CHAPTER 3:

METHODOLOGY

3.1 Computational Identification of *Litopenaeus vannamei* miRNAs

3.1.1 Sequence Collection of miRNAs

Known mature miRNA sequences from invertebrate groups were obtained from the miRBase Registry Release 11.0 (http://microrna.sanger.ac.uk/sequences/index.shtml). Those invertebrate groups included *Arthropoda* (471 sequences), *Nematode* (249 sequences), *Platyhelminthes* (73 sequences) and *Urochordata* (127 sequences). All mature miRNA sequences were aligned using default settings of ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/) to remove any duplicated miRNA mature sequences. After the sorting process, the non-redundant mature miRNA set comprised 604 sequences.

3.1.2 Potential miRNAs Candidates Screening Process

The screening process for potential miRNA candidates involved 4 steps. First, the non-redundant mature miRNAs served as queries to search against *Litopenaeus vannamei* expressed sequence tags (EST) and genome sequence survey (GSS) sequences using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI (http://www.ncbi.nlm.nih.gov/). The parameters used were: wordsize of 7 (Weaver *et. al.*, 2006), expect threshold of 100. EST and GSS showing high similarity [≤ than 4 mismatches (deduced from miRBase, 2008; Norden-Krichmar *et al.*, 2007); or more than 15 nucleotide matches or core seed matches (Fu *et al.*, 2008)] were selected. Next, EST and GSS with repeated accession numbers were manually removed. They were compared

against Rfam database Version 9.0 (http://rfam.sanger.ac.uk/) using BLAST to remove transfer RNA and ribosomal RNA sequences. Following that, the remaining EST and GSS sequences were checked against the non-redundant protein sequence (nr) database using BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). EST sequences with miRNA gene spanning along the protein coding sequences were removed. This was because miRNA resided in the intergenic, intronic region of protein coding sequence or exonic region of non-coding sequence. Those with significant E-value cutoff of $\leq 1 \times 10^{-4}$ and matches with more than 10 amino acid residues were discarded. Finally, using default parameters, mfold Version 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) was applied to predict the secondary folding structure (Zuker, 2003). The EST and GSS sequences that showed the features of miRNA precursors were selected, using the following criteria:

i. The length of animal miRNA precursor is about 60-120 nucleotides (Ambros *et al*., 2003[a]; Lim *et al*., 2003[b]; Tang and Maxwell, 2008).

ii. The candidate miRNA should have unbranched, fold-back (hairpin) secondary structure (Lim *et al*., 2003[b]; Ghosh *et al.*, 2007).

iii. The candidiate miRNA should have hairpin mismatches ≤ 7 nucleotides (Krol *et al*., 2004; Weber, 2005; Tang and Maxwell, 2008).

iv. The candidiate miRNA should preferably start with Uracil on 5' mature miRNA sequence (deduced from miRBase, 2008; Bentwich, 2005; Tang and Maxwell, 2008).

v. The candidiate miRNA should have hairpin matches with minimum of 12 nucleotides (deduced from miRBase, 2008; Tang and Maxwell, 2008).

vi. The candidiate miRNA should have less than 5 pairs of G:U reside at the hairpin stem (Tang and Maxwell, 2008).

vii. Predicted miRNA has same location with the known miRNA on the hairpin arm (Palkodeti *et al*., 2006; Tang and Maxwell, 2008).

viii. The predicted miRNA precursor is usually a minimal free energy (MFE) of -20kcal/mol or lower (Lim *et al*., 2003[b]; Tong *et al*., 2006).

ix. The candidiate miRNA should have well conserved "seed" or "nucleus" regions located at 2-7 nucleotides of the 5' miRNA sequence (Palkodeti *et al*., 2006; Tang and Maxwell, 2008).

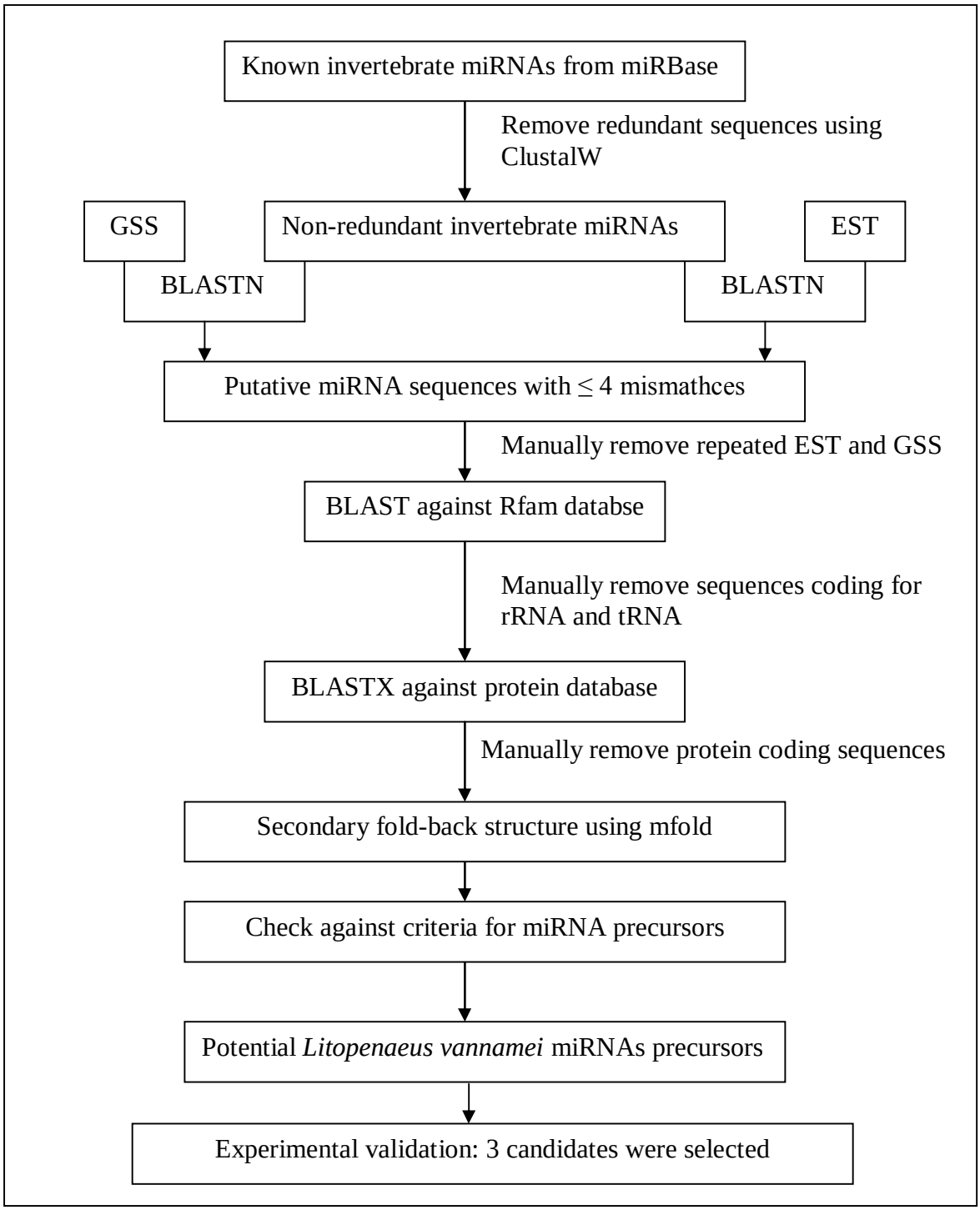

Figure 3.1
Flow Chart of Computational Identification of Potential *Litopenaeus vannamei* miRNA

3.1.3 Primer Design

The three EST candidates that best satisfied the criteria for miRNA precursors were subjected to Primer3 Input 0.4.0 (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 2000) for primer design. The parameters such as primer size, primer melting temperature (T_m), primer GC content and product size were adjusted as follows: The primer size should range from 18 to 25 base pairs; T_m differences between both primers should less be than 5°C; primer GC contents should range from 40-60%; and finally, the PCR product should include the miRNA fold-back precursor. Then, both forward primer and reverse primer were checked for self-complementary and hairpin formation using Oligonucleotide Properties Calculator Version 3.23 (http://www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe, 2007). Next, they were checked for gene specificity by aligning against the *Litopenaeus vannamei* dbEST in the web interface BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI (http://www.ncbi.nlm.nih.gov/). *Litopenaeus vannamei* beta-actin gene was selected as a positive control for reverse transcription PCR (Wang *et al*., 2006). The selected primers were sent to Bioneer Inc., Korea for synthesis. Table 3.1 shows the combination of primers designed to amplify three putative *Litopenaeus vannamei* miRNA precursors. Table 3.2 shows the primer set for *Litopenaeus vannamei* beta-actin gene.

Table 3.1
Primers for Putative *L. vannamei* miRNA Precursors

Putative *L. vannamei* miRNA precursors	Primer	Length for RNA transcript
lva-miR-272	272F: 5'-GAAGGCTGCCAAAGAAAAGA-3' 272R: 5'-CGGCGTCTACATCTTGAACA-3'	233bp
lva-miR-256	256F: 5'-CCCATACCTGCCACCATTAC-3' 256R: 5'-GCTGAAATGTTAAAAGGAAAACTTGA-3'	266bp
lva-miR-1476-3p	1476F: 5'-GAACACTTCTCGGGCTTGAC-3' 1476R: 5'-CAACATGGACCAGACGAATG-3'	371bp

Table 3.2
Primers Set of *Litopenaeus vannamei* Beta-actin Gene

L. vannamei beta-actin gene (AF300705)	Primer	Length for RNA transcript	Reference
β-actin	β-actinF: 5'-TGTGTGACGACGAAGTAGCC-3' β-actinR: 5'-TGGTCGTGAAGGTGTAGCCA-3'	604bp	Wang *et al*. (2006)

3.2 Sample Collection

The edible adult stage of *Litopenaeus vannamei* with an average weight of 25.0g per tail was selected. This was because those potential precursor miRNAs were found in the EST of *Litopenaeus vannamei* adults. About half a kilogram of live and healthy pacific whiteleg shrimps were bought from a sea food restaurant. The pacific whiteleg shrimps were put in ice to reduce pain and stress. Organs including hepatopancreas, brain, eyestalk, leg, gill tissues and muscle tissues were immediately dissected from the shrimps and frozen in

liquid nitrogen. The process was done rapidly to reduce the degradation of RNAs. After that, these samples were then stored at -80°C before RNA and DNA extraction.

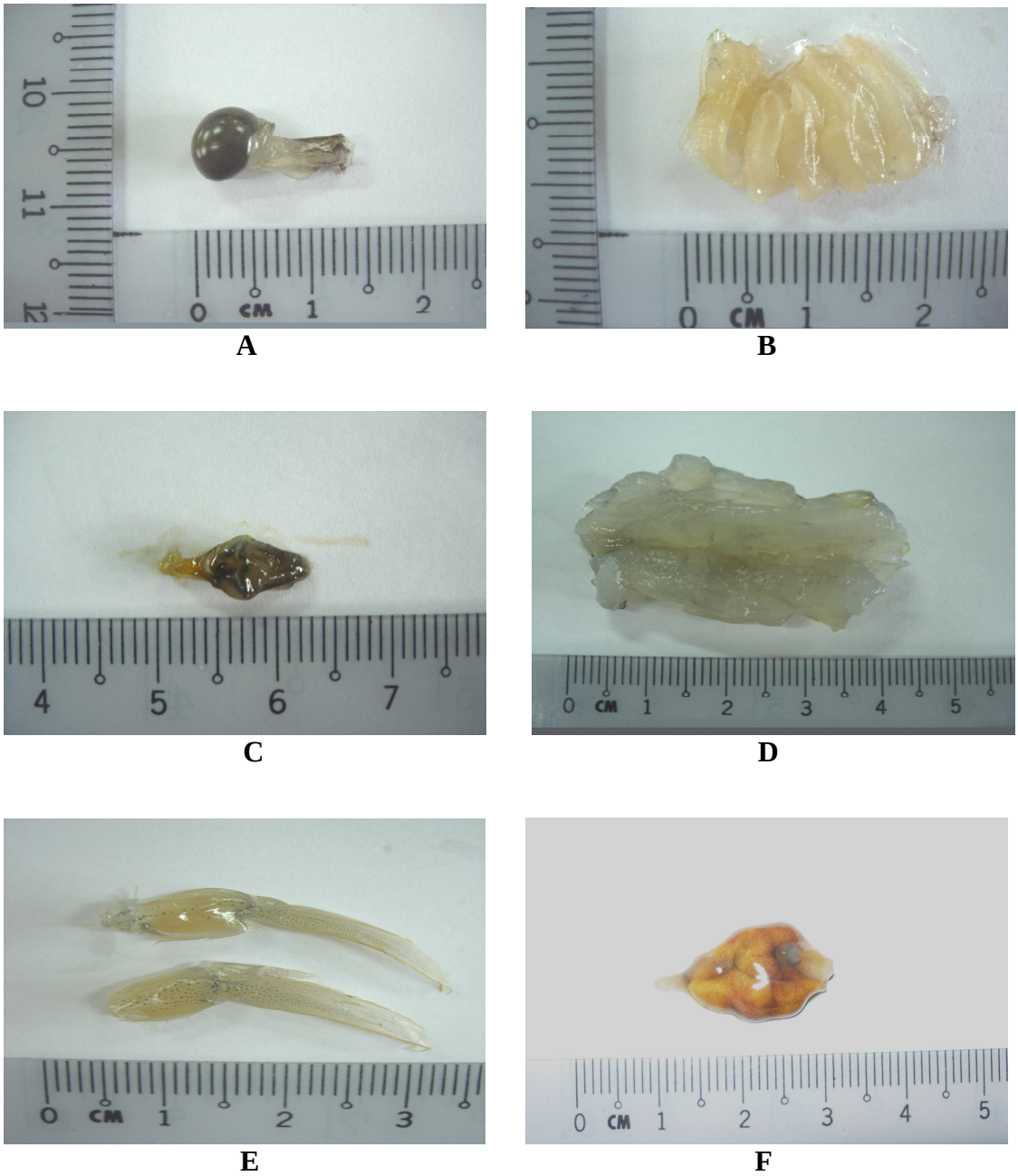

Figure 3.2
Samples for Nucleic Acid Extractions
Description below A: Eyestalk; B: Gill Tissues; C: Brain; D: Muscle Tissue; E: Pleopod Legs; F: Hepatopancreas

3.3 Total RNA Extraction

Total RNA extraction using TRIzol reagent from Invitrogen Corporation, USA was performed. This method has served as the best method in extracting total RNA (Norden-Krichmar *et al.*, 2007; Fu *et al.*, 2008; Su *et al.*, 2008). According to the manufacturer's protocol, all the apparatus should be RNase free to prevent the introduction of RNase during RNA extraction. Besides that, all the reagents that were required were prepared with (diethylpyrocarbonate) DEPC-treated water. The frozen target tissues of approximately 50-100mg were quickly measured and ground to powder in liquid nitrogen before the samples were thawed. Then, they were homogenized in 1mL of TRIzol reagent in a 1.5mL microcentrifuge tube. The sample did not exceed 10% of the TRIzol reagent volume. The microcentrifuge tube was then vortexed for 30 seconds and the homogenized sample was left at room temperature for 5 minutes to allow complete nucleoprotein complexes dissociation. Then, 200µL of chloroform per 1mL of TRIzol was added to the sample. The tube was then shaken vigorously for about 15 seconds to mix the solutions. The mixture was incubated at room temperature for 3 minutes. Following that, the tube was centrifuged at 12000 × g, 4°C for 15 minutes. Next, 500µL of the colorless aqueous solution was pippetted and put in a new microcentrifuge tube containing 500µL of ice-cold isopropyl alcohol. The tube was then inverted for a few times to mix the solutions. It was incubated at room temperature for 10 minutes. Next, the tube was centrifuged at 12000 × g, 4°C for 10 minutes. The supernatant was carefully discarded. Then, the RNA pellet was washed with 1mL of 75% ice-cold ethanol. The tube was spun at 7500 × g, 4°C for 5 minutes. The supernatant was poured out and the pellet was air-dried for about 10 minutes. Finally, the RNA pellet was dissolved in 50µL of DEPC-treated water.

Since some DNA might be carried over during RNA extraction, the RNA samples were treated with Deoxyribonuclease (DNase) I before downstream processes. The RNA concentration and purity was checked using a UV spectrophotometer. According to protocol from the DNase I (Amplification Grade) by Invitrogen, after the DNA digestion, the sample could be directly used for reverse transcription without RNA purification process. This can help to reduce the RNA loss during the purification process. The digestion mixture (in total volume of 10µL) was prepared on ice as in Table 3.3.

Table 3.3
DNase I Digestion Mixture

Reagent/ Solution	Volume
RNA sample	1µg
10X DNase I reaction buffer	1µL
DNase I, Amplification Grade	1Unit /µL
DEPC-treated water	Top up to 10µL

The digestion was performed by incubating the mixtures at room temperature for 15 minutes. Then, the DNase I was inactivated by introducing 2µL of 25mM EDTA solution and heating at 65°C for 10 minutes. Finally, the DNase- treated RNA sample was ready to be used for reverse transcription or it was stored at -80°C for future use. The initial RNA weights for all the organs were made constant at 1µg. So, after the DNase treatment, the final concentration of RNA was 0.1µg/µL. This helped in quantifying the RNA transcripts in the semi-quantitative polymerase chain reaction later.

3.4 DNA Extraction

DNA extraction was performed using GF-1 tissue DNA extraction kit (Vivantis Technologies Sdn. Bhd., Malaysia). Frozen tissues of about 10-20mg were measured and ground to powder in liquid nitrogen. They were put into the supplied 2mL microcentrifuge tube and 250µL of Buffer TL and 20µL of Proteinase K (20mg/mL) were added to the tube. They were mixed thoroughly; then, 12µL of Lysis Enchancer was added and mixed rapidly. The tube was then incubated at 65°C in a water bath until the mixture become clear (approximately 3 hours). During the incubation, the tube was occasionally inverted to obtain complete protein digestion. To obtain RNA-free DNA samples, the aqueous solution were treated with 20µL of RNase A (DNase-free, 20mg/mL). They were mixed and incubated at 37°C for 10 minutes. The digestion reaction was stopped by adding 600µL of Buffer TB and heating at 65°C for 10 minutes. Following that, 200µL of 99% ethanol was added and mixed well using vortex. Approximately 600µL of the mixture was transferred to a filter column in a new collection tube. It was centrifuged at 5000 × g for 1minute. The filtrate was discarded. This step was repeated for the remaining mixture. Next, 750µL of Wash Buffer was added into the filter column and centrifuged at 5000 × g for 1 minute. The flow through was poured off. The washing step was repeated once again. In order to remove all the ethanol traces, the tube was then centrifuged at 10000 × g for 1 minute. The filter column was transferred to a new 1.5mL tube and 100µL of preheated Elution Buffer was added to the filter column. It was left to stand overnight at 4°C to allow complete DNA dissolving. Finally, the tube was centrifuged at 5000 × g for 1 minute to retrieve the eluted DNA and kept at -20°C until use. The DNA concentration and purity were checked with a UV spectrophotometer before proceeding to polymerase chain

reaction. A dilution was made to all the genomic DNA samples to bring the final concentration to 25ng/µL before subjecting to PCR reaction.

3.5 First Strand Complementary DNA (cDNA) Synthesis

First strand cDNA was synthesized using reverse transcription system Promega Pte. Ltd., Singapore. The cDNA synthesis mixture was prepared as below:

Table 3.4
1st Strand cDNA Synthesis Mixture

Component	Final Volume	Final concentration
Magnesium chloride ($MgCl_2$), 25mM	4µL	5mM
Reverse tansciption 10× buffer	2µL	1×
dNTP mixture, 10mM	2µL	1mM
Recombinant RNasin ribonuclease inhibitor	0.5µL	1 Unit /µL
AMV reverse transciptase	0.6µL	15 Unit /µL
Random primer	1.0µL	0.5µg /µL
RNA template	400ng	-
Nuclease- free water	Top up to 20µL	

After preparing the cDNA synthesis mixture, the tube was place in a Multigene Thermal Cycler. A program was created based on the protocol from the manufacturer. It started with incubating the reaction mixture at 25°C for 10 minutes. Then, the temperature increased to 42°C for 15 minutes and followed by 95°C for 5 minutes to disassociate the complementary DNA and RNA template. Finally, it was held at 4°C for 5 minutes. Once the program had finished, the reaction tube was put on ice. For the subsequent application,

amplification of target gene using polymerase chain reaction, 20µL of cDNA product was diluted with 20µL of nuclease-free water. After the dilution, it was vortexed and spun down to obtain homogenized cDNAs.

For PCR using cDNAs as template, the initial weight of RNA template used in the cDNA synthesis was 400ng. After 1st-strand cDNA synthesis, it was diluted at 2-fold.

3.6 Semi-quantitative Polymerase Chain Reaction

The PCR was performed using the GoTaq Flexi DNA polymerase system from Promega Pte. Ltd., Singapore. In the functional assay performed by Promega Pte. Ltd., Singapore, it was tested and found sensitive to amplify as little as 100 molecules (about 0.35ng) of α-1-antitrypsin gene from human genomic DNA. Therefore, this system was chosen in this study. To quantify the expression level of the targeted gene, nucleic acid concentration of each target tissue was determined. All samples were made consistent in PCR by using the same volume at a certain concentration. The total PCR reaction volume was 25µL. A PCR master mix was prepared and aliquoted before adding the templates. The PCR cocktail was as shown in table 3.5.

Table 3.5
PCR Cocktail

Component	Final Volume	Final concentration
Magnesium chloride ($MgCl_2$), 25mM	1.5µL	1.5mM
5× Green buffer	5µL	1×
dNTP mixture, 10mM	0.5µL	1mM
Forward primer	0.5µL	1 pico mole
Reverse primer	0.5µL	1 pico mole

GoTaq DNA polymerase	0.125µL	1.25 Unit
cDNA template or DNA template	100ng or 75ng	- -
Nuclease- free water	Top up to 25µL	-

PCR was performed in a Multigene Thermal Cycler, using the following program: The initial denaturation was at 95°C for 3 minutes, followed by 30 cycles of 1 minute denaturation at 95°C, 1 minute of primer annealing at 59°C and 1 minute of elongation at 72°C; final extension at 72°C for 5 minutes; held at 4°C until removing from the thermal cycler.

3.7 Agarose Gel Electrophoresis

Agarose gel was used as a medium to separate the nucleic acids. It was prepared according to the required percentage in 1X Tris-Borate-EDTA (pH 8.0).

For RNA, 8µL of RNA sample (0.5µg/µL) with 2µL of 6X loading dye were mixed well and loaded into the wells of 1% agarose gel. For DNA, 8µL of DNA sample (1µg/µL) with 2µL of 6X loading dye were mixed. They were loaded into 0.8% agarose gel. The samples were run at 78V, 150mA for about 45 minutes.

For PCR product, 10µL of PCR product were loaded in 2% agarose gel and run at 78V, 150mA for 55 minutes. 100bp DNA ladder (Vivantis) was included as size marker.

3.8 PCR Product Purification

AxyPrep DNA gel extraction kit (Axygen Bioscience, USA) was used to purify the PCR products. The gel containing the expected band was cut under UV illumination. The excised gel was then sliced into small pieces and weight. According to the manufacturer's protocol, 100mg of gel is equal to 100µL volume. After that, the gel slice was transferred to a 1.5mL microfuge tube. Then, 3 times volume (approximately 300µL) of Buffer DE-A was added and mixed well using vortex. The mixture was put in 75°C water bath for about 8 minutes. It was inverted gently every 2 minutes to accelerate gel solubilization. Next, 0.5 times volume (about 150µL) of Buffer DE-B and 100µL of isopropanol were added to the tube. The solutions were mixed well and transferred into an Axyprep column placed in a 2mL microfuge tube. It was centrifuged at 12,000 × g for 1 minute. The filtrate was discarded. Then, 500µL of Buffer W1 was added into the column and centrifuged at 12,000 × g for 30 seconds. Again, the filtrate was discarded and 700µL of Buffer W2 was added. The tube was centrifuged at 12,000 × g for 30 seconds. Washing with Buffer W2 was performed twice in order to remove the salt residual carried over from PCR reaction and gel solubilization reagent. Then, the tube with the AxyPrep column was centrifuged again at 12,000 × g for 1 minute. Next, the column was transferred to a new 1.5mL microfuge tube and 30µL of pre-warmed Eluent was added to the center of the membrane. The tube was stood overnight at room temperature to allow complete nucleic acid solubilization. Then, the tube was centrifuged at 12,000 × g for 1 minute to collect the purified PCR products. Then, sample of 2µL of the purified product was run on 2% agarose gel to check its quality and quantity. Finally, the purified PCR product was ready to be sent for sequencing.

3.9 DNA Sequencing

Sequence of PCR products was determined using a command DNA sequencing service from SolGent Co., Ltd., Korea. DNA sequences of PCR products were subjected to BLASTN program (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) in NCBI (http://www.ncbi.nlm.nih.gov/), with the default parameters, searching against the *Litopenaeus vannamei* dbEST database. Then, the sequence of the precursor miRNA was folded by mfold program to determine the hairpin structure.

3.10 Putative miRNA Target Prediction

To detect the potential target genes, the newly identified *Litopenaeus vannamei* candidate miRNA gene sequences were analyzed using BLASTN against both the *Litopenaeus vannamei* nucleotides database and EST database. Only those that showed reverse complement at nucleotide 1-7 or nucleotide 2-8 of 5' end of predicted mature miRNA sequence and target at 3' UTR were selected as potential miRNA targets (Lewis *et al*., 2005; Gu *et al*., 2007; Lindow and Gorodkin, 2007; Norden-Krichmar *et al*., 2007).

Those *Litopenaeus vannamei* nucleotide sequences and ESTs that satisfied the selection criteria were subjected to BLASTX program to identify their putative functions. Those with significant E-value cutoff of $\leq 1 \times 10^{-4}$ and matches of more than 10 amino acid residues in the protein database were selected as potential protein coding sequences (O'leary *et al*., 2006; Tassanakajon *et al*., 2006).

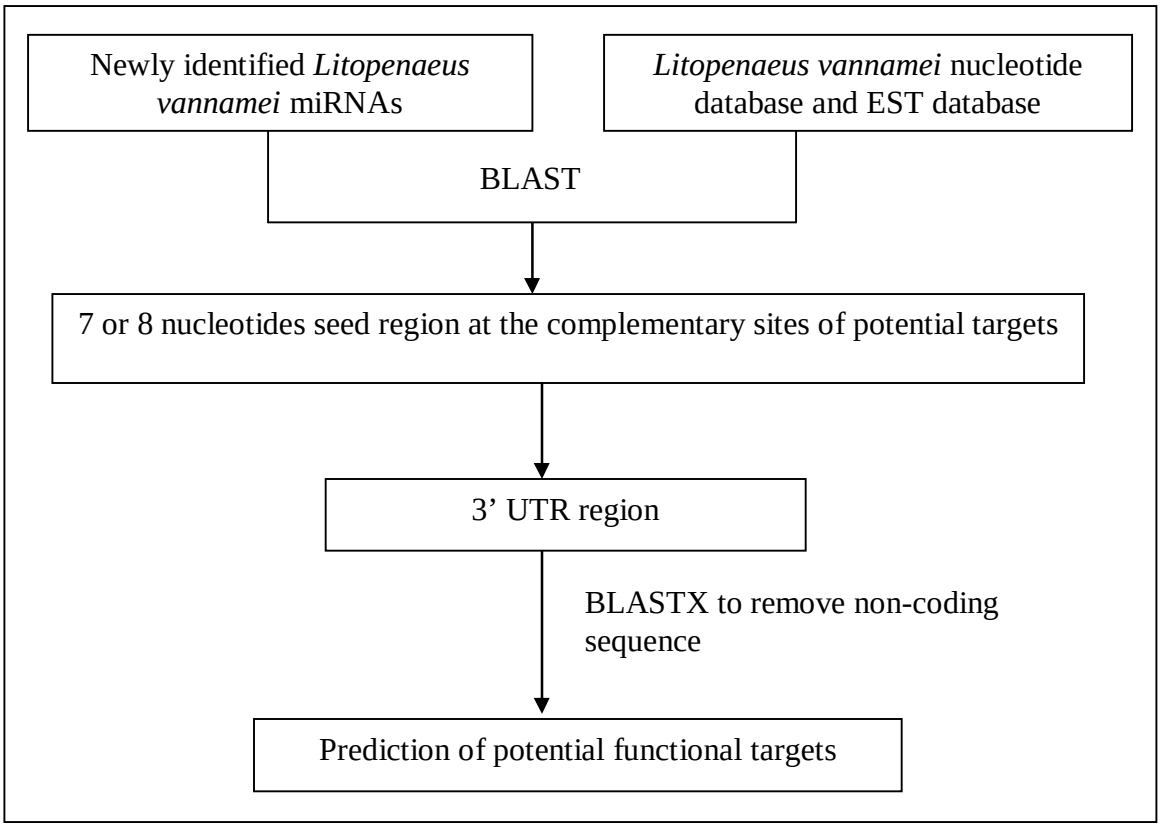

Figure 3.3
Flow Chart of Computational Identification of Potential Targets by *Litopenaeus vannamei* miRNAs