CHAPTER III
MATERIALS

3.1 Shrimp samples

Wild female broodstock of black tiger shrimps (*P. monodon*) at different ovarian stages were either caught from the Gulf of Thailand or purchased from shrimp farms in Thailand.

3.2 Bacterial strains

Escherichia coli strain DH5α {*supE*44, Δ*lac*U169 (ϕ80 *lacZ* ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1}and BL21(DE)pLysS *E. coli* B F^- *dcm ompT hsdS*(rB^- mB^-) *gal* λ(DE3)[pLysS Camr] were used as host for transformation of recombinant plasmid and protein expression in *E. coli*, respectively.

3.3 Mammalian cell

COS-1 cells (kindly provided by Asst. Prof. Witoon Tirasophon) were used as a host for transient DNA transfection.

3.4 Culture medium

3.4.1 Bacterial cell culture medium

Luria-Bertani (LB) broth is composed of 1% (w/v) peptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl and contains 2% (w/v) of Bacto Agar for LB agar preparation. Before use, the medium was autoclaved and added with antibiotics; ampicillin (100 μl/ml) or chloramphenicol (34 μl/ml) for recombinant plasmid selection.

SOB medium composing of 2% (w/v) peptone, 0.5% yeast extract, 0.05% NaCl, 2.5mM KCl) was used for DHα competent cell preparation.

3.4.2 COS-1 cell culture medium

One case of Dulbecco's modified eagle medium (DMEM) were mixed with 3.71 g of sodium hydrogen carbonate ($NaHCO_3$) in 1 liter of autoclaved HPLC water and the pH was adjusted to 7.1-7.2. The medium was filtrated and supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine. Before use, penicillin (100 U/ml) and streptomycin (100 µg/ml) were added to the medium for prevention of bacterial contamination.

3.5 Plasmid vectors

pGEM®-T easy (Promega) served as a vector for recombinant plasmid DNA cloning. pET-17b vector (Novagen) was used for protein expression system driven by T7 RNA polymerase in *E. coli* whereas pcDNA3.1 (+) (Invitrogen) was utilized as a vector for transient DNA transfection in mammalian cells for recombinant protein expression. Description of vector and physical maps was shown in figure 3.1-3.3, respectively.

3.6 Modification and restriction enzymes

ImpromII Reverse transcriptase	Promega
Taq DNApolymerase	Fermentus
Pfu DNA polymerase	Promega
Terminal deoxynucleotidyl transferase	Promega
T4 DNA ligase	Fermentus
Ribonuclease A	Sigma
Lysozyme	Sigma
Deoxyribonuclease I (RQ1 DNase)	Promega

List and detail of restriction enzyme in this thesis are shown in Table 3.1 as follows:

Table 3.1 Description of restriction enzymes (Promega) for recombinant plasmid DNA digestion

Restriction enzyme	Recognition site	Reaction buffer	Incubation Temperature
*Bam*H I	G/GATCC	E	37 °C
*Eco*R I	G/AATTC	H	37 °C
*Hin*d III	A/AGCTT	E	37 °C
Nde I	CA/TATG	D	37 °C

/ indicate the cleavage site

Components of each reaction buffer contain:

Buffer E: 6 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$ and 1 mM DTT

Buffer D: 6 mM Tris-HCl (pH 7.9), 150 mM NaCl, 6 mM $MgCl_2$ and 1 mM DTT

Buffer H: 900 mM Tris-HCl (pH7.5), 500 mM NaCl, 100 mM $MgCl_2$ and 10 mM DTT

3.7 Oligonucleotide primers

Oligonucleotide primers were ordered from Sigma-Proligo (Singapore) and Pacific Sciences. The nucleotide sequences and temperature (Tm) of primers were shown in Table 3.2

Table 3.2 List of nucleotide sequences and Tm of primers

Experiments	Primers	Sequence (5'-3')	Tm (˚C)
Rapid amplification of cDNA ends (RACE)	Oligo dT	CCGGAATTCAAGCTTCTAGAGG ATCCTTTTTTTTTTTTTTT	70
	PM1	CCGGAATTCAAGCTTCTAGAGG ATCC	63
	D1r-F1	CAGACACAGACGCTCAGGAA	62
	D1r-F2	TACTCACGCCTCTACCTCTAC	62
	EX-DP-F	ATGGGCGGCTTGGAGGCGTC	66
	EX-DP-R	TCAAATCGCCGAAATCTGCTC	58
	D1r-R3	AGGTAGAGGCGTGAGTAGATGC	62
	D1r-R4	GAGGATCCGTTCTGGTGGTG	62
mRNA expression amplification	Actin_F	GACTCGTACGTGGGCGACGAG	62
	Actin_R	AGCAGCGGTGGTCATCTCCTG	65
	D1r-F1	CAGACACAGACGCTCAGGAA	62
	i3-DAR-R	CGGGATCCTCACACTGTGGTCG CCGCCTTGG	74
Cloning recombinant plasmid DNA in *E.coli* and COS-1 cell for protein expression	C-ter-DAR-F	GCCATATGAACCAGGAGTTTCA GGAGGC	66
	C-ter-DAR-R	CGGGATCCTCAAATCGCCGAAA TCTGCT	66
	Dar-F	CCCAAGCTTACCATGGGGCGGC TTGGAGGCGTC	73
	Dar-R	ATGAATTCTCAAATCGCCGAAA TCTGCTC	62

3.8 Miscellaneous

Tri reagent®	Molecular Research Center
Diethyl pyrocarbonate (DEPC)	USB corporation
Deoxynucleotide Triphosphates (dNTPs)	Promega
100 bp + 1.5 kb DNA marker	SibEnzyme
Lambda *Hin*d III DNA marker	Promega
QIAquick Gel Extraction Kit	QIAGEN
QIAprep Spin Miniprep Kit	QIAGEN
Polyvinylidene difluoride (PVDF) membrane	Bio-Rad
Broad range SDS PAGE standards	Bio-Rad
Ampicillin	Sigma
Chloramphenical	Sigma
Lipofectamine™ 2000	Invitrogen
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Carl Roth
3-Isobutyl-1-methyxanthine (IBMX)	Sigma
Forskolin	Sigma
Cyclic AMP XP™ Assay Kit	Cell signaling technology

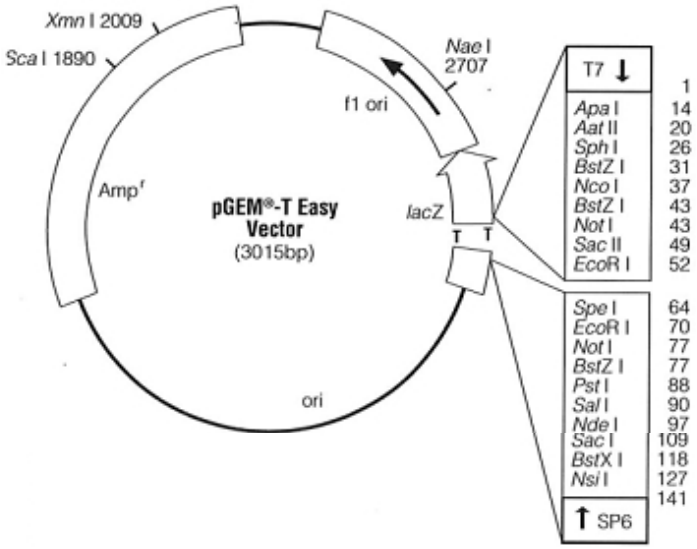

Figure 3.1 Physical map of pGEM®-T Easy vector (Promega)

pGEM®-T Easy vector is the linearized vector with thymidine at both 3' ends that is suitable for cloning of PCR product and contains ampicillin resistance gene. The multiple cloning region is located between T7 and SP6 promotors and flanked by the coding sequence of β-galactosidase gene for blue-white colony selection.

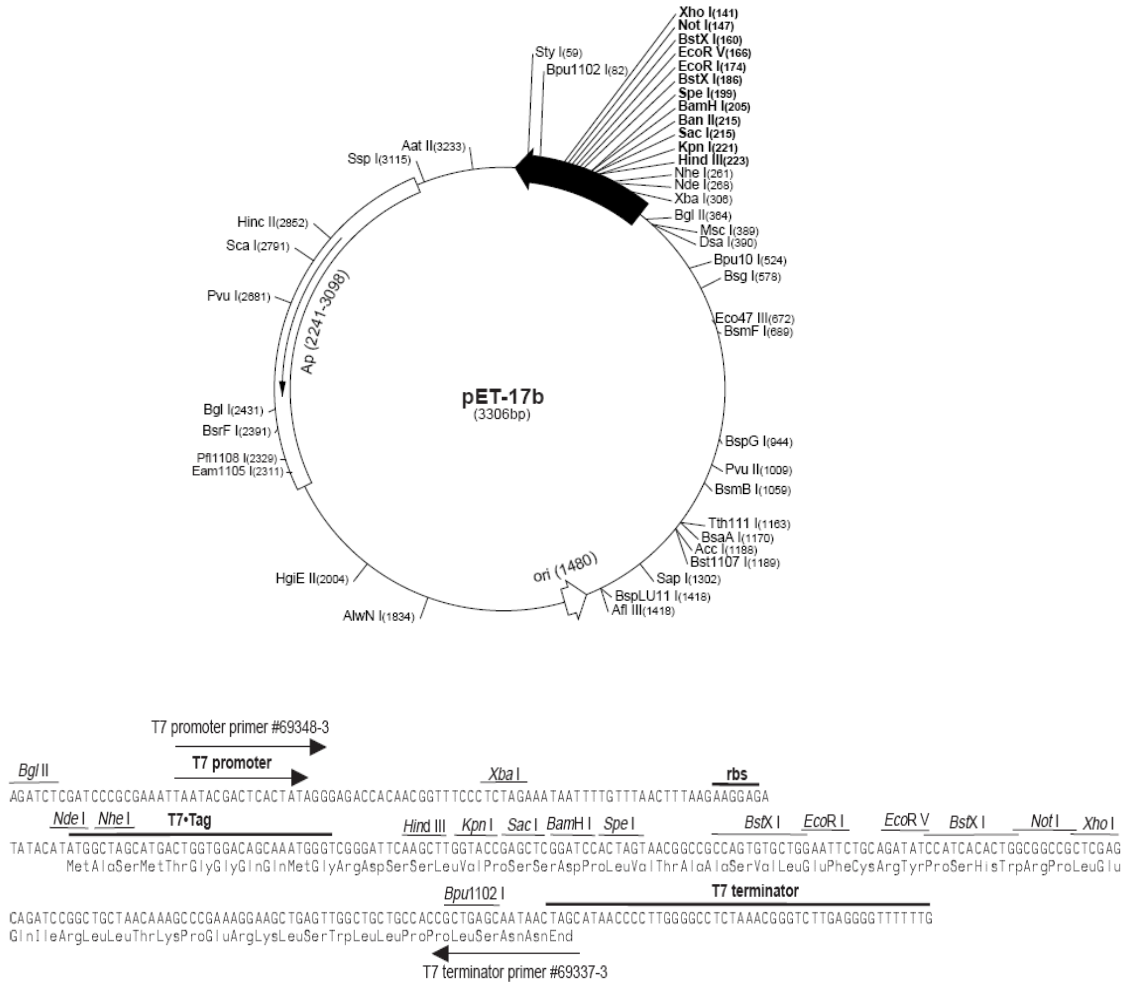

Figure 3.2 Physical map of pET-17b vector map (Novagen)

pET-17b carries an N-terminal T7 tag sequence followed by multiple cloning sites. The vector contains an origin of replication from pBR322 (Ori) and an amplicilin resistance (*bla*) gene (Ap). The position of other sequences are indicated as follows;

T7 promotor	333-349
T7 transcription start	332
T7 tag coding sequence	237-269
Multiple cloning site (Hind-XhoI)	141-228
T7 terminator	28-74
pBR322 origin	1480
Bla coding sequence	2241-3098

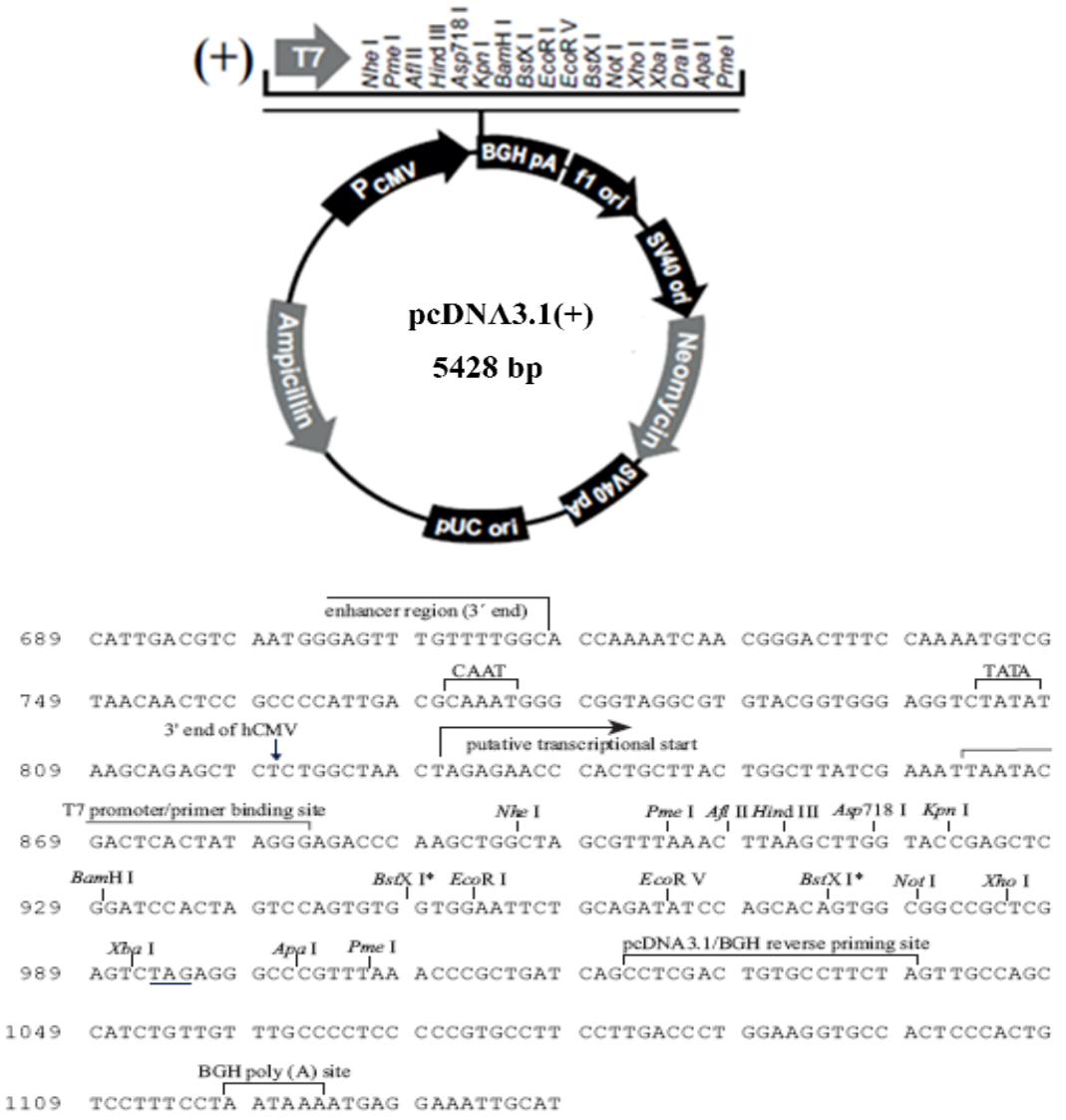

Figure 3.3 Physical map of pcDNA3.1 (+) vector and multiple cloning sites (Modified from Invitrogen)

An immediate-early/enhancer of Human cytomegalovirus (CMV) and T7 promotor is used as a promoter/priming site. BGH is bovine growth hormone polyadenylation signal. F1 ori is f1 origin replication. SV40 ori is early promoter and origin. SV40 pa is SV40 early polyadenylation signal. pUC ori is pUC origin replication. Neomycin and amplicilin are antibiotic for selection of stable transfections in mammalian cells and in *E. coli*, respectively.