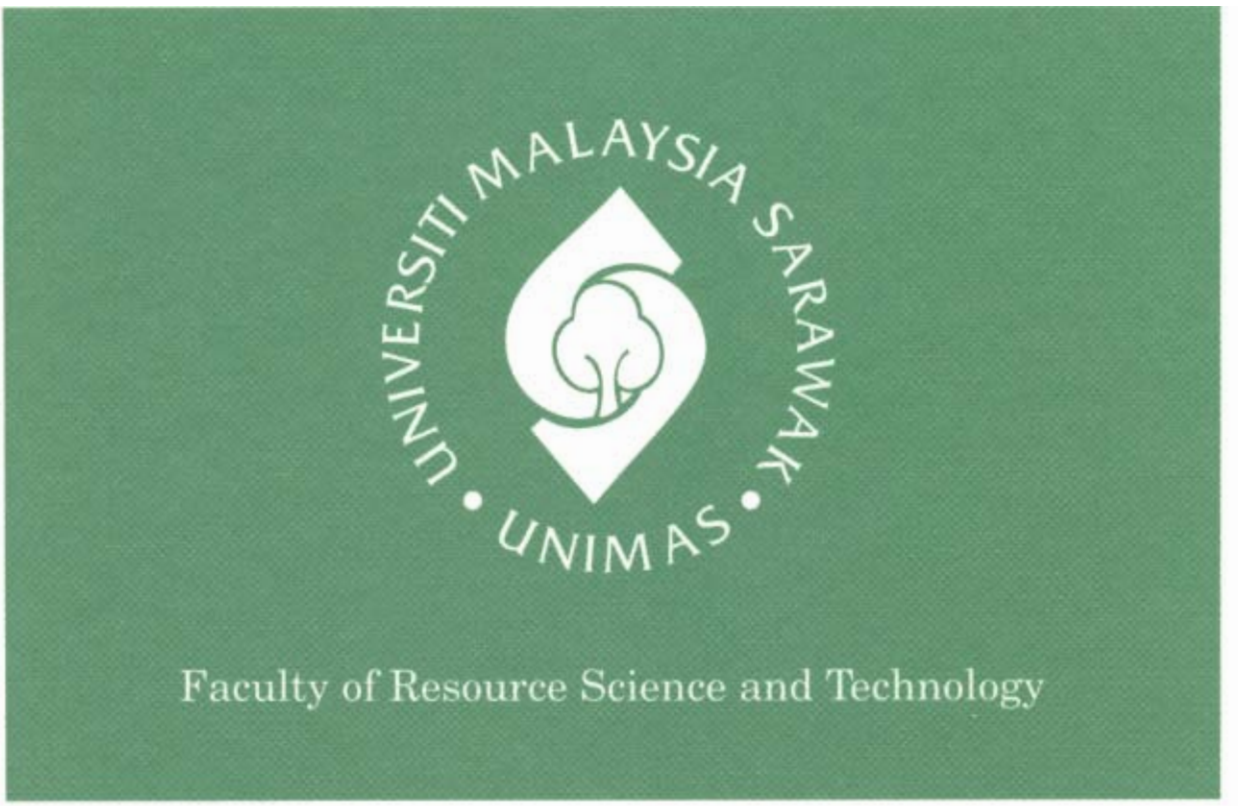

GENERATION OF EXPRESSION CONSTRUCT HARBOURING HUMAN TUMOUR SUSCEPTIBILITY GENE, TSG101 cDNA

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Bachelor of Science with Honours
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GENERATION OF EXPRESSION CONSTRUCT HARBOURING HUMAN TUMOUR SUSCEPTIBILITY GENE, *TSG101* cDNA

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This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours
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Generation of Expression Construct harbouring Human Tumour Susceptibility Gene *TSG101*, cDNA

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ABSTRACT

Human tumour susceptibility gene 101 (*TSG101*) is located on chromosome 11. This region was proposed to contain tumor suppressor gene. The proteins have an influence in cell growth and differentiation, and act as a negative growth regulator. Functional studies of *TSG101* should be performed to understand its function and expression in tissues. In this report, cDNA fragments of *TSG101* from human normal and tumor colorectal tissue are isolated by RT- PCR for the purpose of amplification of the gene fragments, clone into pTargeT™ Mammalian Expression Vector System and sent to DNA sequencing analysis. Information generated from DNA sequencing analysis is used to confirm the successful construction of the *TSG101* into expression vector. Expression constructed of *TSG101* into pTargeT™ vector can be used for further functional studies. The fragment of *TSG101* gene with a predicted size of 1315 bp was successfully amplified. However, cloning of *TSG101* in pTargeT™ vector was unsuccessful due to several problems. Therefore, DNA sequencing analysis was not performed.

Key words: *TSG101* gene, pTargeT™ Mammalian Expression Vector System, Reverse Transcription Polymerase Chain Reaction (RT- PCR), colon tissue, total RNA.

ABSTRAK

Tumour susceptibility gene 101 (TSG101) manusia adalah berada pada kromosom ke-11. Tempat ini adalah dicadangkan mempunyai gen penindas tumor. Proteinnya mempunyai kesan dalam pertumbuhan sel and pembahagian, dan bertindak sebagai pengawalaturan pertumbuhan negatif. Kajian di peringkat pengekspresan protein TSG101 di dalam sel mamalia perlu dijalankan untuk mengenalpasti dengan lebih lanjut fungsinya dan sistem pengekspresan yang terlibat. Oleh itu dalam laporan ini, fragmen cDNA bagi TSG101 diasingkan daripada sel usus normal dan tumor dengan menggunakan kaedah RT- PCR dan diamplifikasikan, diklonkan ke dalam pTargeT™ Mammalian Expression Vector System dan kemudian dianalisa dari segi jujukan nukleotidanya untuk memastikan gen yang diklonkan ke dalam vektor mengandungi jujukan nukleotida yang tepat. Fragmen daripada gen TSG101 bersaiz 1315 bp telah berjaya diasingkan. Walau bagaimanapun, fragmen ini tidak berjaya diklonkan ke dalam vektor pTargeT™. Maka analisis jujukan nukleotida periostin tidak dapat dijalankan.

Kata kunci: gen TSG101, pTargeT™ Mammalian Expression Vector System, Reverse Transcription Polymerase Chain Reaction (RT- PCR), tisu usus, keseluruhan RNA

CHAPTER 1

INTRODUCTION

1.1 Introduction

Human tumour susceptibility gene 101 (*TSG101*) is located on chromosome 11, subbands p15.1- 15.2, a site that shows loss of heterozygozity (LOH) in a variety of human malignancies, primarily breast cancer. This region was proposed to contain tumor suppressor gene (Xu *et al.*, 1998). The *TSG101* DNA size is about 46,63 kb and has 10 exons. It encodes a 381-amino acid polypeptide of 42.8 kD which is 94% identical to the mouse protein (Li *et al.*, 1997) and belongs to a group of apparently inactive homologs of ubiquitin-conjugating enzymes (Koonin *et al.*, 1997). The gene product contains a coiled-coil domain that interacts with stathmin, also known as oncoprotein which is a cytoplasmic phosphoprotein implicated in tumorigenesis. The protein may have an influence in cell growth and differentiation and act as a negative growth regulator (Bennett *et al.*, 2001). Recent study showed *in vitro* steady-state expression of *TSG101* was important for maintenance of genomic stability and cell cycle regulation (Bennett *et al.*, 2001).

My study goal is to clone *TSG101* and validate the premise that *TSG101* able to interact or function with control isolated environment. By this, generation of expression construct was needed. However, because of the problems associated with introns that may interrupt the expression in mammalian cells, construction of cDNA from mRNA by reverse transcriptase is essential. The cDNA is the DNA molecule corresponding roughly to the mRNA sequence. It is a double stranded molecule included in plasmid for expression in most cases. Reverse transcriptase was discovered by Howard Temin and David Baltimore in the 1970s. This technique uses reverse transcriptase enzyme that purified from cells infected with Moloney Murine Leukemia Virus (MMLV) (Alcamo, 1996). RT-PCR typically perform by used a reverse transcriptase to generate a single strand complementary DNA (cDNA) copy of mRNA molecules. This step is followed by polymerase chain reaction (PCR) to amplify the cDNA, to synthesis the corresponding second strand of a DNA molecule to restores the real cDNA molecule (Wikipedia., 2000).

In my study, pTARGET (5.7 kb) expression vector was used for cloning PCR-amplified genes and for expression of cloned PCR products in mammalian cells. pTARGET™ have single 3'-T overhang at the insertion site to improve the efficiency of ligation of a PCR product into the plasmid (pTARGET Mammalian Expression Vector System, Promega Corp., USA). Many thermostable polymerase (e.g., *Taq*) add a single deoxyadenosine, in a template independent fashion, to the 3'-end of amplified products. The amplified product is then directly ligated into a T-vector, which is a linearized vector containing a single thymidine at the 3'-ends. Thermostable polymerases that have a 3'--

>5' exonuclease activity generate blunt-ended products that also can be easily cloned into a T-vector by the addition of an "A-tailing" step following amplification (Brondky, 1996). The presence of inserted cDNA can be analyzed by restriction enzyme characterization procedure. Sanger dideoxy termination sequencing will be used to verify whether inserted gene is in the correct orientation for expression.

The objective of generating expression construct is to construct and maintain the *TSG101* gene in the expression vector, and to verify the insertion site. Verification of insertion site by DNA sequencing is to design primers that can be used to specifically sequence the ligation sites. The correct insertion site downstream from the driving promoter will lead to production of correct recombinant protein when transfected into mammalian cell line. The isolated *TSG101* cDNA are placed under the monitoring system in order to ensure the *TSG101* gene can function in the isolated environment. In this study, *TSG101* was used as model to answer the question on whether the *TSG101* cDNA can be inserted successfully in the pTARGET expression vector to construct an *in vitro* expression system. Upon successful cloning, the expression construct will be amenable for functional studies of *TSG101* gene in mammalian cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 LITERATURE REVIEW

The tumor susceptibility gene 101 (*TSG 101*) was initially identified in mouse cells in a screen for potential tumor suppressors using insertional mutagenesis in immortalized fibroblasts (Krempler *et al.*, 2002). *TSG101* is located on chromosome 11 band p15, a site that shows loss of heterozygozity in a subset of sporadic breast cancers and other human malignancies (Xu *et al.*, 1998). This was further supported by evidence that introducing a normal chromosome 11 or segments of this chromosome into breast cancer cells reverses their metastatic potential (Li *et al.*, 2001). Frequent abnormalities at 11p15 have been identified in human breast cancers and also in a variety of other human cancers, including lung cancer, testicular cancer and male germ cell tumor, stomach cancer, Wilms' tumor, ovarian cancer, bladder cancer, myeloid leukemia, malignant astrocytomas and other primitive neuroectodermal tumors, and infantile tumors of adrenal and liver (Hampl *et al.*, 1998).

The protein encoded by *TSG101* gene belongs to a group of apparently inactive homologs of ubiquitin-conjugating enzymes. The function of the gene product remains unknown, but on the basis of structural features it has been postulate that it may

conjugate with ubiquitin and prevent ubiquitin-dependent degradation of inhibitors of cyclin-dependent kinases required for cell cycle progression (Micheal *et al.*, 1998). The gene product contains a coiled-coil domain that interacts with stathmin, an oncoprotein that has cytoplasmic phosphoprotein implicated in tumorigenesis. The protein may play a role in cell growth and differentiation and act as a negative growth regulator (O'Boyle *et al.*, 2002). The presence of DNA-binding motifs in the *TSG101* protein and a proline-rich region near the leucine zipper DNA-binding motifs suggests that *TSG101* may be a transcription factor that is a downstream effector of stathmin action (Li *et al.*, 1997).

Recent studies have showed *in vitro* steady-state expression of *TSG101* gene appears to be important for maintenance of genomic stability and cell cycle regulation (Bennett *et al.*, 2001). *TSG101* is expressed in all tissues throughout development, but strong overexpression of *TSG101* also leads to the inhibition of cell division and cell death, thus proposing that the amount of *TSG101* within a cell is essential for its function (Krempler *et al.*, 2002). According to Li and co-workers (1997), mutations in two *TSG101* alleles were identified by both genomic DNA analysis and cDNA analysis in three breast cancers, and by cDNA analysis in a fourth tumor. There is no *TSG101* abnormalities observed in transcripts or genomic DNA of matched normal breast tissue from these breast cancer patients (Carter, 2002). This suggested that mutations and alternative splicing in the *TSG101* gene occur in high frequency in breast cancer and suggest that defects occur during breast cancer tumorigenesis or progression (Balz *et al.*, 2002). Aberrant products from this *TSG101* gene are observed quite frequently in human breast cancer. The occurrence of these abnormal products is tightly associated with tumor

grade and the mutation status of another important tumor-suppressor gene called *p53*. To date, the actual and detailed biological role of *TSG101* in normal and cancer cancerous cells is still unknown (Krempler *et al.*, 2002).

Insertion of *TSG101* cDNA into expression vector and verification of insertion sites in order to study the function of the *TSG101* gene leads to generate of a *TSG101* expression construct. As known, not all the inserted gene will be compatible with the expression vector. In order to construct, maintain the *TSG101* gene in the expression vector and verifying the insertion site, generation of expression construct is needed. An isolated *TSG101* cDNA are placed under the monitoring system in order to ensure the *TSG101* gene can function in an isolated environment.

An expression construct is defined as the expression vector with the inserted cDNA at the T overhang (Ridgway, 2001). An expression vector is a relatively small DNA molecule that is used to introduce and express a specific gene into a target cell. Once the expression vector is inside the cell, the protein that is encoded by the gene is produced by cellular transcription and translation machinery (Wikipedia, n.d.). In general, cloning vectors that are used in many molecular biology gene cloning need not result in the expression of protein. Expression vector are often specifically designed to contain regulatory sequences that act as enhancer and promoter regions, and lead to efficient transcription of the gene that is carried on the expression vector (Wikipedia, n.d.).

The pTARGET™ mammalian expression vector system is a system for cloning PCR product and for expression of cloned PCR product in mammalian cells (pTARGET Mammalian Expression Vector System, Promega Corp., USA). In this study, pTARGET™ was used as expression vector because of its several advantages. The single A residues at the ends of a PCR product can serve as a one-base overhang to facilitate ligation, when the complementary thymidine (T) nucleotide will be added to the 3' ends of the pTARGET™. It carries the human cytomegalovirus immediate-early (CMV) promoter that allows strong, constitutive expression in many cell types. Transient transfection is an introduction of episomal expression vector into mammalian tissue culture cells or short term expression experiments (Brondyk, 1996). In transgenic mice, expression of the chloramphenicol acetyltransferase (CAT) gene under the regulation of CMV promoter was observed in 24 of the 28 tissues examined. The vector is maintained as an episome in cells expressing the SV40 large T antigen, leading to even higher levels of expression. The presence of an intron near the cDNA insert is critical for high level of expression of the majorityof cDNA insert; the exact level of expression enhancement will depend upon the gene that is cloned (Promega). The pTARGET expression vector also have blue/white screening to allow the easy identification of recombinant clones and the single digest removes the inset DNA (pTARGET Mammalian Expression Vector System, Promega Corp., USA).

In cloning of RT-PCR products, the protruding ends anneal to form a hybrid molecule in two distinct steps. First, an intermolecular reaction occurs between one end of the vector and insert to generate a linear molecule. Second, an intramolecular reaction

occurs between the two free ends of each linear DNA to generate a circular molecule. The insert may have two orientations relative to the vector (forward and reverse) and this orientation can be critical. When cloning into a pTARGET expression vector, the gene may be expressed as sense or antisense, depending on the orientation relative to the promoter in the vector (McLaren, 1990). The problems is the orientation of the cDNA insert cannot be controlled, this may be cause by nonrecombinants due to vector molecules escaping T addition or false recombinants due to contaminating exonucleases removing the overhanging T residue (Newton and Graham, 1997). It is absolutely essential that cDNA be placed in the sense orientation downstream from the promoter. In other words, the poly (T) strand or (-) strand of the cDNA should be 3'to 5' downstream from the driving promoter in order to make 5'to 3' mRNA. Insert orientation obviously affects protein expression when transfected into mammalian cells. Expression is possible only if the start codon sits adjacent to the promoter region present on the plasmid. Reverse orientation will juxtapose stop codon with the promoter and yield a negative protein expression (Enthusiast, 2005). The purpose of verification of insertion site by DNA sequencing is to design primers that can be used to specifically sequence the ligation sites to ensure that the sequence at the insertion sites is not altered, including a potential missing base, base addition, or base substitution (Wu *et al.*, 1997). Verification of insertion sites is principal step going ahead to further transfection of mammalian cells with the pTARGET™. This study will facilitate further investigate of expression of the *TSG101* gene in mammalian cell lines in order to fully understand its function.

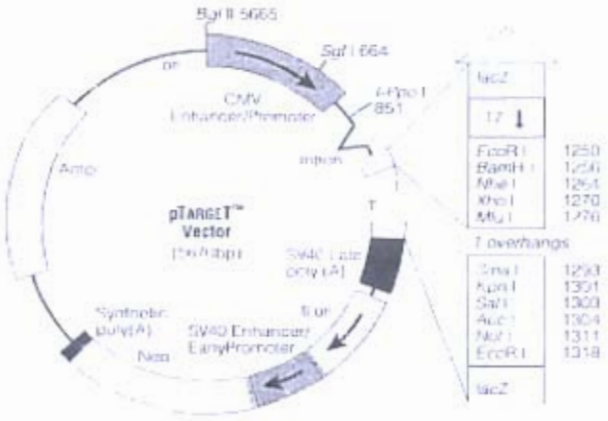

Figure 1: pTARGET expression vector (Taken from pTARGET Mammalian Expression Vector System, Promega).

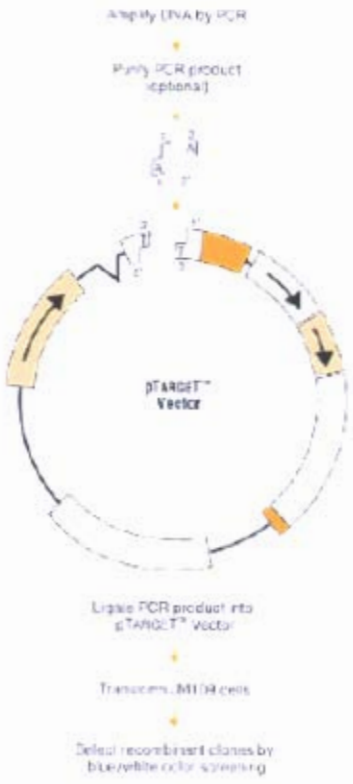

Figure 2: Flow diagram of preparation and cloning of DNA using pTARGET™ Vector (Taken from pTARGET TM Vector: A New Mammalian Expression T-Vector, Promega Notes Magazine Number 58, 1996, p. 02).

CHAPTER 3

MATERIALS AND METHODS

3.1. RNA Isolation

The commercially available total RNA (normal and tumor) from the human colorectal tissue was provided by post-graduated student (Ma Xiang Ru, Human Molecular Genetic Lab, UNIMAS).

3.2. Reverse Transcriptase (RT) Reaction

Total RNA (1μL) was added to a sterile eppendorf tube along with 1 μL oligo dT primers (0.25mg/ml) (Cat no. C1101, Promega Corp., USA) and 11.5 μL nuclease free water. The reaction was heated to 70°C for 10 minutes and immediately transfers to ice and centrifuged. After that, 4 μL of 5X first strand buffer, 1 μL 10 mM dNTP mix (Cat no. U1515, Promega Corp., USA) and 0.5 μL RNase inhibitor (Cat no. N2611, Promega Corp., USA) was added. The reaction was mixed gently and incubated at 42°C for 2 minutes. A volume of 1 μL MMLV reverse transcriptase (Cat no. M1701, Promega Corp., USA) was added. The reaction was incubated at 42°C for 50 minutes and inactivated by heating at 70°C for 15 minutes. The total volume of 20 μL cDNA produced was stored at -4°C.

3.3. Polymerase Chain Reaction (PCR)

PCR was performed by using MJ DNA Engine (Model N0.: PTC 2000) in total volume of 25 μL reaction mixture containing 10x PCR buffer (Cat no. EP0404, Fermentas,USA); 25mM $MgCI_2$; 10mM dNTPs mix (Cat no.U1515, Promega Corp., USA); each 25pmol/μL reverse and forward primers; 5unit/μL Fermentas Taq polymerase (recombinant) (Cat no. EP0404, Fermentas, USA); cDNA template and sterilized ddH_2O. Besides, the Go buffer (Cat no. M3001, Promege Corp., USA) also be used. The control (without template cDNA) was also prepared. The forward primer is GTGCCGACTTCCTGTTGTTT and reverse primer is CCTCCAGCTGGTATCAG AGAA. The parameters for amplification cycles was carried out under the thermal cycling profile of: 1 minutes of initial denaturing at 96°C, 50 seconds of denaturing at 95°C, 1 minute 30 seconds of annealing at optimum annealing temperature of primers, 1 minute 30 seconds of elongation at 72°C and 10 minutes of final elongation at 72°C, with the total of 39 cycles. After the PCR reaction was completed the products was stored in fridge at 4°C until electrophoresis. The diagrams below showed the stock, final concentration and final volumes used in both different reactions.

	Stock concentration	Final concentration	Volume	Control
PCR buffer	10 x	1 x	2.5 µL	2.5 µL
MgCI2	25mM	1mM	1 µL	1 µL
dNTP mix	10mM	0.2mM	0.5 µL	0.5 µL
Forward primer	25 pmol/µL	1 pmol/µL	1 µL	1 µL
Reverse primer	25 pmol/µL	1 pmol/µL	1 µL	1 µL
Fermentas Taq polymerase	5 u/µL	0.02 u/µL	0.1 µL	0.1 µL
sterilized ddH_2O			16.9 µL	18.9 µL
cDNA			2 µL	-
Total volume			**25 µL**	**25 µL**

Table 1: Ingredients and their appropriate amount used in each PCR reaction.

	Stock concentration	Final concentration	Volume	Control
Go buffer	10 x	1 x	5 µL	5 µL
MgCI2	-	-	-	-
dNTP mix	10mM	0.2mM	0.5 µL	0.5 µL
Forward primer	25 pmol/µL	1 pmol/µL	1 µL	1 µL
Reverse primer	25 pmol/µL	1 pmol/µL	1 µL	1 µL
Fermentas Taq polymerase	1 u/µL	0.02 u/µL	0.5 µL	0.5 µL
sterilized ddH_2O			15.0µL	17.0µL
cDNA			2 µL	-
Total volume			**25 µL**	**25 µL**

Table 2: Ingredients and their appropriate amount used in each PCR reaction.

3.4. Analysis of RT-RCR Products: Agarose Gel Electrophoresis

3.4.1. Preparing 50X TAE buffer and agarose gel

A 242g of tri-base was added together with 57.1g acetic acid glacial, and with 100 mL 0.5M EDTA solution. A mixture was topped up to 1 Liter with distilled water. The pH was adjusted to pH7.2 using acetic acid. To prepare 2 Liter 1X TAE buffer, an amount of 40 mL 50X TAE buffer was added and top up to 2 Liter with distilled water. For 1% agorose gel, 0.4g of agarose powder was added. An amount of 40 mL of 1X TAE buffer was added to agarose powder. The mixture was dissolved in a microwave oven for 1 minute and poured into the tank (250-300mL). When the gel was solidifying after 30 minutes, the tank was filled with 1X TAE buffer as running buffer. The staining solution was prepared by adding 500 mL distilled water with 25 µL of ethidium bromide.

3.4.2. Agarose gel electrophoresis

A 10 µL of each PCR product was mixed with 2 µL of commercially available dye (6x Promega) and loaded into the wells on a 1.0% agarose (Promega) gel with 1x TAE as running buffer. Electrophoresis was carried out from cathode to anode with a constant voltage of 90V for 30 minutes at room temperature. A 1kb DNA ladder (Cat no. SM 0311, Gene Ruler) was used as molecular weigh standards. Electrophoresis was stopped when the dye had reached the end of the gel. After staining with ethidium bromide (EtBr)

for 30 minutes, the amplified DNA fragment in the gel was visualized under UV illumination. A photograph was taken by using a Polaroid camera.

3.4.3. Gel Extraction

Prior to downstream applications of RT-PCR products as in cloning and sequencing, the products were purifies using the Viogene® Gel extraction System according to manufacturer's instruction. A brief summary of the protocol is as follows: A clean, sharp scalpel or razor blade was used to excise the gel slice containing the DNA fragment of interest. The gel was placed into a sterile 1.5 mL centrifuge tube. A 500 µL GEX buffer was added to it. The mixture was incubated at 58°C for 10 minutes until the gel is completely dissolved. The tube was mixed by inversion every 1-2 minutes during incubation. Incubation was stopped when the gel has been completely dissolved. The gel mixture was allowed to cool down to room temperature. A Gel-M column was placed onto a collection tube. No more than 700 µL dissolved gel mixture was loaded into a column. The mixture was centrifuged for 1 minute and the flow-through was discarded. The column was washed once with 500 µL of WF Buffer centrifuged for 1 minute and the flow-through was discarded. Then, the column was washed once with 700 µL WS Buffer centrifuged for 1 minute and the flow-through discarded. The column was centrifuged at full speed for another 3 minutes to remove residual ethanol. The column is placed onto a new 1.5 mL centrifuge tube. A 30 µL of Elution Buffer was added onto the centre of the membrane. The column was let to stand for 2 minutes, and centrifuged for 2 minutes to elute the DNA.

3.4.4. Sequencing of PCR product

The purified recombinant plasmid was sent for sequencing at First BASE Laboratories Sdn Bhd (604944 –X).

3.5. Cloning of the DNA fragment

3.5.1. Ligation into pGEMT Easy Vector Syatem and pTARGET™ expression vector

RT-PCR products were cloned using pGEMT Easy Vector System (Cat no. A1380, Promega Corp., USA). The RT-PCR products also cloned using pTARGET™ vector (Cat no. A1410, Promega Corp., USA). The vector and control insert DNA tubes were be briefly centrifuged to collect the contents at the bottom of the tube. The ligation reactions were set up as described below (Table 3 and 4).

Reagent	Standard reaction	Positive control
2X Rapid Ligation Buffer, T4 DNA ligase	5 µL	5 µL
pGEMT Easy Vector (50ng)	1 µL	1 µL
PCR product	3 µL	-
Control insert DNA	-	2 µL
T4 DNA ligase	1 µL	1 µL
Deionized water to a final volume of	10 µL	10 µL

Table 3: Set up of ligation reactions for pGEMT vector

Reagent	Standard reaction	Positive control
T4 DNA Ligase 10X Buffer	1 µL	1 µL
pTARGET™ vector (60ng)	1 µL	1 µL
PCR product	3 µL	-
Control insert DNA	-	2 µL
T4 DNA ligase	1 µL	1 µL
Deionized water to a final volume of	10 µL	10 µL

Table 4: Set up of ligation reactions for pTERGET expression vector

The reactions were prepared in 0.5ml tube that has low DNA binding capacity. They were mixed by gentle pipetting and then incubated for 1 hour at room temperature.

Following ligation, the ligated vectors were transformed into *E.coli* JM109 competent cells. These cells were prepared following the protocol by Sambrook *et al.* (1989).

3.5.2. Transformation of recombinant pGEMT vector and pTARGET™ vector into JM109 competent cells

The recombinant pGEMT and pTARGET™ were transformed into *E. coli* JM109 competent cells. These cells were prepared following the protocol by Sambrook *et al.* (1989). Two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction were prepared. The plates were equilibrated to room temperature prior to plating. Basically, the tubes containing the ligation reaction were centrifuged to collect the contents at the bottom of the tube. 2 µL of each ligation reaction were added to a sterile 1.5 mL microcentrifuge tubes on ice. The frozen JM109 High Efficiency Competent Cells were removed from -70°C storage and placed on ice until just thawed. The cells were mixed by gently flicking

of the tube. Then, 50 µL of cells were carefully transferred into each tube. The tubes were gently flicked to mix and placed on ice for 20 minutes. The cells were subjected to heat shock for 45-50 seconds at exactly 42°C in a water bath. The tubes was immediately returned to ice for 2 minutes. After that, 950 µL of room temperature SOC medium were added to the tubes containing cells transformed with ligation reaction. The tubes were incubated for 1.5 hours at 37°C with shaking (~150 rpm). A 100 µL of each transformation reaction was plated into duplicate antibiotic plates. The plates will be incubated overnight (16-24 hours) at 37°C to obtain desired colonies of transformants.

3.5.3. Screening for colonies harbouring PCR product

Insertion of DNA fragment into pGEMT-T Easy Vectors and pTARGET expression vectors respectively will disrupt the nucleotide sequence that codes for ß-galactosidase hence, recombinants can be identified by blue-white screening on plates containing X-gal and IPTG. With few expectations as in the case of DNA fragments cloned in frame with *lacZ* gene or introduction of mutations, colonies that contain the desired PCR products are usually white in color (Promega, 1999).

Single white colonies that were suspected to have incorporated the desired RT-PCR product were inoculated into 5 mL of LB broth and incubated overnight with shaking at 200 rpm after which plasmid isolation using Eppendorf's FastPlasmid™ Mini was performed. The plasmid isolation protocol is as follows: 1.5 mL of fresh bacterial culture

was pelleted by centrifugation at 14000 rpm for 1 minute in 2 mL Culture Tube provided by the manufacturer after which the medium is removed by decanting. Each culture tube is added with 400 µL of ice-cold Complete Lysis Solution, and mixed thoroughly by constant vortexing at the highest setting for 30 seconds. The lysate were incubated at room temperature for 3 minutes, transferred into a Spin Column Asembly by pipetting and centrifuged for 60 seconds at 14000 rpm. Following centrifugation, 400 µL of diluted Wash Buffer was added into each Spin Column Assembly which was then subjected to further centrifugation at 14000 rpm for 60 second. An additional centrifugation step at 14000 rpm was carried out to dry the Spin Column Assemblies before the Spin Columns were transferred into Collection Tube. Then, 50 µL of Elution Buffer was added directly to the center of each Spin Column membrane. The Collection Tubes holding the Spin Columns were centrifuged again at 14000 rpm for 60 seconds and subsequently, the Spin Columns were removed. A 5 µL of the eluted plasmid DNA were used for Restriction Digestion analysis while the rest were stored at -20 °C.