrophotometer. pPICZαA was digested with *Eo*RI and *Not*I restriction enzymes as mentioned above (Section 2.5.2).

2.5.2.1. Preparation of Low Salt Luria-Bertani (LSLB) broth and agar media

The components of LSLB broth medium (1.0% tryptone, 0.5% yeast extract and 0.5% sodium chloride) were dissolved in distilled water and poured in 10ml glass vials. For solid LSLB medium 1.5% agar powder was added into the broth medium. 1N sodium hydroxide was used to adjust pH medium to 7.5. The two media were autoclaved on liquid cycle at 15lbs/sq.in., 120°C for 20min. Then, the agar medium was cooled down to at least 50 °C and an antibiotic Zeocin^{TM} (Invitrogen, Netherlands) was added to a final concentration of 25µg/ml. Agar medium was poured into Petri dishes and left to solidify at room temperature then kept at 4°C for using in space of two weeks. These media provide salt concentration < 90 mM and pH 7.5 for Zeocin^{TM} to be active.

2.5.2.2. Preparation of Top10F *E. coli* competent cells

Top 10F strain of *E. coli* (Invitrogen, Netherlands) was kept as glycerol stock in -80°C and used to prepare competent cells for transformation. To get single colonies, inoculums of 10µl was plated on LSLB-agar plate without antibiotic and grown overnight. From the growing colonies, single colony was used to inoculate 10ml of LSLB broth and grown overnight to prepare the competent cells using $CaCl_2$ as mentioned previously in section 2.4.2.

2.5.2.3. Ligation reaction

Digested pPICZαA plasmid (1µl) was mixed with 3µl of digested *adiponectin* fragments in 5µl of 2X ligase buffer. One microliter of Ligase enzyme (New England Biolabs, UK) was added and the mixture was mixed by the pipette and incubated overnight at 4°C.

2.5.2.4. Transformation

Deep storage *E. coli* competent cells (TOP 10F) was thawed on ice and mixed with 5µl of ligation mixture. After applying heat shock, the cells were plated on LSLB agar containing Zeocin^{TM} (Invitrogen, Netherlands) as mentioned previously in section 2.5.1.2.

2.5.2.5. Colony selection

All colonies which grew on agar surface were white colonies because pPICZαA plasmid doesn't contain *Lac* gene. Each colony was transferred to grid LSLB- agar-Zeocin^{TM} plate to prepare master library. The transferring loop each time was rinsed into 0.5ml tube containing 50µl dH_2O to prepare DNA template for PCR reaction with pPICZαA plasmid, α-factor and 3AOX primers as mentioned in section 2.5.1.3.

2.5.2.6. Plasmid isolation

From the positive colonies, recombinant pPICZαA–*APDs* and pPICZαA–*APDws* plasmids were isolated by inoculating a single colony in 10ml LSLB broth containing 25µg/ml Zeocin$^{TM.}$ The inoculated medium was grown overnight and the glycerol stock was prepared. The rest of the culture was transferred to 15ml plastic tube for plasmid isolation as described earlier in section 2.5.1.4. The concentration of purified recombinant plasmid was adjusted to be 300ng/µl and sequenced using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA).

2.5.2.7. Linearization of the recombinant plasmid

The recombinant plasmid was linearized to increase transformation efficiency into *P. pastoris* cells and integration within the host cells chromosomes. One unit of the restriction enzyme *Sac*I (Fermentas, USA) was mixed with 1µg of the recombinant plasmid in 1X reaction buffer and the mixture was incubated at 37°C for 3 hours. The linearized plasmid was checked by loading 5 µl of the reaction mixture into agarose gel. The double bands of circular plasmid would appear as a single band after plasmid linearization.

2.5.3. Cloning into *Pichia pastoris*

The linearized plasmid was transformed into X-33 strain of *P. pastoris* according to the transformation and expression protocol of EasySelect™ *Pichia* Expression Kit (Invitrogen, Netherlands).

2.5.3.1. Preparation of *P. pastoris* broth and agar media

To prepare Yeast Extract Peptone Dextrose (YPD) broth, 1% yeast extract and 2% peptone were mixed with dH_2O. The mixture was poured into 10 ml glass tubes and autoclaved on liquid cycle at 15lbs/sq.in., at 121°C for 15min. Before using this broth, 2% filtered and sterile dextrose should be added. For YPDS-Zeocin™- agar medium (YPDS), 1 M sorbitol and 2% agar were added to the previous mixture and autoclaved at the same condition. After cooling to 50°C, 2% filtered and sterile dextrose and Zeocin™ (final concentration of 100µg/ml) were added and poured into Petri dishes. The plates containing Zeocin™ can be stored at 4°C for up to two weeks.

2.5.3.2. Preparation of *P. pastoris* competent cells

Single colony of X-33 strain *P. pastoris* was used to inoculate 10ml of YPD broth. The inoculated broth was grown overnight at 30°C and shaking 250rpm. The glycerol stock was prepared by adding 150µl of 30% glycerol to 850µl of the culture and stored in -80°C. The cells from the overnight culture were diluted to an OD_{600} of 0.1 -0.2 in 10ml YPD broth. Then, the cells were grown at the same condition until OD_{600} reached 0.6-1.0 through 4 to 6 hours. The cells were harvested by centrifugation at 500*xg* for 5min at room temperature. Next, the cells were resuspended in 10ml of solution I (provided in the EasySelect™ *Pichia* Expression Kit [Invitrogen, Netherlands]). Without incubation period, the mixture was centrifuged at 500*xg* for 5min at room temperature and the supernatant was discarded. Finally, the cells were resuspended in 1ml of solution I and transferred into autoclaved 1.5ml tubes as aliquots of 50 to 200µl per each tube. At this point, the cells can be kept at room temperature and used directly for transformation or frozen for future use. Before freezing, competent cells tubes should be wrapped with several layers of paper towels and placed in -80°C to freeze down slowly without snap-freezing in liquid nitrogen.

2.5.3.3. Transformation

Pichia pastoris competent cells were thawed at room temperature and aliquoted (50µl) into sterile microcentrifuge tube. One tube will be used as control without DNA and another tube will received 3µg of linearized DNA. The volume of DNA should not exceed 5µl to increase transformation efficiencies. Fresh competent cells can be used directly in this step as mentioned above in section 2.5.3.2. Then, 1ml of solution II was added to the transformation mixture with mixing by brief vortex. The next step was incubation for transformation reaction for one hour at 30°C with mixing

every 15min by brief vortexing to increase transformation efficiency. Mean while, the water bath was set up to 42°C to carry out the heat shock process for 10min. After that, the transformation reaction was divided into two microcentrifuge tubes (approximately 525µl per tube) and 1ml of YPD medium was added to each tube. In order to allow expression of Zeocin ™ resistance, the cells were incubated at 30°C for one hour. Next, the cells were centrifuged at 3000*xg* for 5min. at room temperature, resuspended in 500µl of solution III (provided in kit) for each tube and combined into one tube. The cells were centrifuged again at 3000*xg* for 5min at room temperature and resuspended in 100µl of solution III. The entire transformation was plated on YPDS-Zeocin™- agar medium and incubated for 2 to 4 days at 30°C. Solution II and III were provided in the EasySelect™ *Pichia* Expression Kit (Invitrogen, Netherlands).

2.5.3.4. Selection of positive colonies

2.5.3.4.1. Colony selection via PCR

Normal PCR was used to detect the Zeocin™ resistance colonies that are carrying *adiponectin* fragments. First, new YPD agar- Zeocin™ plate was gridded to use as a library. Second, part of each Zeocin™ resistance colony was taken by sterile loop to inoculate the library plate and the loop was then rinsed into 50µl d H2O to use as a DNA template. Normal PCR was performed using *adiponectin* fragments specific primers and pPICZαA specific primers (α-factor forward primer and 3-AOX reveres primer) as mentioned previously in section 2.5.1.3. Finally, according to the expected size of PCR product, the positive colony was chosen to carry on protein expression.

2.5.3.4.2. Colonies capacity of the Zeocin™ resistance

In order to select colonies with high copy number of expression cassette , the Zeocin™ resistance colonies were transferred to two of new YPD agar- Zeocin™ plate were gridded to use as libraries contain different concentration of Zeocin™ (final concentration of 150μg/ml and 200μg/ml). The libraries were incubated for two days at 30°C to select the most resistant colony that ideally contains high copy number of the xpression cassette.

2.5.3.4.3. Differentiation between Mut^+ and Mut^S phenotypes

For Mut^+ conformation, the colonies were transferred to two different plates. The first plate was YPD-Zeocin™- agar that contains dextrose as a source of carbon. The second plate was YPM-Zeocin™- agar that contains methanol as a source of carbon. The colonies on the two plates were grown for two days at 30°C. The colonies that grow on the YPD-Zeocin™- agar and YPM-Zeocin™- agar were the Mut^+ phenotype.

2.5.3.5. Preparation of expression media

For expression experiment, Buffered Glycerol-complex Medium (BMGY) and Buffered Methanol-complex Medium (BMMY) were prepared. The first medium contained glycerol as a source of carbon to increase biomass and the second medium contained methanol as a source of carbon to induce expression. The two types of the media were designed to control the pH of the medium and to decrease protease activity. BMGY medium consisted of 1% yeast extract, 2% peptone, 100mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, $4x10^{-4}$g/L biotin, and 1% glycerol. BMMY medium was consisted of the same component except glycerol which

was replaced by methanol. Yeast extract and peptone were dissolved in conical flasks (125ml and 250ml) and autoclaved on liquid cycle at 15lbs/sq.in., at 121°C for 15min and then the other components of BMGY and BMMY media were added.

2.5.3.6. Protein expression and optimization

In order to determine the success of protein expression, some of the positive colonies were used to inoculate some of flasks (125ml) each containing 10ml of BMGY medium. The cultures were grown overnight at 30°C in a shaking incubator (225rpm) until OD_{600} was 2 to 6. The cells were precipitated by centrifugation at 3000*xg* for 5min at room temperature and resuspended into some of new baffled flasks (250 ml) each one contained 50 ml of BMMY media. The cultures were grown for 2 days with induction by adding 100% methanol to a final concentration of 0.5% every 24 hrs. The supernatant of each culture was analysed by SDS-PAGE (see section 2.4.7.)

To optimize protein expression, BMGY media (10ml) were prepared into three baffled flasks (125ml) and each flask was inoculated with single colony of recombinant *P. pastoris.* The cultures were grown for18 hours at 30°C in a shaking incubator (225rpm) until OD_{600} was 2 to 6. The cells were precipitated by centrifugation at 3000*xg* for 5min. at room temperature and resuspended into three baffled flasks (250ml) each one contained 50ml of BMGY media. After incubation in a shaking incubator (225rpm) at 30°C for 18 hrs, the cells were resuspended and divided into 10 baffled flasks (250ml) contained 50ml of BMMY medium to an OD_{600} approximately 1. The induction of expression had been performed by adding 100% methanol to a final concentration of 0.5% every 12 hrs. At each time points (0, 12, 24, 36, 48, 60, 72, 86 and 96 hrs) one flask was processed to analysis protein expression and the culture pH and OD_{600} were measured as well. The culture in each flask was transferred into 50 ml

plastic tube, centrifuged and the supernatant was transferred to plastic bottle (200ml) containing 3 volumes of ice cold acetone and the mixture was kept in -20°C overnight. Later, the mixture was centrifuged for 30min at 4°C and 10000rpm and the precipitated protein was resuspended into phosphate buffered saline **(**PBS) for subsequent purification.

2.5.3.7. Protein purification

Protein samples were purified by His GraviTrap^{TM} Flow (Amersham Biosciences, USA) column containing precharged Ni Sepharose^{TM} 6 Fast. The column was normalized with 8ml of phosphate buffer (20mM sodium phosphate buffer and 500mM NaCl, pH 7.4). The sample (4 ml) was loaded into the column and the column was washed with 10ml of binding buffer (phosphate buffer containing 20mM imidazole, pH 7.4). The recombinant protein was eluted with 4ml of elution buffer (phosphate buffer containing 200mM imidazole, pH 7.4). The fractions (1ml) were collected to subject for SDS-PAGE and Western blot analysis as mentioned in section 2.4.7 and 2.4.8. Protein concentration was determined using Bradford assay (section 2.4.11).

2.5.3.8. Scale- up expression of recombinant adiponectin

Each one of five flasks (125ml) containing 10ml of YPD medium were inoculated by a single colony of recombinant *P. pastoris.* Inoculated flasks were incubated at 30°C for 16-18 hours and shaking 250rpm until OD_{600} was 2 to 6. Then, the harvested cells were transferred to five flasks (250ml) containing 50ml BMGY media and incubated further in the same condition until OD_{600} was 2 to 6. These two steps was performed to increase yeast biomass by growing yeast on BMGY medium that contained glycerol as a source of carbon. Next, the harvested cells were

resuspended into five 1L flasks containing 200ml BMMY medium and OD_{600} was approximately 1. In order to achieve continues induction of the Alcohol Oxidase promoter (AOX1), 100% methanol was added to final concentration of 0.5% every 12 hrs with continuous shaking (250-300rpm at 30°C) until the optimal time of induction was reached 60 hours. To precipitate the secreted recombinant protein, three volumes of ice cold acetone was added to the collected supernatant and kept at -20°C overnight. The dissolved protein was resuspended with PBS buffer, purified by affinity chromatography and quantified using Bradford method as described previously in section 2.5.3.6.

2.5.4. Bioactivity tests

Three experiments were designed to assess the bioactivity of the recombinant adiponectin that was expressed by *E. coli* and *P. pastoris*. In all experiments female ICR mice were used as a model system. Recombinant adiponectin was injected intraperitoneally and blood glucose was measured with a glucometer (Contour™ Bayar, Hong Kong). Blood concentration of triglyceride (TG), total cholesterol (CHOL) (Siemens, USA), low density lipoprotein (LDL) and high density lipoprotein (HDL) (Dade Behring, USA) were also measured using available commercial kits.

2.5.4.1. Effect of recombinant adiponectin produced by *P. pastoris* and *E. coli* on blood glucose and lipids.

This experiment was designed to compare the biological activity of adiponectin expressed by the prokaryotic expression system (*E. coli)* and eukaryotic expression system (*P. pastoris)*. The overnight fasted animals (four groups n=6 each) were gavaged with (V=0.1% BW) high fat-sucrose diet (6g butter, 6g sunflower oil, 10g nonfat dry

milk, 10g sucrose, 12ml distilled water). Simultaneously, the first group was injected with 1.8mg/kg BW recombinant adiponectin expressed by *P. pastoris* (P-ADP), the second group was injected with 1.8mg/kg BW recombinant adiponectin expressed by *E. coli* (E-ADP), and the third group injected equal volume (0.3ml) of saline as control. These doses were divided equally into two injections, the first injection at the time of feeding and the second injection after one hour of feeding. Blood glucose was measured at each hour for four hours of the experiment by making an incision at the tail vein. Also, blood concentration of triglyceride (TG), cholesterol (CHOL), low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured at the end of the fourth hour of the experiment by sacrificing the animals.

2.5.4.2. High and low doses effects of recombinant adiponectin on blood glucose and lipid profile.

This experiment was designed to study the effect of high and low dose of recombinant adiponectin (P-ADP) on blood glucose and lipids. Three groups (n=6 each) of animals were fasted overnight and simultaneously gavaged (V=0.1% BW) with high fat-sucrose diet (Section 2.5.4.1). Recombinant adiponectin (P-ADP) was injected intraperitoneally at 1.25mg/kg BW as low dose, 2.5mg/kg BW as high dose and one group injected with the same volume of saline as control. Blood glucose was measured at each hour of the four hours of the experiment by tail vein incision. Also, blood concentration of triglyceride (TG), cholesterol (CHOL), low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured at the end of the fourth hour of the experiment by sacrificing animals.

2.5.4.3. Oral glucose tolerance test

This experiment was designed to determine P-ADP potential in lowering blood glucose. Two groups of ICR female mice were fasted overnight and then subjected to oral glucose challenge (2g/kg BW, via gavage). At same time injected 2.5mg/kg BW of P-ADP and blood glucose measured by tail vein insicion at 1, 2 and 3 hours after P-ADP injection.

2.5.5. Assessment of genes expression

This experiment was done to assess the changes in the gene expression of genes encode glucagon, insulin and leptin receptors. Two groups of mice (n=4 each) were fasted overnight and then fed high fat /sucrose diet. One group was injected with P-ADP (2.5mg/kg BW) and another group was injected saline as control (as described in section 2.5.4.2). The animals were sacrificed after four hour from feeding and dissected to collect liver, skeletal muscle, abdominal fat tissue and kidney. The dissected tissues were snap frozen in liquid nitrogen and kept in -80°C until use.

2.5.5.1. Total RNA extraction

Total RNA was isolated from the liver, abdominal fat tissue, skeletal muscle and kidney using Trizol™ reagent, according to the manufacture's procedure (Invitrogen, Netherlands). All required equipments were treated with DEPC-dH_2O overnight and autoclaved on liquid cycle at 15lbs/sq.in., 121°C for 45min. The frozen tissue (50-100mg) was transferred to autoclaved pestle and ground until a layer of very fine dust was all that was left. Then, RNase-free spatula was used to transfer the dust to fresh 1.5ml tubes containing 1ml of Trizol™ solution and the mixture was shaken vigorously. Next, 200µl chloroform was added for each tube and the tubes vigorously shaken for

15s and incubated for 3min at room temperature. The mixture was centrifuged for 15min at 4°C and 12000*xg*. The colour less upper aqueous phase that contained RNA was carefully removed (avoiding the interface) and placed into a new 1.5ml microcentrifuge tube. At this step 600µl of aqueous phase was recovered. Then, 0.5ml of isopropanol was added, mixed and incubated at room temperature for 15min. The mixture was centrifuged for 15min at 4°C and 12000*xg*. The supernatant was discarded and 1ml of 75% ethanol was added, vortex and centrifuged again for 3min, at 4°C and 7500*xg*. This step was repeated as washing step and the white pellet was left to dry for 10min at room temperature. The pellet was resuspended with 50µl DEPC-dH_2O, vortex, placed on ice for 10min, heated at 65°C for 10min and placed on ice again before storage at -80°C. The agarose gel electrophoresis was performed to detect ribosomal RNA bands as an indicator for successful RNA extraction.

2.5.5.2. DNase treatment

After reading total RNA concentration at $A_{260}/A_{280,}$ DNase treatment was performed to remove any contaminating genomic DNA. The RNase-free reaction components were mixed into 0.5ml microcentrifuge tube on ice as follows:

- 1µg RNA sample
- 1µl 10X DNase I reaction buffer (Invitrogen, Netherlands)
- 1µl DNase I, Amp Grade, 1U/µl (Invitrogen, Netherlands)
- DEPC-treated water to 10µl

The reaction mixture was incubated at room temperature for 15min. Then, DNase I was inactivated by adding 1µl of 25mM EDTA and incubating for 10min at

65°C. Finally, the agarose gel electrophoresis was performed to check removal of DNA residuals by comparing with control sample without DNase I enzyme.

2.5.5.3. cDNA preparation

The RNA samples were converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The first step was preparation of 2X RT master mixtures (Table 2.4).

The second step was adding 10 µl of RNA sample into each tube containing same amount of master mix and mixed by pipetting. The tubes were briefly centrifuged to spin down the contents and to eliminate any air bubbles. The tubes were placed in the PCR machine and the following thermal cycle conditions were applied (Table 2.5).

Table 2.4: The components of 2X RT master mix

Component	**Volume (µl)**
10X buffer	2.0
25X dNTP Mix (100mM)	0.8
10 X RT random primers	2.0
MultiScribe™ Reverse transcriptase	1.0
RNase inhibitor	1.0
Nuclease – free water	3.2
Total per reaction	10.0

Table 2.5: Thermal cycle conditions of RT reaction

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min.	120 min.	5 min.	--

2.5.5.4. Real-Time PCR

Rreal-Time PCR (RT-PCR) was performed by ABI 7500 instrument using Syber green™ fluorescence dye (Applied Biosystems, USA.) to assess the expression of glucagon, insulin and leptin receptors. The relative gene expression was quantified using *β- actin* as an endogenous gene control. The primers were designed using ABI 7500 instrument software, SDS2.1. The total volume of RT-PCR reaction was 12.5 µl and the components are shown in Table 2.6.

There were four replicates for each unknown sample or target gene and endogenous gene. All replicates were given in the same thermal cycling conditions of RT- PCR (Table 2.7). For each tissue for treatment and control groups, the expression of three target genes were quantified and normalized with *β*-actin as an endogenous gene. The design of RT-PCR experiment was shown in Figure 2.1.

Table 2.6: The components of RT-PCR reaction

Reaction component	**µl/sample**	**Final concentration**
2X syber mix.	6.5	1X
Primers mix.	1.25	50 to 900 nM
cDNA	3.0	50 to 100 ng
Nuclease free water	2.0	--
Total	12.5	--

Table 2.7: Thermal cycle conditions of RT-PCR reaction

PCR stages	**Enzyme activation**	**Melt temperature**	**Anneal/extend**
Stage 1	10 sec. at 95°C	-----	-----
Stage 2	------	3 sec. at 95°C	20 sec. at 60°C

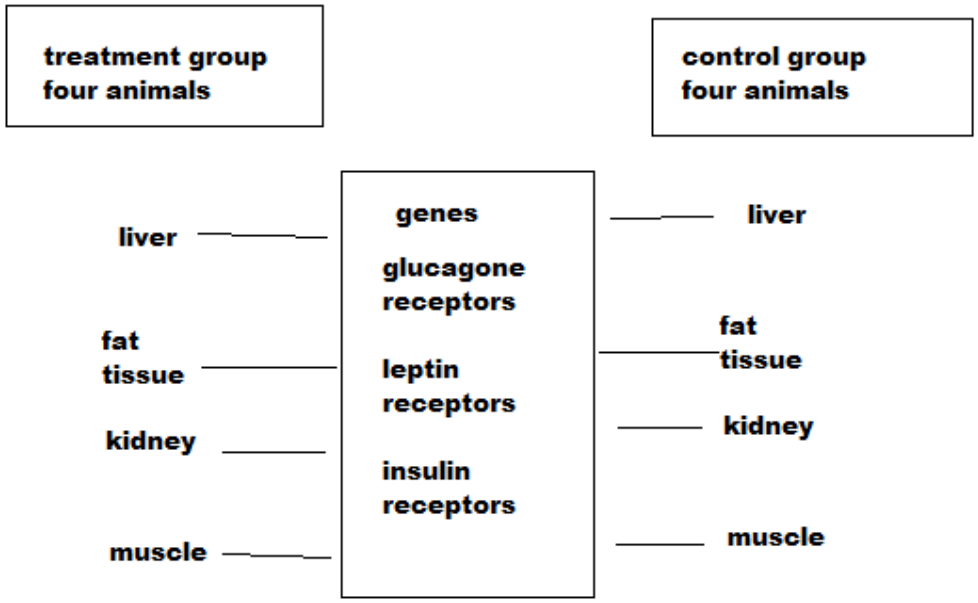

Figure 2.1: The design of RT-PCR experiment.

2.5.4.4.1. Primers design and validation

The full sequence of *insulin* (NM_010568.2), *glucagon* (NM_008101.1), *leptin* (NM_010704.2) receptors and *β-actin* (NM_007393.2) genes were obtained from GenBank. Forward and reverse primers were designed for each gene of interest using ABI 7500 instrument software, SDS.2.1. to amplify 80 to 100 bp fragment length from each gene (Table 2.8). Applied Biosystems primer design software creates 50 set of primer for each gene sequence, and the convenient set of primers was selected based on less probability of primer dimer and hair pin formation.

The primers specificity was determined by melt curve analysis for each set of primers (Figure 2.2). According to the melt curve (dissociation curve) the one peak curve indicates that one product was obtained through PCR reaction and presence of any other peaks would indicate the presence of products due to non specific annealing of primers resulting to different PCR products. After complete PCR reaction, the samples was applied for dissociation curve and the real time PCR software(ABI 7500 instrument software, SDS.2.1.) determines the amplicon melting temperature (Tm°C) that appears as a single peak. Therefore, the dissociation curves of all sets of primers were checked as a single peak curves (Figure 2.2).

Table 2.8: Forward and reverse primers for each gene of interest and an endogenous gene.

Genes	Primers	TM °C
Insulin receptor forward	5-GTCAGGAGAGGAACGACATTGC-3	61.9
Insulin receptor reverse	5-TGTCCGAATGAAAGAAAATTTAAGC-3	54.7
Glucagon receptors forward	5-GCTGGTGGCTGTTCTCTACTGTT-3	62.2
Glucagon receptors reverse	5-CTTCTTGCCATTGCCTCCAA-3	59.5
Leptin receptors forward	5-CCTGGGCACAAGGACTGAA-3	62.5
Leptin receptors reverse	5-CAAATATCACTGATTCTGCATGCTT-3	55.4
β-actin receptors forward	5-ACTGCCGCATCCTCTTCCT-3	62.6
β -actin receptors reverse	5-AACCGCTCGTTGCCAATAGT-3	59.5

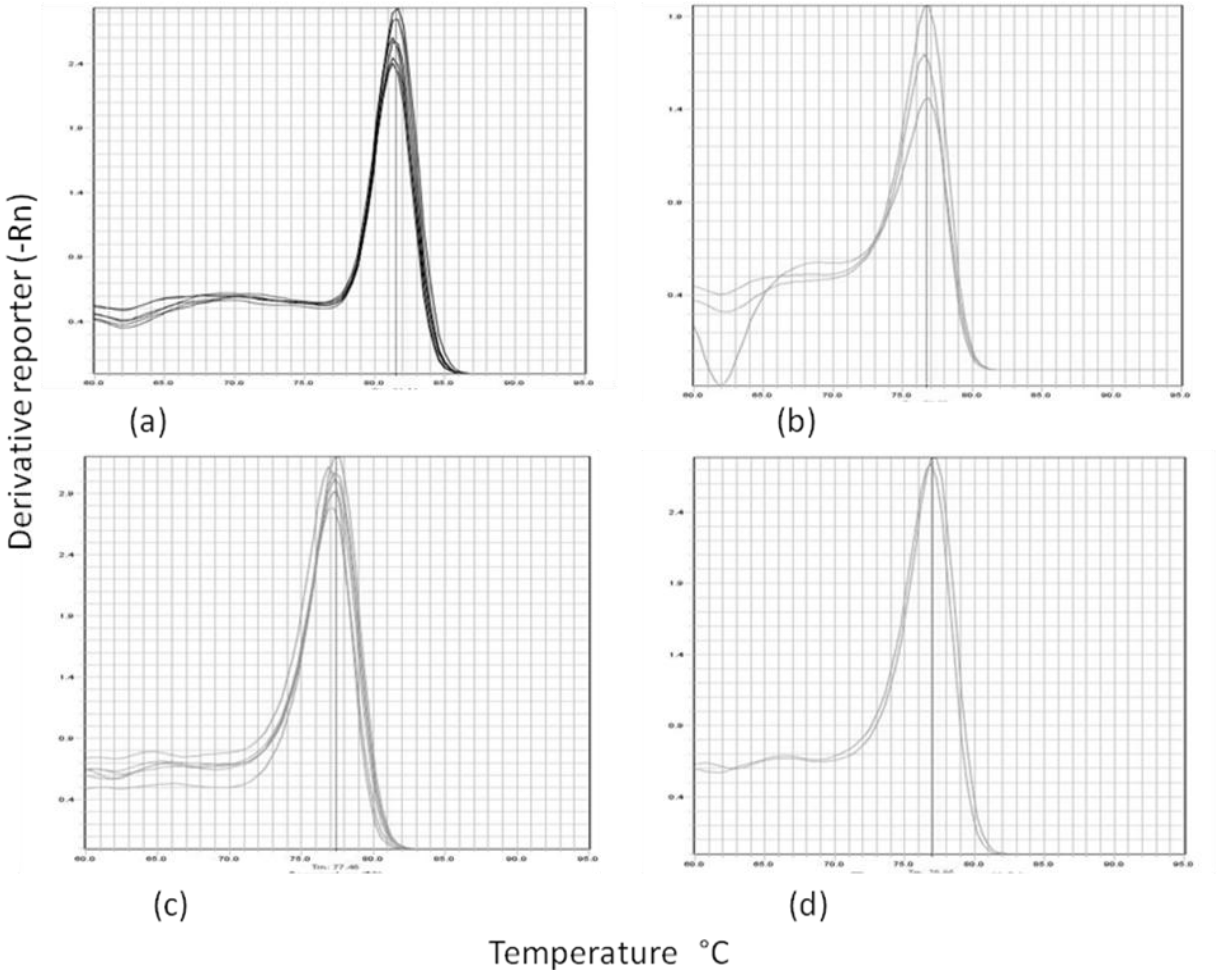

Figure 2.2: Melting curves of each gene of interest and an endogenous gene. *β –actin* receptors gene (a), insulin receptors gene (b), glucagon receptors gene (c), and leptin receptors gene (d). The amplicon melting temperature (Tm°C) for each gene appears as a single peak indicating high specific primers.

2.5.4.4.2. Data analysis of Real-Time PCR

To determine the mRNA level in each unknown sample, the genes of interest (*insulin*, *glucagon* and *leptin* receptors genes) was normalized to the reference gene (*β-actin* gene) in order to account for cDNA loading differences using comparative *Ct*, or as called *ΔΔCt* method. The target gene replicates and endogenous gene replicates were applied in the same thermal cycle conditions of RT-PCR. The calculations of this experiment were performed as described by Bookout and Mangeladorf (Bookout & Mangeladorf, 2003).

The average (*avg*) cycle time (*Ct*) as a mean for four replicates, and the standard deviation (*stdev*) were calculated by the software (Applied Biosystem software) for each sample. In addition, the coefficient of variation values (*CV*) for each sample that was more than 4% was removed from the calculations according to the following formula:

$$CV = stdev / avg$$

By the RT-PCR software, the average *Ct* value for each sample ($avg.Ct_s$) was normalized with the average *Ct* value of endogenous gene ($avg.\ Ct_e$) to calculate *ΔCt* value for each sample. The following formula was applied to calculate *ΔCt* value for each sample:

$$\Delta Ct = avg.Ct_s - avg.\ Ct_e$$

In addition, the standard deviation for each sample *ΔCt* value was calculated based on the following formula:

$$stdev_{\Delta Ct} = \sqrt{(stdev\ of\ Ct_s)^2 + (stdev\ of\ Ct_e)^2}$$

The biological group contains four samples for the same tissue that was divided to control and treatment groups. The mean comparative *ΔCt* value standard deviation and mean standard error was calculated for each biological group by SPSS statistical analysis software.

Then, to calculate the relative quantification value (*RQ* value) or the fold of induction the target gene expression was calibrated with one of control group's gene expression to get *ΔΔCt* value by which *RQ* value was calculated. The following formula was used to calculate *ΔΔCt* value:

$$\Delta\Delta Ct = \Delta Ct\ target \ \_ \ \Delta Ct\ calibrator$$

In addition, the standard deviation of *ΔΔCt (ΔΔCt stdev*) is the same as standard deviation of *ΔCt* (*stdev ΔCt*). Then, to calculate relative quantification (*RQ* value) for each target gene the following formula was applied:

$$RQ\ value = 2^{(-\Delta\Delta Ct)}$$

Therefore, the statistical analysis was applied to explain the significance of differences between control group and treatment group for each biological group. In this case, *t-test* statistical analysis was chosen to explain the significance of differences. It is necessary to calculate standard deviation for *RQ* values as the following formula:

$$stdev_{RQ} = (ln2)(stdev\ \Delta\Delta Ct)(2^{(-\Delta\Delta Ct)})$$

Finally, *t-test* was applied for each treatment biological group comparing with the control biological group using *RQ* values and standard error means (*SEM*) using SPSS statistical analysis software. The biological group standard error means (*SEM*) was calculated according to the following formula:

$$SEM = stdev\ of\ group/\sqrt{number\ of\ samples\ in\ group}$$