# Chapter 4

# Results and Discussion

In this study, 11 *Litopenaeus vannamei* miRNA (lva-miR) candidates have been identified in silico. Three of them were experimentally validated using PCR and sequencing methods. In addition to that, their potential mRNA targets were also identified. The 11 lva-miR candidates were found to target 20 mRNA sequences and 27 EST protein coding sequences.

## 4.1 Identification of potential *Litopenaeus vannamei* miRNAs

Out of 604 non-redundant mature miRNAs extracted from the invertebrates miRBase (2008) blasted against 155558 sequences of *Litopenaeus vannamei* EST database, 318 EST sequences passed the first round of screening. Invertebrate groups were selected as the pacific whiteleg shrimp was also classified under invertebrate. Both homolog and paralog miRNAs should be able to be identified across species (Kim and Num, 2006). miRNA candidates were EST sequences with ≤ than 4 mismatched from the known invertebrate miRNAs (deduced from miRBase, 2008; Norden-Krichmar *et al.*, 2007); or EST sequences showing more than 15 nucleotide or core seed matches to a known invertebrate miRNA (Fu *et al*., 2008). However, no sequences with similarity were found in the GSS database which was not surprising because the GSS database contained only two DNA sequences of *Litopenaeus vannamei* clones (NCBI, 2008). Neither GSS sequence was classified into either exon trapped product nor gene trapped product. Sequences with complete matches or one mismatch were not found against the known invertebrate mature miRNA sequences. On the other hand, two or more than two mismatches against the known

invertebrate mature miRNAs could be found. Although miRNA is said to be evolutionarily conserved (Ambros *et al*., 2003[b]; Lim *et al*., 2003[a]), nucleotide polymorphisms have been observed in miRNA families for example the lin-4 family, let-7 family and others in human and *C. elegans* (Lim *et al*., 2003[b]). Palakodeti *et al*. (2006) showed nucleotide polymorphisms in the bantam family, *let-7* family, *lin-4* family, *miR-1* family and others in *Schmidtea mediterranea*, human, *Drosophila* and *C. elegans*. Following that, the 318 sequences were screened for redundancy and Rfam search. This was done to eliminate transfer RNA and ribosomal RNA sequences (Chen *et al*., 2005; Yao *et al*., 2007). No repeated EST sequences were identified and no similarity was found when the EST sequences were blasted against Rfam database. This meant that these 318 RNA transcripts did not encode transfer RNA or ribosomal RNA. The third round of screening was to remove protein-coding EST sequences. MicroRNA sequences residing within or spanning along protein-coding EST sequences were eliminated. This is because animal miRNAs had been observed to reside in intronic regions of coding sequences or intergenic regions (Rodriguez *et al*., 2004; Tang and Maxwell, 2008), so, would not be present in mRNA encoding proteins. There were 38 EST sequences that were found to potentially encode proteins and these were discarded from further analysis. The remaining 233 EST sequences were then subjected to mfold to predict their secondary structure. During the screen of potential miRNA precursors, candidates were evaluated according to the features of miRNA precursors. Among the 233 sequences, only 17 EST sequences were found to pass the filters. In these 17 EST sequences, 11 lva-miR candidates were identified: lva-miR-33, lva-miR-79, lva-miR-256, lva-miR-261, lva-miR-272, lva-miR-279, lva-miR-281, lva-miR-315, lva-miR-752, lva-miR-1476-3p and lva-miR-36*.

Homology searching using EST data can serve as a method to computationally identify miRNA candidates. This method is fast compared to large scale small RNA cloning methods. However, this method can only determine homologous miRNA genes. If the EST database is limited, very few miRNA candidates will be detected. However, if homology searches are performed against the complete genome sequence of an organism, a large number of potential miRNA genes can be identified. For instance Grad *et al*. (2003) were able to predict computationally 214 miRNA candidates from the *C. elegans* genome using sequence conservation and structural similarity of known miRNA. Palakodeti *et al*. (2006) have determined 71 miRNA genes from the planarian *Schmidtea mediterranea* genome where 17 appeared to be *Schmidtea mediterranea* specific miRNA genes. Zhou *et al*. (2008) reported their findings in the dog genome, 300 miRNAs were found homologous to human miRNAs whereas 57 candidates were known as novel miRNAs. Tang and Maxwell (2008), using homology searching, managed to identify 142 potential miRNA genes from the genome of frog, *Xenopus tropicalis*. In addition to that, the miRNA candidate can be identified whether it resides in an intronic or intergenic region of protein coding sequence, or an exonic region of non-protein coding sequence (Grad *et al*., 2003; Lin *et al*., 2006; Tang and Maxwell, 2008). High throughput sequencing methods such as small RNA library sequencing can discover novel miRNA genes, quantify their expressions within a short period; and provide the complete profile of miRNA genes available in the organism which later can be used to produce miRNA arrays (Hafner *et al*., 2008). Using the sequencing by synthesis approach developed by Illumina, Galzov *et al*. (2008) not only has identified most of previously known chicken miRNA genes and miRNA* sequences, his research group has also identified 361 new miRNA genes and 88 new miRNA candidates from different developmental stage of chicken embryos. Morin *et al*. (2008) whose group applied the same technology on the human embryonic stem cells and embryoid bodies,

managed to identify 334 known miRNA genes and 104 novel miRNA genes. In addition to that, their group was also able to observe 171 known and 23 new miRNA genes showing significant expression variations between human embryonic stem cells and embryonic bodies. However, the cost for this service is quite expensive. Besides that, the bioinformatics pipeline is also required to analyze the mass data input. For other method, Grundhoff *et al*. (2006) has combined the bioinformatics and microarray based technology without cloning, managed to identify 18 new pre-miRNAs that gave rise to 22 mature miRNA sequences which encoded by human gamma-herpesvirus.

**Table 4.1**
***Litopenaeus vannamei* miRNA Candidates**

| BLAST search against *Litopenaeus vannamei* EST database | | | |
|---|---|---|---|
| Known miRNA | EST Accession Number | Identities | Strand |
| cel-miR-256 | FE118488 | 18/21 | + |
| cel-miR-261 | FE175367 | 16/19 | + |
| cin-miR-33 | CV468691 | 17/19 | + |
| odi-miR-1476-3p | FE146432 | 18/22 | + |
| | FE136124 | 18/22 | + |
| | FE143167 | 18/22 | + |
| odi-miR-281 | FE053314 | 17/21 | + |
| | FE099869 | 17/21 | + |
| | FE181745 | 17/21 | - |
| ame-miR-79 | FE134721 | 17/21 | + |
| | FE134913 | 17/21 | + |
| cel-miR-272 | FE085435 | 16/18 | + |
| | FE085434 | 16/18 | - |
| bmo-miR-279 | FE075623 | 16/19 | - |
| sme-miR-36* | FE094497 | 17/20 | - |
| sme-miR-752 | FE055542 | 15/18 | - |
| miR-315 | FE165299 | 18/22 | - |

**Table 4.2**
**Secondary Structure and Folding Energy of lva-precursor-miRNA Candidates**

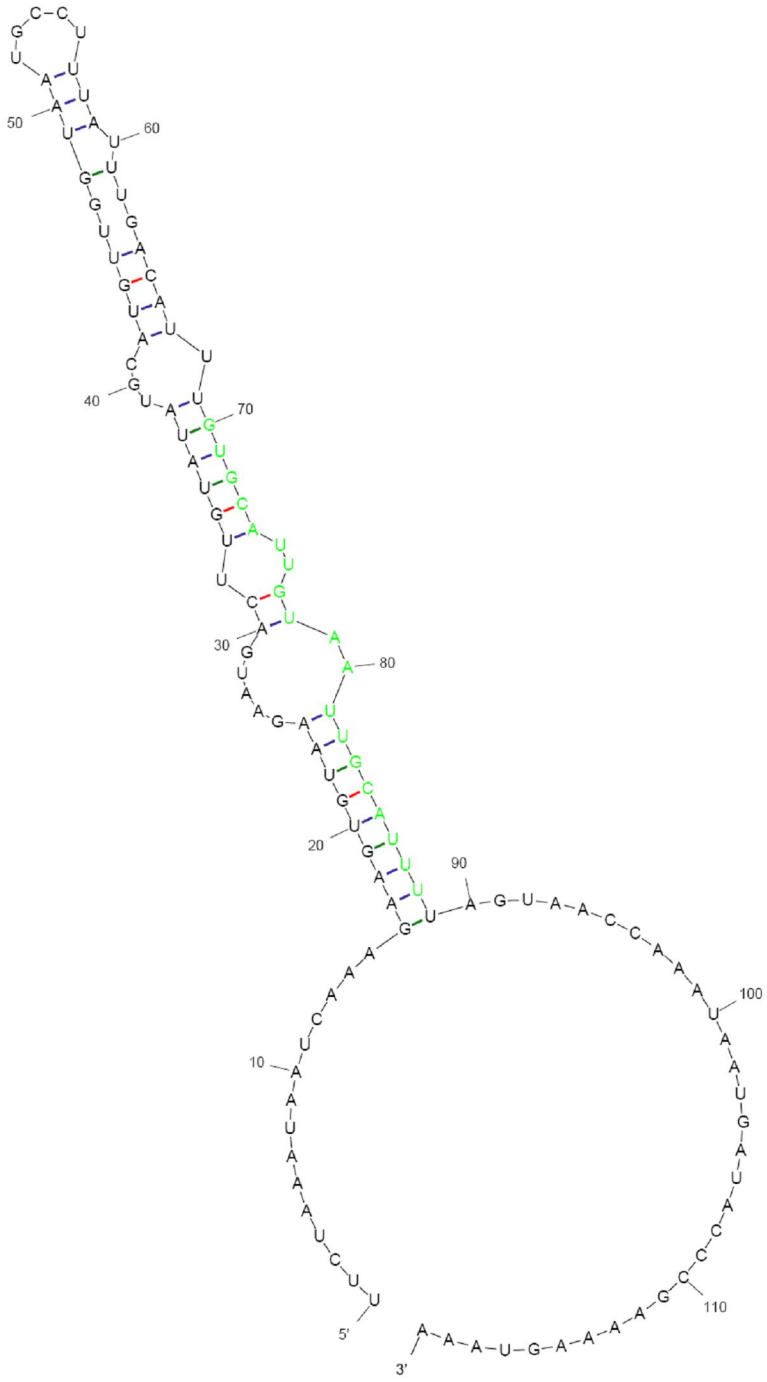
lva-miR-33 GUGCAUUGUAAUUGCAUUU 19

lva-pre-miR-33

UUCUAAAUAAUCAAAGAAGUGUAAGAAUGACUUGUAUAUGCAUGUUGGUAAUGCCUUUAUUUGACA

UUUGUGCAUUGUAAUUGCAUUUUAGUAACCAAAUAAUGAUACCCGAAAAGUAAA

Minimal folding energy = -16.00kcal/mol



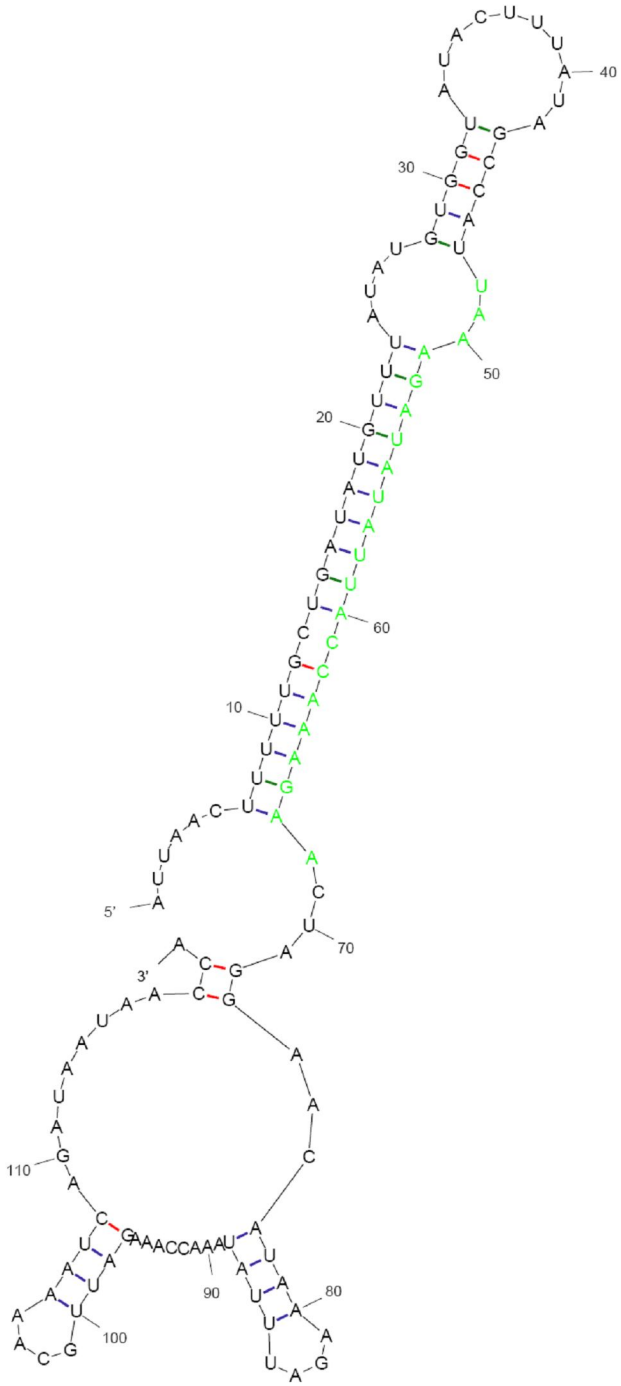
lva-miR-79 UAAAGAUAUAUUACCAAAGAA 21

lva-pre-miR-79

AUUAACUUUUUGCUGAUAUGUUUAUAUGUGGUAUACUUUAUAGCCAUUAAAGAUAUAUUACCAAAG

AACUAGGAACAUAAAGAUUUAUAAACCAAAGAUUGCAAAAUCAGAUAAUAACCA

Minimal folding energy = -17.90kcal/mol



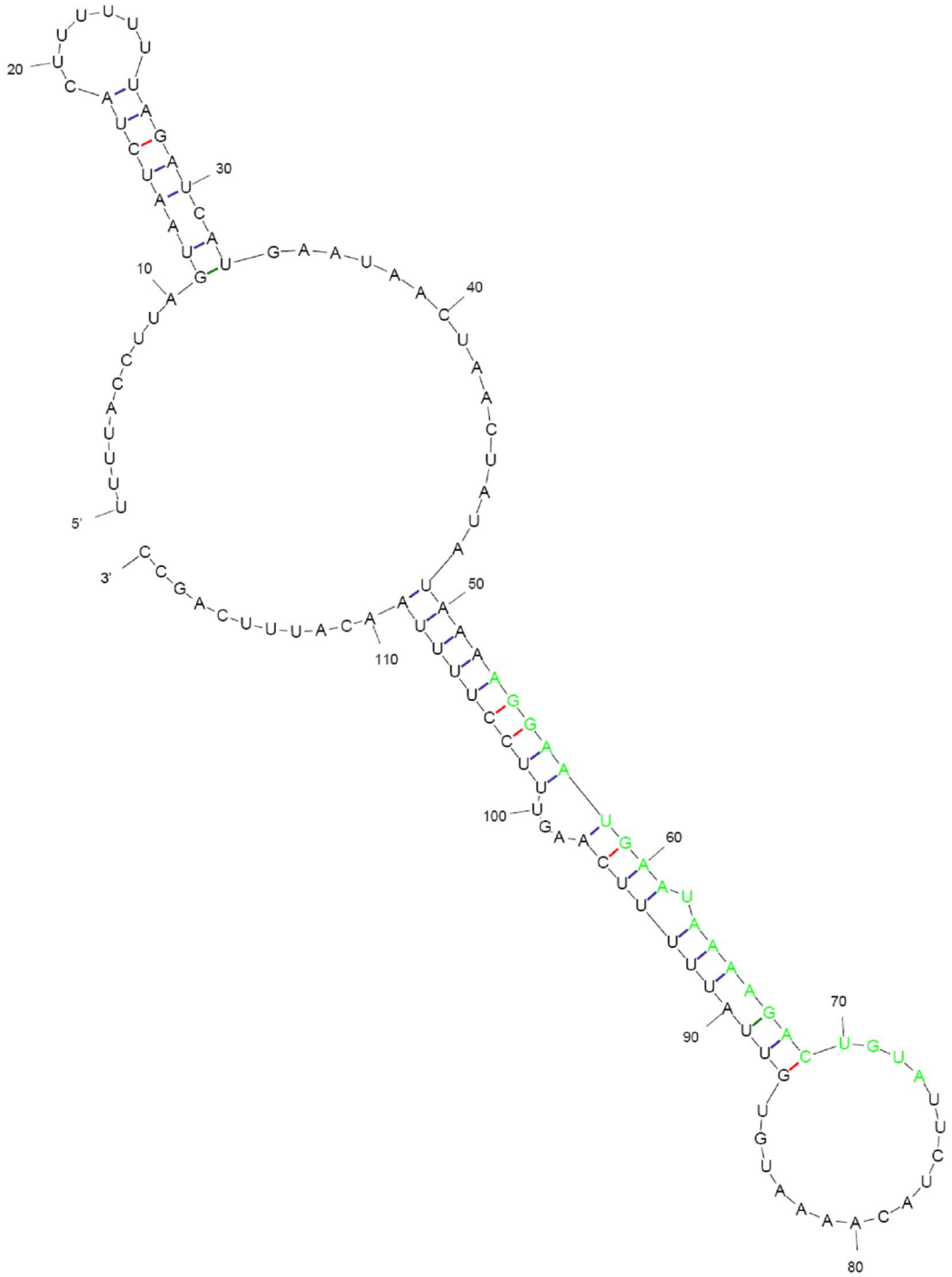
lva-miR-256 AGGAAUGAAUAAAAGACUGUA 21

lva-pre-miR-256

UUUUACCUUAGUAAUCUACUUUUUUUAGAUCAUGAAUAACUAACUAUAUAAAAGGAAUGAAUAAAA

GACUGUAUUCUACAAAAUGUGUUAUUUUUCAAGUUUCCUUUUAACAUUUCAGCC

Minimal folding energy = -11.50kcal/mol



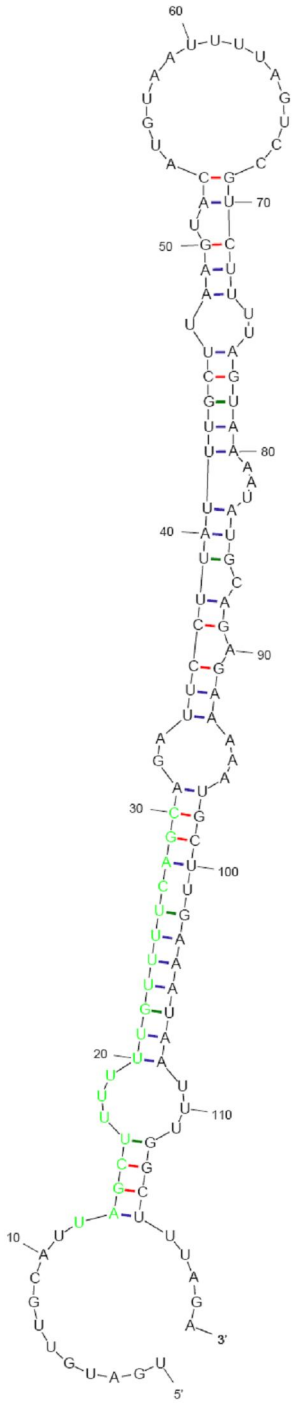
lva-miR-261 UAGCUUUUUUGUUUUCAGC 19

lva-pre-miR-261

UGAUGUUGCAUUAGCUUUUUUGUUUUCAGCAGAUUCCUUAUUUGCUUAAGUACAUGUAAUUUUAGU

CCGUCUUUUAGUAAAAUAUGCAGAGAAAAAUGCUUGAAAUAAUUUGGCUUUAGA

Minimal folding energy = -17.60kcal/mol

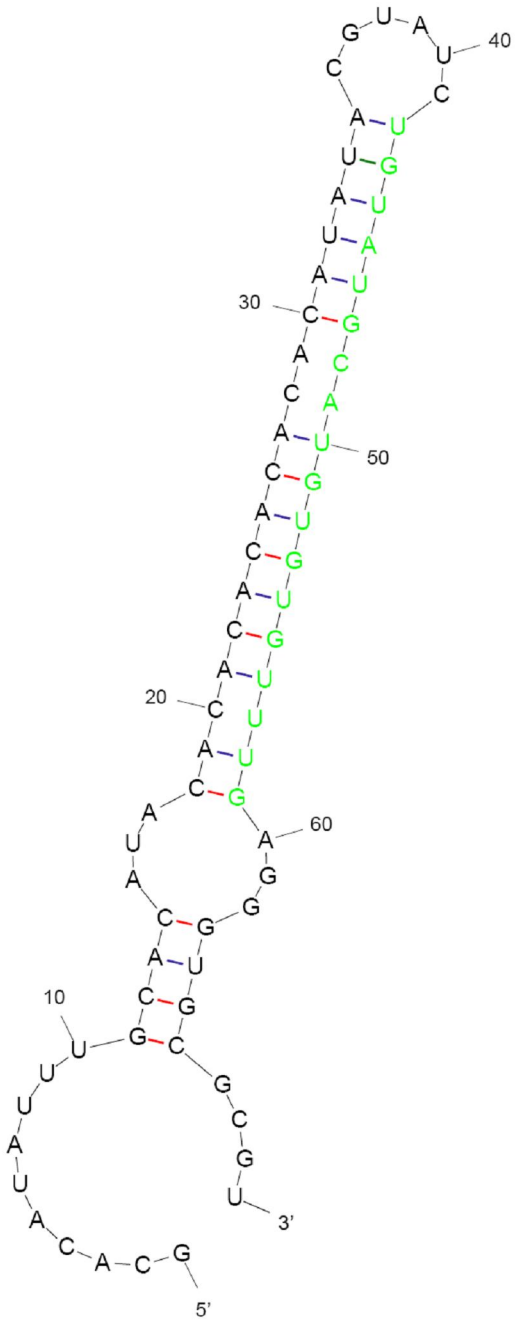


lva-miR-272 UGUAUGCAUGUGUGUUUG 18

lva-pre-miR-272

GCACAUAUUUGCACAUACACACACACACACAUAUACGUAUCUGUAUGCAUGUGUGUUUGAGGGUGC GCGU

Minimal folding energy = -21.90kcal/mol



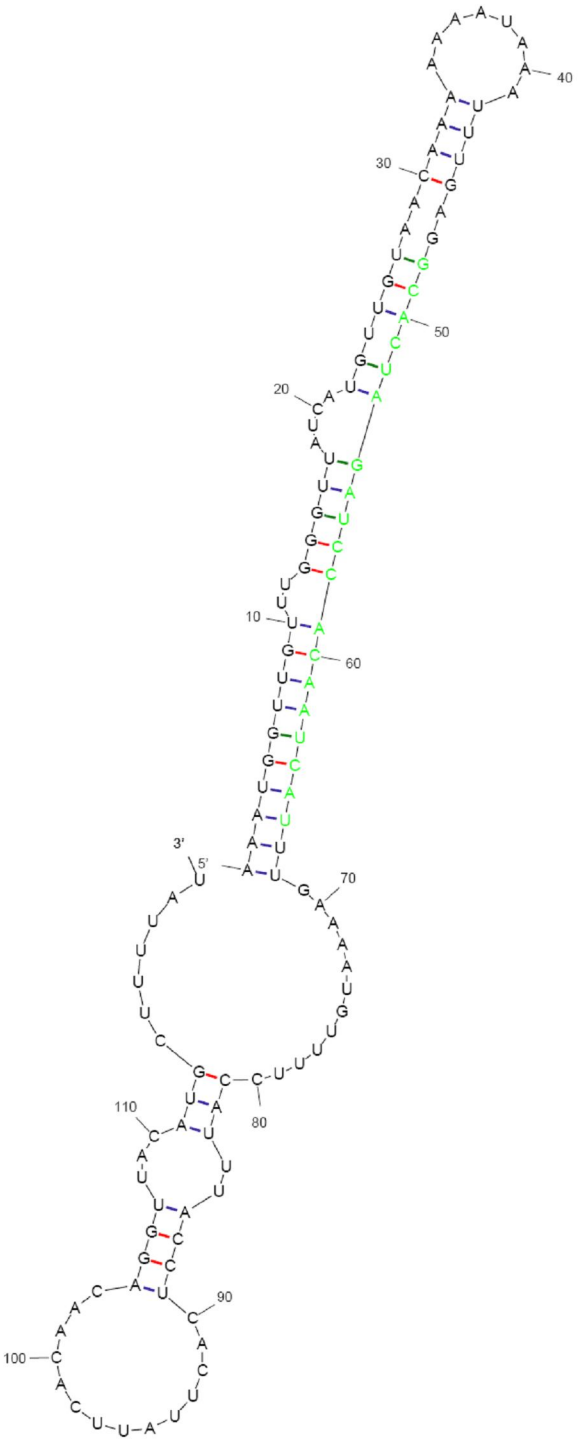
lva-miR-279 GCACUAGAUCCACAAUCAU 19

lva-pre-miR-279

AAAUGGUUGUUUGGGUUAUCAUGUUGUAACAAAAAAAUAAAUUUGAGGCACUAGAUCCACAAUCAU

UUGAAAAUGUUUUCCAUUUACCUCACUUAUUCACAACAGGUUACAUGCUUUUAU

Minimal folding energy = -16.00kcal/mol



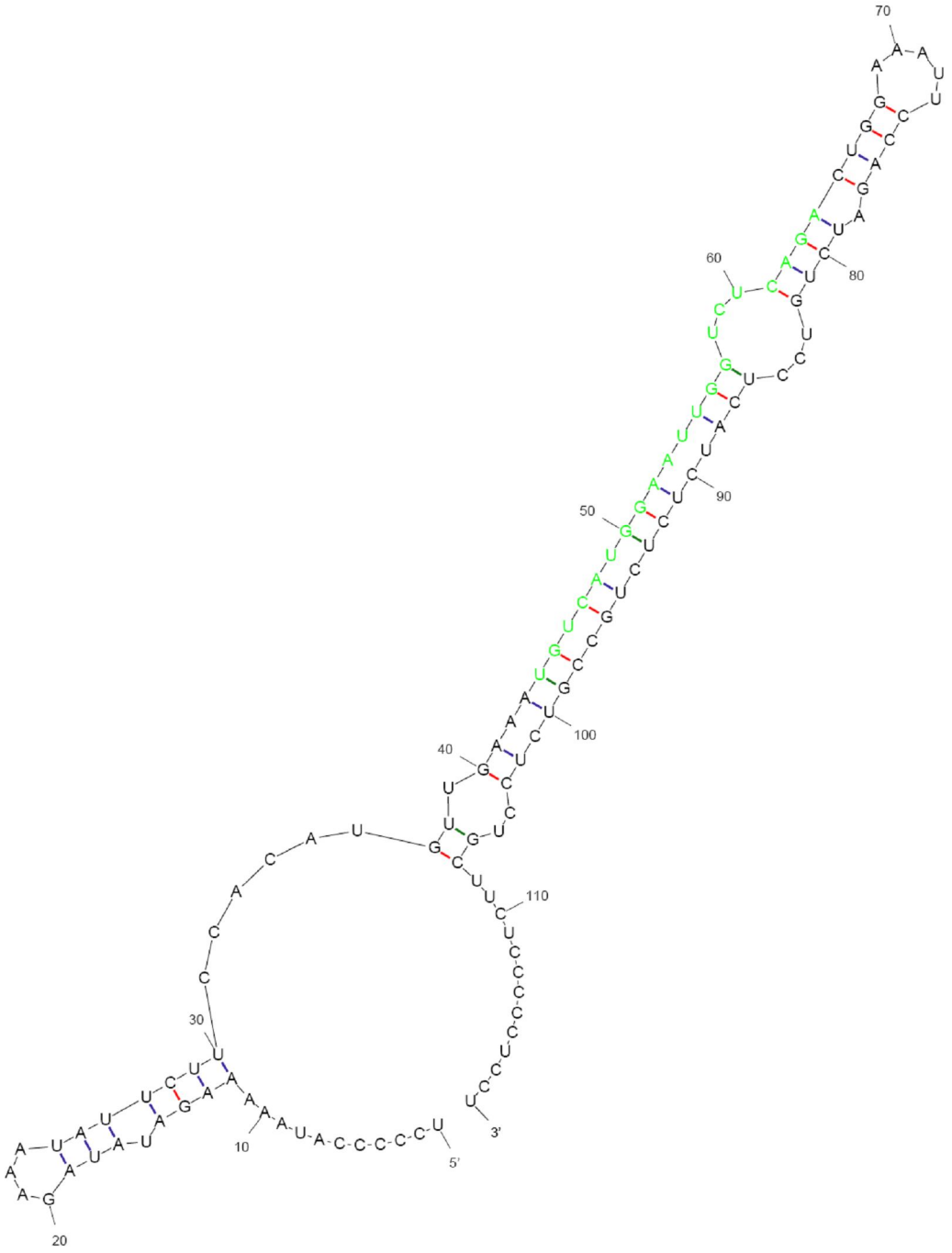
lva-miR-281 UGUCAUGGAAUUGGUCUCAGA 21

lva-pre-miR-281

UCCCCCAUAAAAAGAUAUAGAAAUAUUCUUCCACAUGUUGAAAUGUCAUGGAAUUGGUCUCAGACU

GGAAAUUCCAGAUCUGUCCUCAUCUCUCUGCCGUCUCCUGCUUCUCCCCCUCCU

Minimal folding energy = -21.00kcal/mol

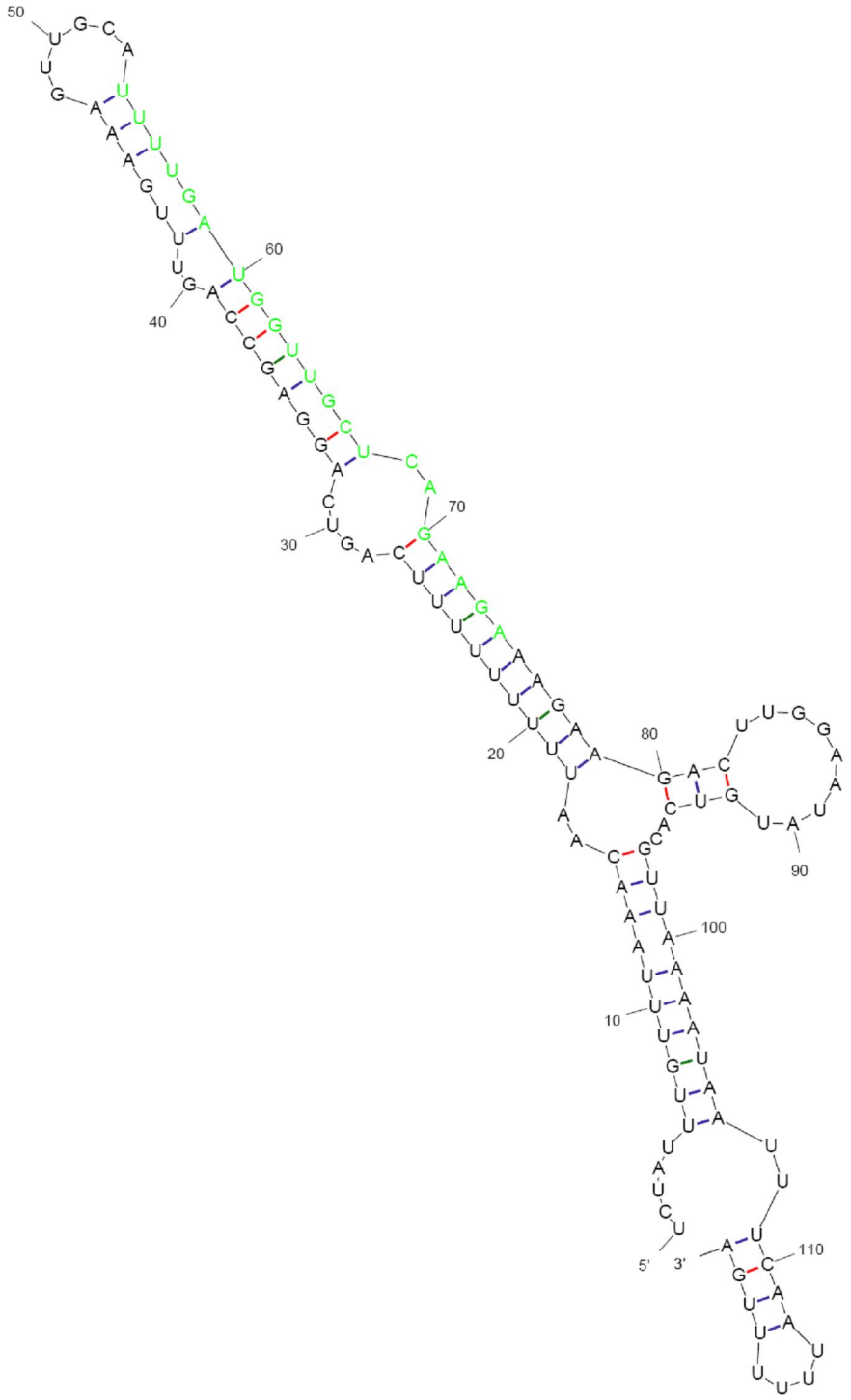


lva-miR-315 UUUUGAUGGUUGCUCAGAAGA 22

lva-pre-miR-315

UCUAUUUGUUUAAACAAUUUUUUUUUCAGUCAGGAGCCAGUUUGAAAGUUGCAUUUUGAUGGUUUG CUCAGAAGAAAGAAGACUUGGAAUAUGUCACGUUAAAAUAAUUUCAAUUUUUGA

Minimal folding energy = -20.80kcal/mol



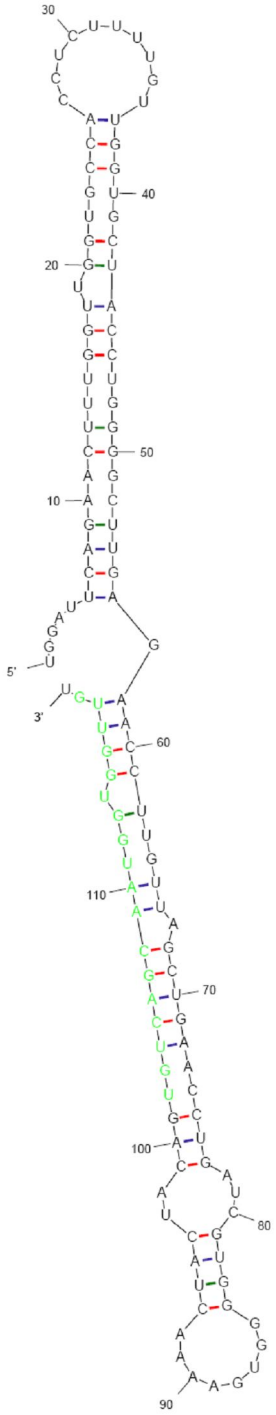
lva-miR-752 UGUCAGCAAUGGUGGUUG 18

lva-pre-miR-752

UGGAUUCAGAACUUUGGUUGGUGCCACCUCUUUUGUUGGUGCUACCUGGGGCUUGAGAACCUUGUU

AGCUGAACCUGAUCGUGGGGUGAAAACUACUACAGUGUCAGCAAUGGUGGUUGU

Minimal folding energy = -36.20kcal/mol

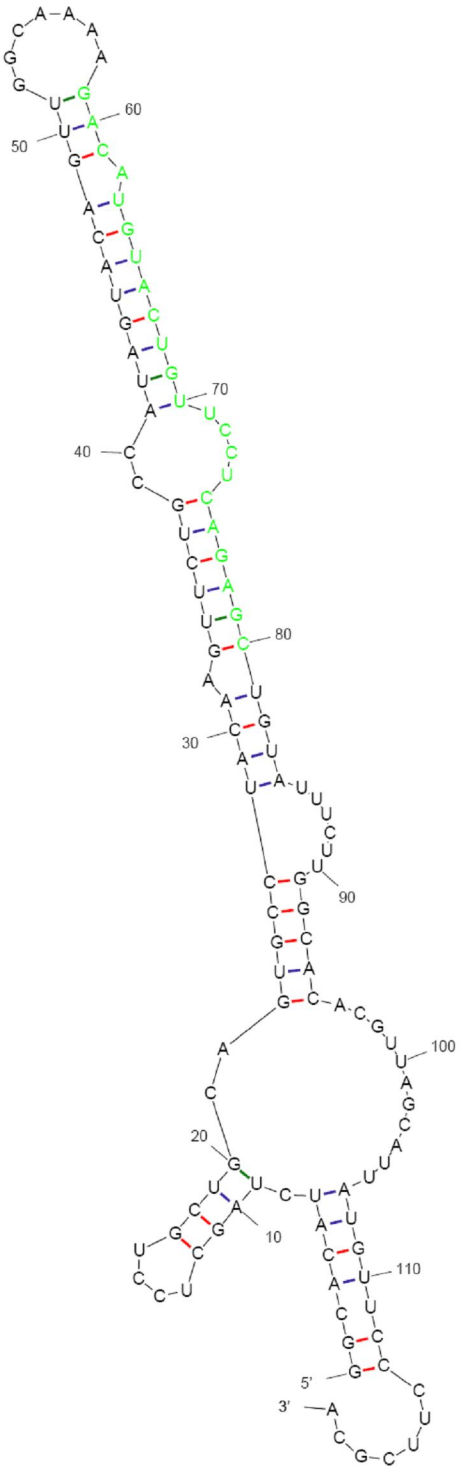


lva-miR-1476-3p GACAUGUACUGUUCCUCAGAGCU 22

lva-pre-miR-1476-3p

GGCACAUCUAGCUCCUGCUGCAGUGCCUACAAGUUCUGCCAUAGUACAGUUGGCAAAAGACAUGUA CUGUUCCUCAGAGCUGUAUUUCUUGGCACACGUUAGCAUUAUGUUCCCUUCGCA

Minimal folding energy = -33.40kcal/mol

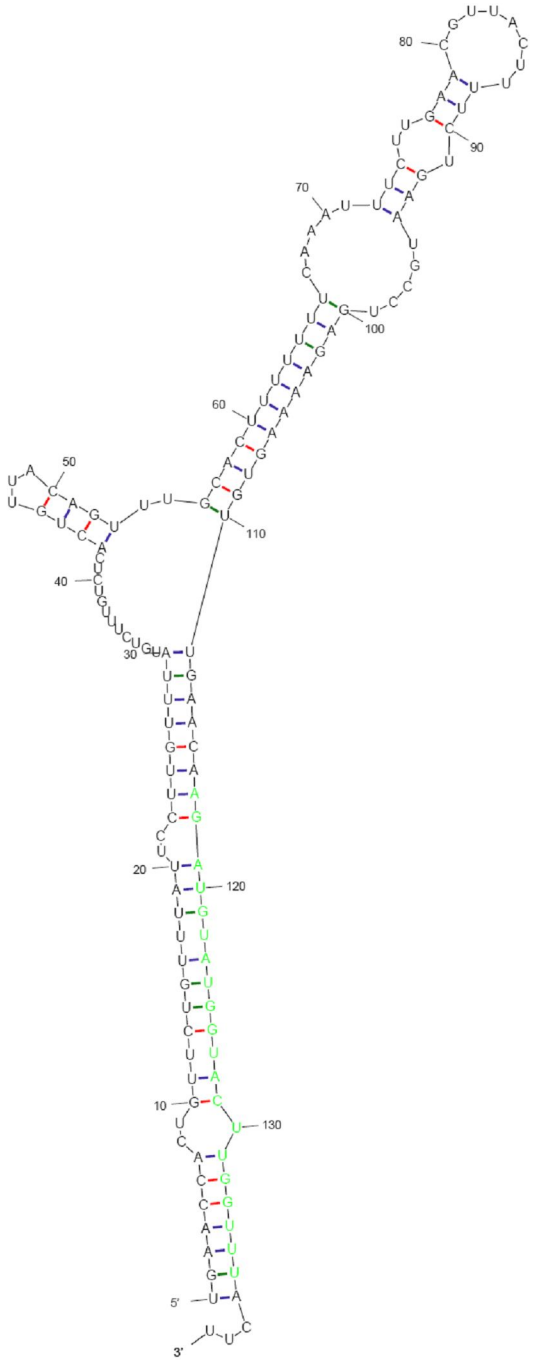


lva-miR-36* AGAUGUAUGGUACUUGGUUU 20

lva-pre-miR-36*

UGAACCACUGUUCUGUUUAUUCCUUGUUUAUGUCUUUGUCUCACUGUUACAGUUUGCACUUUUUUU
CAAAUUUCUUGAACGUUACUUUUCUGAAUGCCUGAGAAAAGUGUUGAACAAGAUGUAUGGUACUUG
GUUUACUU

Minimal folding energy = -28.00kcal/mol



During this study, the folding energy varied from -11.50 to -36.20kcal/mol for the miRNA precursors candidates. In previous studies, Lim *et al*. (2003[b]) and Tong *et al*. (2006) defined the minimal folding energy for precursor miRNA ranged from ≤ -20 to 25kcal/mol. However, Tang and Maxwell (2008) found that the minimal folding energy for *Xenopus tropicalis* precursor miRNA 31b (Accession number in miRBase: MI0006264) was -15.70kcal/mol when folding with mfold. In addition to that, Ambros *et al*. (2003[b]) in their study found that folding energy of *Caenorhabditis elegans* precursors miRNAs predicted by mfold ranged from -12.6 to -57.1kcal/mol. These were probably due to the fact that length of the miRNA precursor affects the folding energy. Brameier and Wiuf (2006) found that free energy of 6 human miRNAs fell below -15kcal/mol when the central 70 nucleotides in 100 nucleotides scanning window was folded. The length of precursor miRNA was set at window of 120 nucleotides. This is because animal precursor miRNA ranges from 60 to 120 nucleotides (Ambros *et al*., 2003[a]; Lim *et al*., 2003[b]; Tang and Maxwell, 2008). The length of all identified lva-pre-miR candidates were 120 nucleotides except lva-pre-miR-272 and lva-pre-miR-36*. Lva-pre-miR-272 was found to be 70 nucleotides whereas lva-pre-miR-36* was found to be 140 nucleotides in length. In addition to that, the G:C, A:U and G:U pairing would also influence the folding energy (Zuker, 2003) .

## 4.2 Experimental Validation of Potential miRNA Precursors

Having computationally identified 11 miRNA candidates from the *Litopenaeus vannamei* EST database, 3 candidates were selected for experimental validation using PCR and sequencing methods. The main purpose was to verify the presence of precursor miRNA candidates in the *Litopenaeus vannamei* cells. Forward primer and reverse primer were

designed from the EST flanking miRNA candidate region. Semi quantitative RT-PCR was performed in each targeted tissue to observe the miRNA candidate expression. The house keeping gene beta actin served as a control for gene expression. It was relatively quantified against the lva-pre-miR candidates. All PCR products were sent for sequencing to validate the sequences of miRNA genes. The results could be obtained in the Table 4.3 and Appendix.

**Table 4.3**
**BLAST Search against Sequencing Results**

| Query | Identity | EST Accession Number |
|---|---|---|
| Beta-actin gene from the mRNA transcripts of muscle tissue, hepatopancreas, gill tissue, leg, eyestalk and brain (rnaBT, rnaBH, rnaBG, rnaBL, rnaBE, rnaBB) | 100% | gb\|AF300705.2\| *Litopenaeus vannamei* beta-actin mRNA, complete cds |
| Precursor miRNA 256 from hepatopancreas tissue transcripts (rna256H) | 96% | gb\|FE118488.1\| LV_HC_RA068N13r *Litopenaeus vannamei* hemocyte cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |
| Precursor miRNA 256 from gill tissue transcripts (rna256G) | 98% | gb\|FE118488.1\| LV_HC_RA068N13r *Litopenaeus vannamei* hemocyte cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |
| Precursor miRNA 256 from leg tissue transcripts (rna256L) | 97% | gb\|FE118488.1\| LV_HC_RA068N13r *Litopenaeus vannamei* hemocyte cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |
| Precursor miRNA 256 from eyestalk tissue transcripts (rna256E) | 97% | gb\|FE118488.1\| LV_HC_RA068N13r *Litopenaeus vannamei* hemocyte cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |

| | | |
|---|---|---|
| Precursor miRNA 256 from brain tissue transcripts (rna256B) | 97% | gb\|FE118488.1\| LV_HC_RA068N13r *Litopenaeus vannamei* hemocyte cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |
| Precursor miRNA 256 from gill tissue DNA (dna256G) | 95% | gb\|FE118488.1\| LV_HC_RA068N13r *Litopenaeus vannamei* hemocyte cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |
| Precursor miRNA 272 from gill tissue transcripts (rna272G) | 96% | gb\|FE085434.1\| LV_GL_RA29M12r *Litopenaeus vannamei* gills cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |
| Precursor miRNA 272 from gill tissue DNA (dna272G) | 97% | gb\|FE085434.1\| LV_GL_RA29M12r *Litopenaeus vannamei* gills cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |
| Precursor miRNA 1476-3p from hepatopancreas tissue transcripts (rna1476H) | 100% | gb\|FE146432.1\| LV_HP_RA50B22f *Litopenaeus vannamei* hepatopancreas cDNA library *Litopenaeus vannamei* cDNA, mRNA sequence. |

RNA transcripts were used as templates for RT-PCR, to validate the expression of candidate miRNA precursors in vivo. The PCR products generated from the RNA transcripts were sent for sequencing and blasted against the *Litopenaeus vannamei* EST database to retrieve the same accession numbers from where lva-miRNA gene candidates were predicted. As shown in Figure 4.2 and 4.3, the expected size of lva-miR1476-3pFR was 371bp whereas beta-actin was 604bp; lva-miR-272FR was 233bp; lva-miR-256FR was 266bp. For beta actin gene, 100% identities to AF300705 (complete coding sequence for *Litopenaeus vannamei* beta-actin gene) were retrieved in all targeted samples. For primers

of lva-miR-256, the amplified samples showed more than 95% identity to the FE118488 and expression of the lva-miR-256 gene was observed for all samples. For primers of lva-miR-272, 96% identity was obtained compared to FE085434 and lva-miR-272 gene was found present in the transcript sequence. Last but not least, lva-miR-1476-3p primers allowed 100% identities to be retrieved from FE146432 where the presence of lva-miR-1476-3p gene was retained. It was found that lva-miR1476-3pFR was only amplified from hepatopancreas tissue whereas lva-miR-272FR was amplified from gill tissue. Lva-miR1476-3p was found in hepatopancreas and lva-miR-272 was found in gills. This indicated that both miRNA candidates could have tissue-specific expressions and roles. No amplification was observed in other target tissue as their expression in prawn might be too low to visualize on an agarose gel. Lva-miR-256FR showed its amplification at each target tissue except muscle tissue. Lva-miR-256 was computationally found in hemocyte. Organs such as hepatopancreas and gill tissues were highly saturated with blood vessels; therefore, lva-miR-256 expressions were higher in these 2 organs compared to leg tissues, eyestalk and brain. However, unlike mammalian muscle tissues surrounded by tiny blood vessels, the prawn muscle tissue consisted of flesh. Again, the expression of lva-pre-miR-256 available in muscle tissue might be too low to be seen on an agarose gel even after polymerase chain reaction amplification.

The designed primers were also tested for their ability to amplify product from genomic DNA. The lva-miR-272FR and lva-miR-256FR primers amplified products of 233bp and 266bp respectively (refer to Figure 4.1). This suggests that there was no intron between the regions that primers targeted. The sequencing results were blasted against the *Litopenaeus vannamei* EST database to retrieve the accession number for the computationally predicted lva-miRNA genes. Lva-miR-256 primer amplified product

matched the lva-miR-256 gene with 95% identity to FE118488. As for the primers for lva-miR-272, the amplified DNA fragment showed 96% identity to FE085434. Lva-miR-272 gene was found to be present in genomic DNA. However, surprisingly, as shown in Figure 4.1, lva-miR1476-3pFR gave PCR products at about 1.4kb whereas beta-actin produced amplicons at about 710bp. For lva-miR-1476-3p primers, PCR products amplified from genomic DNA were sequenced forward and reverse as its length was quite long (1.4kb) (refer to Figure 4.1). The lva-miR-1476-3p gene was able to be identified from the reverse sequence. However, homology search against FE146432 resulted in low identity since it was most probably spanning unknown splice junctions. For the beta-actin primers, low identity was found for the DNA sample against AF300705 (complete coding sequence for *Litopenaeus vannamei* beta-actin gene). This was probably due to the presence of introns within the targeted regions. The sequences of PCR products for genomic DNA were not as similar as from RNA transcripts. This could be due to the heterozygous genotypes where 2 different alleles present in the genomic DNA. Besides that, it could be due to the targeted DNA sequences containing repeat sequences, which may have led to sequencing errors.

Therefore, the beta-actin primers are quite useful as an indicator of genomic DNA contamination in the RNA samples. If an RNA sample was not treated with DNase and subjected to PCR using beta-actin primers, there would be 2 bands observed on the agarose gel: 710bp and 604bp. The 710bp band indicated amplicons of the beta-actin gene from genomic DNA whereas the 604bp band indicated amplicons of beta-actin gene RNA transcript (cDNA) (refer to Figure 4.1 and 4.2).

Apart from that, the miRNA gene expression level in different individuals was also studied (as shown in Figure 4.4). Total RNAs from six individuals of adult stage were

extracted and subjected to semi-quantitative reverse transcription PCR. As shown in Figure 4.4, the beta actin gene served as the control. The beta actin gene in each target tissue was constantly and consistently expressed at the adult stage. The validated miRNA genes also showed consistent amplification, however, their intensities varied for each individual. This could be due to poor quantification of RNA concentration using UV spectrophotometer. Another possibility could be related to the expression of miRNA genes in different tissues. Since this research was the fundamental study for the search of *Litopenaeus vannamei* miRNA candidates, the miRNA genes involved in different stages are yet to be looked into. Therefore, it led to potential further miRNA studies involving in different developmental stages of *Litopenaeus vannamei*. For example, *let-7* regulates temporally at the 3' untranslated region (UTR) of the *lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12* genes (Reinhart *et al*., 2000). On the other hand, Aravin *et al*. (2003) discovered that miR-3, miR-309 and miR-7 were expressed during *Drosophila melanogaster* development. Therefore, the miRNA expression is elucidated according to its functions in regulating the mRNA targets since miRNA genes were found involving in developmental timing, cell proliferation, apoptosis, metabolism, morphogenesis, cell differentiation, neurobiology, cancer and virus (Houbaviy *et al*., 2003; Ambros, 2004; Stark *et al*., 2005; Esau and Monia, 2007). It has been estimated that 20-30% of animal genes are regulated by miRNA genes (Wienholds and Plasterk, 2005; Stark *et al*., 2005).

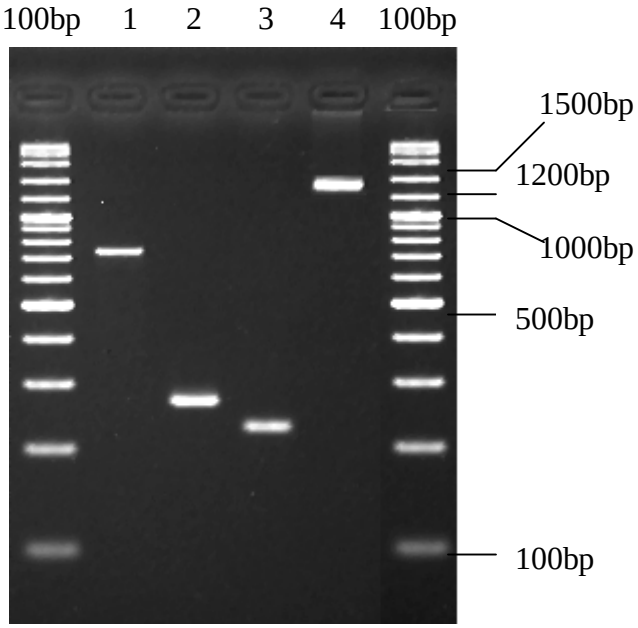



**Figure 4.1**
**PCR Products from Genomic DNA**

100bp was 100bp plus DNA ladder (Vivantis); Lane 1: beta-actin (710bp); Lane 2: lva-miR-256FR (266bp); Lane 3: lva-miR-272FR (233bp) and Lane 4: lva-miR1476-3pFR (1.4kb)

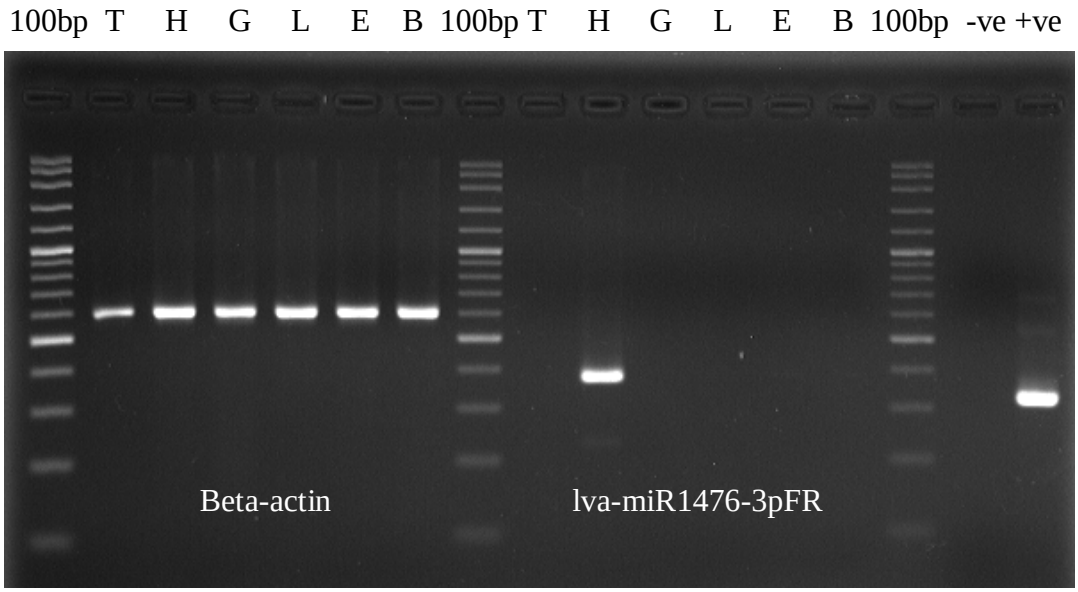



**Figure 4.2**
**PCR Products from Total RNA (Beta-actin and lva-miR 1476-3pFR)**

T-muscle tissues; H -hepatopancreas; G -gill tissue; L -leg tissue; E -eyestalk and B –brain tissue; 100bp plus DNA ladder; -ve control -distilled water and +ve control -kanamycin positive control RNA

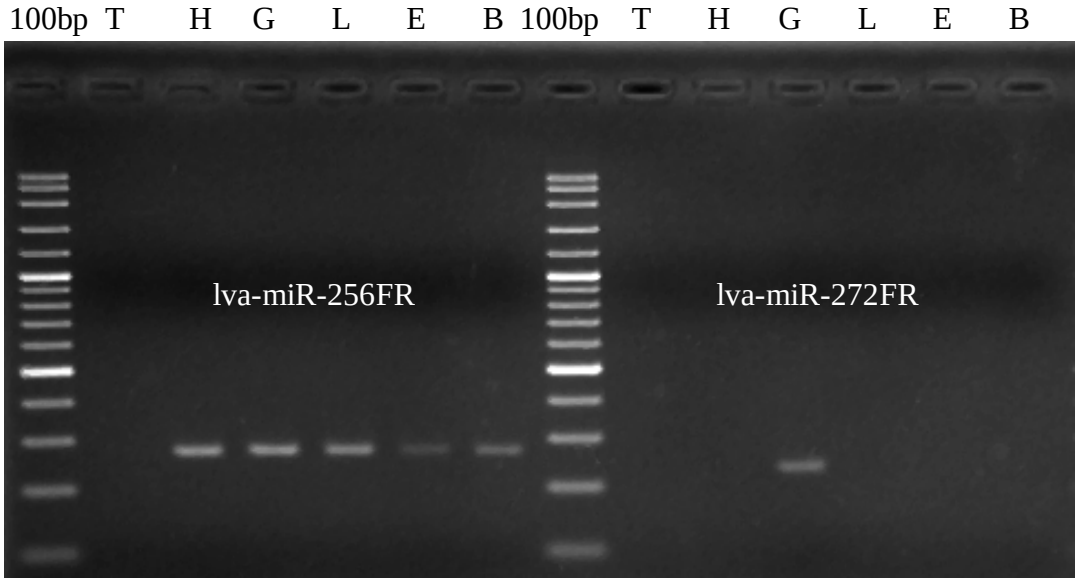
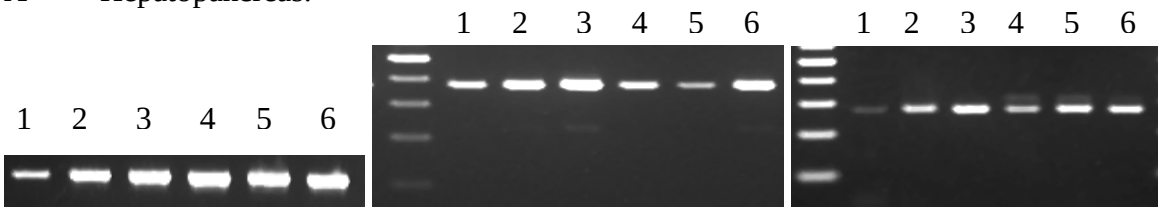


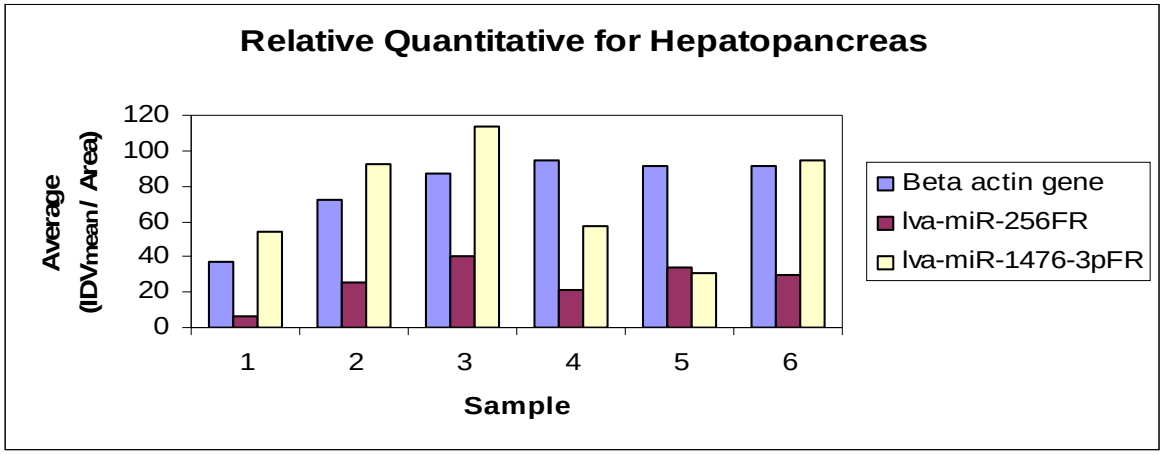

**Figure 4.3**
**PCR Products from Total RNA (lva-miR-256FR and lva-miR-272FR)**
T -muscle tissues; H -hepatopancreas; G - gill tissue; L -leg tissue; E -eyestalk and B -brain tissue; 100bp plus DNA ladder

A Hepatopancreas:

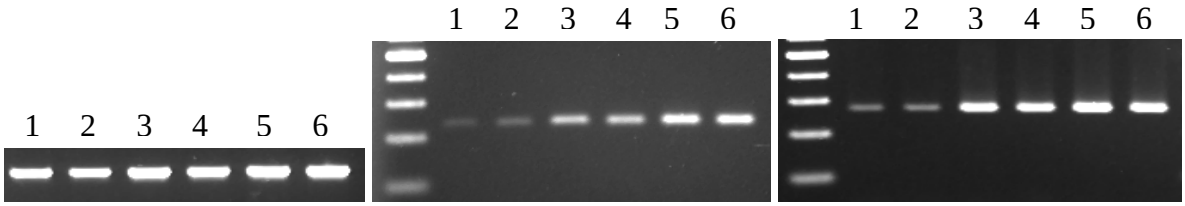



Beta actin lva-miR-1476-3pFR lva-miR-256FR

| Beta actin | | | | | |
|---|---|---|---|---|---|
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 18000 | 3.95 | 480 | 37.5 | |
| 2 | 34560 | 7.6 | 480 | 72 | |
| 3 | 41760 | 9.2 | 480 | 87 | +/- 1.173 |
| 4 | 45120 | 9.95 | 480 | 94 | |
| 5 | 43680 | 9.6 | 480 | 91 | |
| 6 | 43680 | 9.6 | 480 | 91 | |
| lva-miR-1476-3pFR | | | | | |
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 26160 | 6.15 | 480 | 54.5 | |
| 2 | 44160 | 10.4 | 480 | 92 | |
| 3 | 54480 | 12.85 | 480 | 113.5 | +/- 1.947 |
| 4 | 27600 | 6.5 | 480 | 57.5 | |
| 5 | 14880 | 3.5 | 480 | 31 | |
| 6 | 45120 | 10.65 | 480 | 94 | |
| lva-miR-256FR | | | | | |
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean (based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 3120 | 2.05 | 480 | 6.5 | |
| 2 | 12240 | 8.1 | 480 | 25.5 | |
| 3 | 19200 | 12.75 | 480 | 40 | +/- 1.291 |
| 4 | 10320 | 6.85 | 480 | 21.5 | |
| 5 | 16080 | 10.65 | 480 | 33.5 | |
| 6 | 14400 | 9.55 | 480 | 30 | |

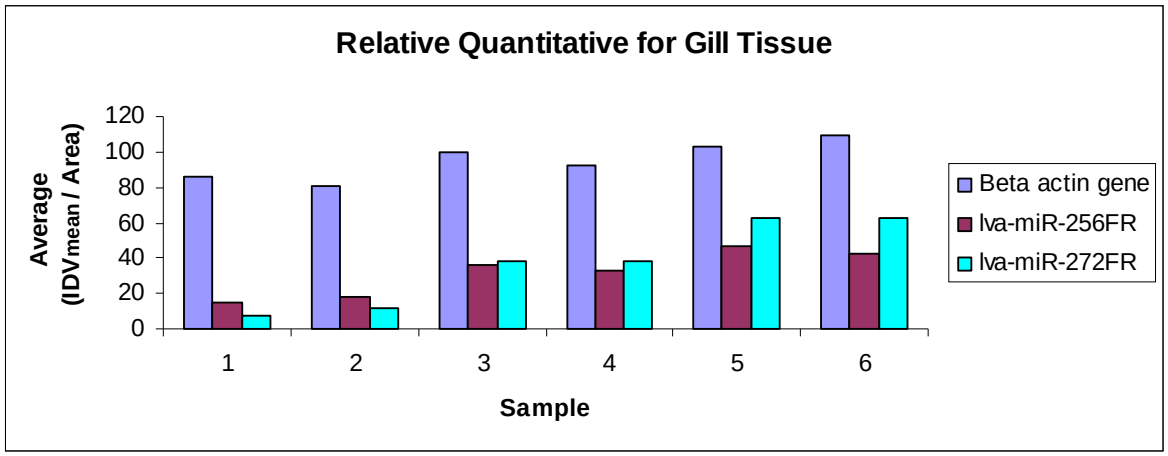


B Gill Tissue:

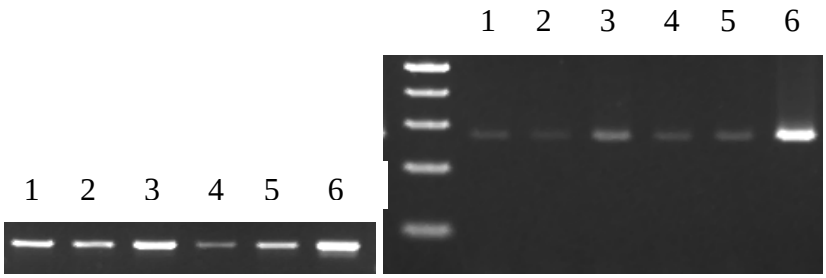



Beta actin lva-miR-272FR lva-miR-256FR

| Beta actin | | | | | |
|---|---|---|---|---|---|
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 41280 | 7.5 | 480 | 86 | |
| 2 | 38640 | 7.05 | 480 | 80.5 | |
| 3 | 47760 | 8.75 | 480 | 99.5 | +/- 2.282 |
| 4 | 44400 | 8.1 | 480 | 92.5 | |
| 5 | 49200 | 9 | 480 | 102.5 | |
| 6 | 52560 | 9.6 | 480 | 109.5 | |
| lva-miR-272FR | | | | | |
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 3360 | 3.2 | 480 | 7 | |
| 2 | 5760 | 5.4 | 480 | 12 | |
| 3 | 18240 | 17.2 | 480 | 38 | +/- 0.289 |
| 4 | 18480 | 17.4 | 480 | 38.5 | |
| 5 | 30240 | 28.5 | 480 | 63 | |
| 6 | 30000 | 28.3 | 480 | 62.5 | |
| lva-miR-256FR | | | | | |
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean (based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 7200 | 3.95 | 480 | 15 | |
| 2 | 8640 | 4.7 | 480 | 18 | |
| 3 | 17520 | 9.55 | 480 | 36.5 | +/- 1.658 |
| 4 | 15600 | 8.5 | 480 | 32.5 | |
| 5 | 22560 | 12.3 | 480 | 47 | |
| 6 | 20160 | 11 | 480 | 42 | |

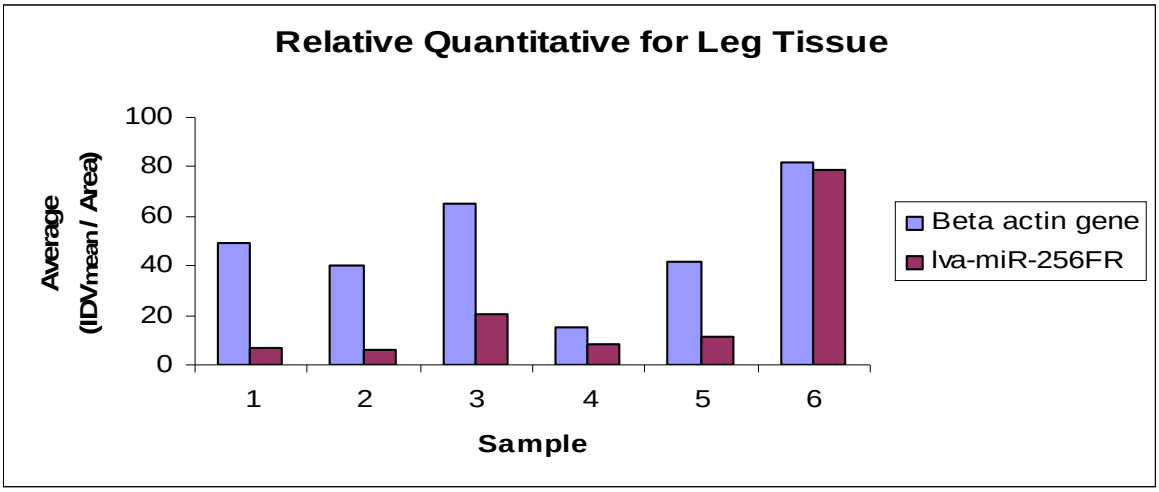


C Leg Tissue:

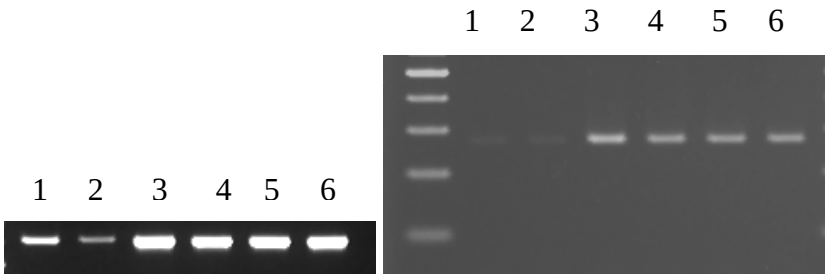



Beta actin lva-miR-256FR

| Beta actin | | | | | |
|---|---|---|---|---|---|
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 23520 | 8.4 | 480 | 49 | +/- 1.514 |
| 2 | 19200 | 6.8 | 480 | 40 | |
| 3 | 31440 | 11.2 | 480 | 65.5 | |
| 4 | 7200 | 2.55 | 480 | 15 | |
| 5 | 19920 | 7.1 | 480 | 41.5 | |
| 6 | 39120 | 13.95 | 480 | 81.5 | |
| lva-miR-256FR | | | | | |
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 3120 | 2.5 | 480 | 6.5 | +/- 0.408 |
| 2 | 2880 | 2.3 | 480 | 6 | |
| 3 | 9840 | 7.8 | 480 | 20.5 | |
| 4 | 3840 | 3.1 | 480 | 8 | |
| 5 | 5520 | 4.4 | 480 | 11.5 | |
| 6 | 37680 | 30 | 480 | 78.5 | |

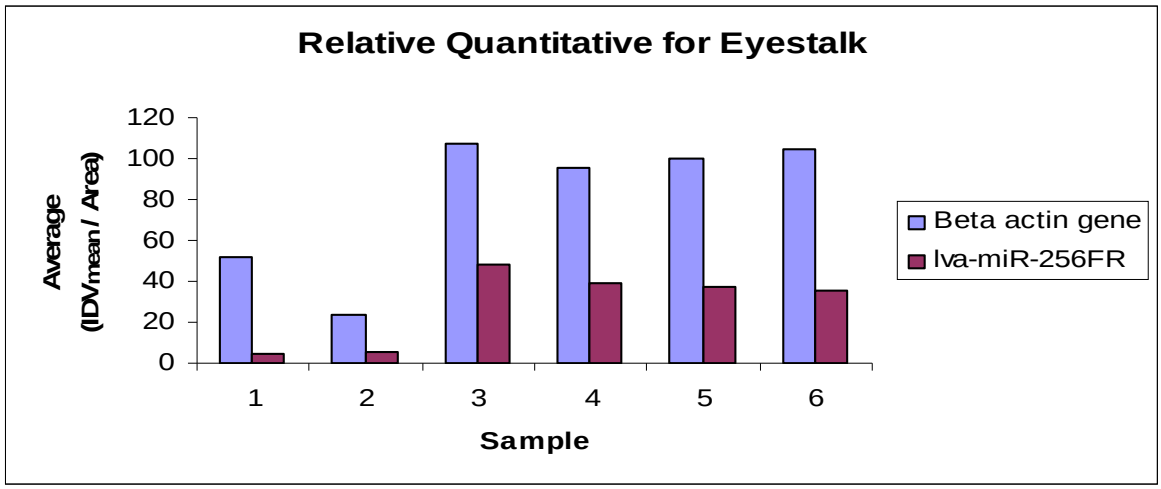


D Eyestalk:

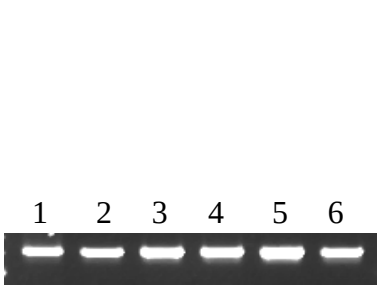



Beta actin lva-miR-256FR

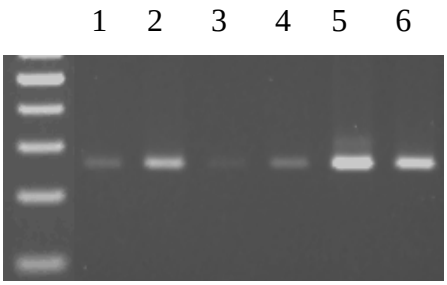
| Beta actin | | | | | |
|---|---|---|---|---|---|
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 24960 | 5.4 | 480 | 52 | +/- 1.429 |
| 2 | 11520 | 2.5 | 480 | 24 | |
| 3 | 51360 | 11.1 | 480 | 107 | |
| 4 | 45600 | 9.8 | 480 | 95 | |
| 5 | 48000 | 10.4 | 480 | 100 | |
| 6 | 50160 | 10.85 | 480 | 104.5 | |
| lva-miR-256FR | | | | | |
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 2400 | 1.5 | 480 | 5 | +/- 1.947 |
| 2 | 2640 | 1.65 | 480 | 5.5 | |
| 3 | 23280 | 14.2 | 480 | 48.5 | |
| 4 | 18720 | 11.4 | 480 | 39 | |
| 5 | 17760 | 10.85 | 480 | 37 | |
| 6 | 17040 | 10.45 | 480 | 35.5 | |



E Brain Tissue:




Beta actin




lva-miR-256FR

| Beta actin | | | | | |
|---|---|---|---|---|---|
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 35280 | 7.05 | 480 | 73.5 | +/-1.061 |
| 2 | 36480 | 7.3 | 480 | 76 | |
| 3 | 44400 | 8.85 | 480 | 92.5 | |
| 4 | 43200 | 8.65 | 480 | 90 | |
| 5 | 51360 | 10.3 | 480 | 107 | |
| 6 | 39120 | 7.85 | 480 | 81.5 | |
| lva-miR-256FR | | | | | |
| Sample | Integrated Density Value Mean ($IDV_{mean}$) | % Mean(based on $IDV_{mean}$) | Area | Average ($IDV_{mean}$/ Area) | Standard Deviation |
| 1 | 8400 | 9.05 | 480 | 17.5 | +/-0.456 |
| 2 | 17760 | 38.3 | 480 | 37 | |
| 3 | 3840 | 4.15 | 480 | 8 | |
| 4 | 10560 | 11.4 | 480 | 22 | |
| 5 | 29280 | 31.5 | 480 | 61 | |
| 6 | 23040 | 24.8 | 480 | 48 | |

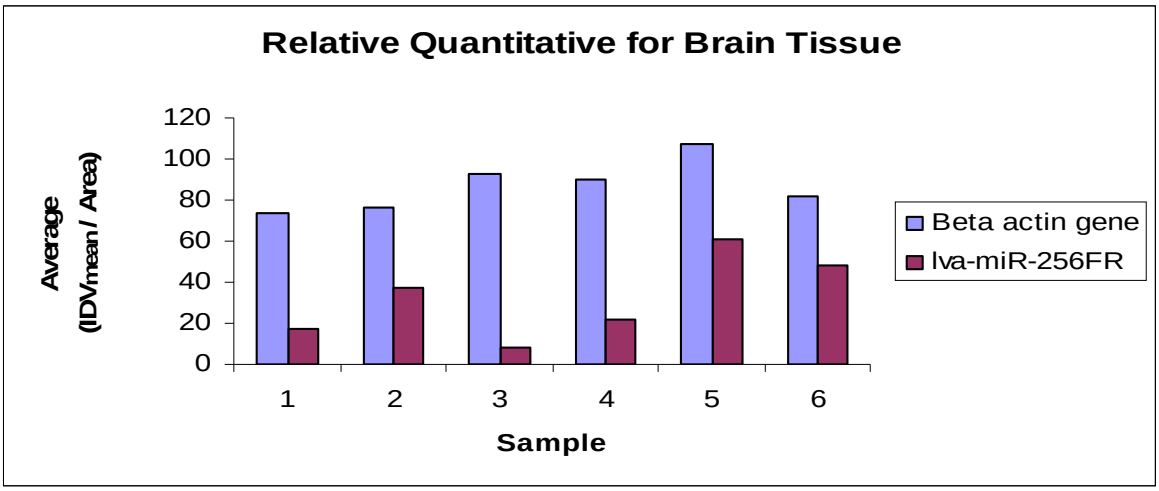



**Figure 4.4**
**A, B, C, D and E: The Relative Expression of Beta Actin against lva-miR-1476-3pFR, lva-miR-272FR and lva-miR-256FR in each target tissue.**

## 4.3 Potential miRNA Target Prediction

The putative miRNA genes were compared against the *Litopeneaus vannamei* mRNA and EST database using BLAST (NCBI, 2008). Unfortunately, predicted miRNA targets were limited since only 244 *Litopenaeus vannamei* mRNAs were available in NCBI and the complete genome has not yet been published. After passing the screening criteria, there were 20 mRNA and 27 EST sequences found to be potential miRNA targets. miRNA candidates could target partially at the 5' UTR, coding region and 3' UTR of genes. However, in this study, only those which were found to have targets within the 3' UTR of mRNA were selected since they are, the most likely to be regulated by miRNAs in animals (Gu *et al*., 2007; Lindow and Gorodkin, 2007; Norden-Krichmar *et al.*, 2007). In addition to that, Norden-Krichmar *et al*. (2007) reported a strong indicator of functional binding site is within the conserved sequences of untranslated regions of orthologous genes. The well conserved core region is usually located at nucleotide 1-7 or 2-8 (Kloosterman *et al*., 2006; Norden-Krichmar *et al.*, 2007). This was because many found that the strongest base-pairing between the miRNA and mRNA was located at the first 8 or 9 nucleotides of 5' end of the miRNA (Nilsen, 2005; Palkodeti *et al*., 2006; Norden-Krichmar *et al*., 2007; deduced from miRBase Targets, 2009). Animal miRNAs are found to inhibit translational process (Kloosterman *et al*., 2006; Lindow and Gorodkin, 2007) or cause mRNA degradation Bagga *et al*. (2005) although imperfect matches of miRNA: mRNA target were observed.

As shown in Table 4.4, five mRNA sequences were predicted to be targeted by 2 different miRNA genes. These have been observed in zebrafish miR-430 in regulating early development morphogenesis; and directing maternal mRNAs deadenylation and clearance (Giraldez *et al*., 2006). Cel-let-7 was found to be temporally regulated at the 3' UTR of the

*lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12* genes (Reinhart *et al*., 2000). In the current study, lva-miR-256 and lva-miR-79 were predicted to bind at different locations within the 3' UTR of EU373096 (prophenoloxidase gene); lva-miR-315 and lva-miR-1476-3p were predicted to target the 3' UTR of AY170126 (lysozyme gene); lva-miR-79 and lva-miR-1476-3p could target at the 3' UTR of DQ923424 (toll protein); lva-miR-33 and lva-miR-279 were predicted to bind to 2 different sites within the 3' UTR of EF467169 (secretory leukocyte proteinase inhibitor); and finally, lva-miR-33 and lva-miR-281 each was predicted to bind to different locations within the 3' UTR of the penaeidin genes (DQ206401, DQ206402, DQ206403, and DQ211701) (refer to Table 4.4). In addition to that, lva-miR-256 was predicted to target at repeat region residing at the 3' UTR (refer to Table 4.4). Comparing both mRNA and EST as potential miRNA targets, mRNA helps to determine which part (5' UTR, coding region or 3' UTR) the miRNA gene can target at whereas EST sequences cannot. This will eliminate false positives of target finding as many have found that animal miRNAs targeted imperfectly at the 3' UTR of mRNA for down regulation. Lee *et al*. (1993), Wightman *et al*. (1993) and Olsen and Ambros (1999) found miRNA *lin-4* impeded the translation process of *lin-14* mRNA through imperfect complementary at *lin-14* 3' UTR during their miRNA discovery in *C. elegans*. *hbl-1* (*C. elegans* hunchback homolog) was found to be regulated by *let-7* and *lin-4* at the 3' UTR of *hbl-1* since the cis-regulatory elements in *hbl-1* 3' UTR contained the complementary site for miRNA *let-7* and *lin-4* (Lin *et al*., 2003). Vella *et al*. (2005) reported that cel-let-7 bound partially to the 3' UTR of the *lin-41* gene (ring finger-B box-coiled coil protein for proper temporal control of post embryonic cell fate) to down regulate the LIN-41 protein. On the other hand, Bagga *et al*. (2005) found that *lin-41* target mRNA degradation occurred when miRNA *let-7* partially complemented to its 3' UTR regulatory sequences. Most of *Litopenaues vannemi* mRNA sequences in the NCBI database are related to prawn immune

genes such as heat shock protein 60 (HSP60), chitinase, crusacyanin, penaeidin and others since there are lots of studies carried out to fight against viral or bacterial diseases in prawn (O'Leary *et al*., 2006). For EST target sequences, it was hard to determine which part of the mRNA the miRNA can target, as most EST represent partial mRNA sequences.

The candidate lva-miR-256 was predicted to target nucleoporin, troponin C and crustacean hyperglycemic hormone- ion transport peptide (as shown in Table 4.4). Nucleoporin is the major nuclear pore protein in eukaryotic cells, and helps to transport mRNA and protein between the nucleus and cytoplasm (Zhang *et al*., 1999; Pfam 23.0, 2008). Chakraborty *et al*. (2008) reported that nucleoporin helped to regulate cell cycle progression and gene-specific expression in humans. Troponin C is the calcium ($Ca^{2+}$) binding subunit of troponin complex (Potter *et al*., 1995; Pfam 23.0, 2008). Potter *et al*. (1995) discovered that when calcium bound to troponin C: it released troponin inhibitory protein and interacted with troponin T to activate actomyosin ATPase in muscle contraction. From the miRBase Target Database (2008), cel-miR-256 was predicted to target mup-2 gene which coded for muscle contractile protein troponin T. Prophenoloxidase (proPO) is part of the immuno-recognition process of defense system in invertebrates which directing melanization and hemolymph coagulation; phenoloxidase enzyme is produced to catalyze the oxidation of phenolic compounds during melanin formation (Lai *et al*., 2005[b]; Cerenius and Söderhäll, 2004). Its functions involve both oxidoreductase activity and as oxygen transporter (deduced from UniProtKB, 2008). *Litopnenaus vannamei* crustacean hyperglycemic hormone-ion transport peptide (LvCHH-ITP) is known to regulate osmo-regulation (Tiu *et al*., 2007) and its molecular function is in neuropeptide hormone activity where this peptide hormone can be found in the central nervous system (UniProtKB, 2008). Nucleoporin, troponin C, prophenoloxidase and ion transport peptide regulate cellular,

developmental and metabolic process. So, lva-miR-256 possibly participates in controlling cellular, developmental and metabolic processes.

The candidate lva-miR-272 was predicted to regulate the beta chain of T-cell receptor beta chain ANA 11, alpha-2-macroglobulin and hemocyanin (refer to Table 4.4). T-cell receptor beta chain ANA 11 is found to be involved in immune responses where its functions interact with protein complex and merging with extracellular or intracellular product to protect the cell (UniProtKB, 2008). Alpha-2-macroglobulin ($\alpha_2$M) is an immune related gene. It contains multiple reactive sites with functions involved in binding, acting as carrier and targeting foreign proteins or pathogens in the immune system (Borth, 1992). Its functions are being annotated in UniProtKB (2008) as both interacting with protein complex and controlling the endopeptidase activity. Hemocyanin is a colorless cuprum containing protein (Voet and Voet, 2004). In prawn, hemocyanin helps to carry oxygen to cells. Based on selective Gene Ontology analysis (Marine Genomics Project, 2008), predicted targets of lva-miR-272 are involved in cellular process. So, lva-miR-272 is potentially involved in regulating cellular processes.

The candidate lva-miR-1476-3p was predicted to target gamma-interferon-inducible-lysosomal thiol reductase (GILT), toll protein, lysozyme and cyclin B. GILT, toll protein and lysozyme, involved in the immune system (as shown in Table 4.4). GILT was identified by Phan *et al*. (2000) and it helps to catalyze disulfide bond reduction in antigen-presenting cells. Toll protein was found to induce innate immune responses in *Drosophila* (Medzhitov *et al*., 1997). Its functions are mainly involved in transmembrane receptor and binding activity (UniProtKB, 2008). Lysozyme is an antimicrobial protein which helps in destroying bacterial peptidoglycan and is often found in animal secretions (Madigan and

Martinko, 2006). Cyclin is a family of proteins that regulating the cell cycle (Voet and Voet, 2004). So, lva-miR-1476-3p is most involved in regulation of cellular and developmental process.

The candidate lva-miR-33 was predicted to target penaeidin and secretory leukocyte proteinase inhibitor at 3' UTR (refer to Table 4.4). Penaeidin (PEN) is a newly described family of antimicrobial peptides which is found specifically in penaeid shrimps and plays a major role in shrimp innate immunity. This peptide with chitin-binding activity is synthesized and kept in shrimp granulocytes (Destoumieux *et al*., 1997). When pathogens present in the environment, penaeidins will be released to hemolymph to regulate exocytosis (Destoumieux *et al*., 2000). Secretory leukocyte proteinase inhibitor is found in vertebrates and invertebrates acting as an immune-associated protein where its main function is to protect cells by inhibiting proteases such as chymotrypsin, trypsin, elastase, and cathepsin from damaging healthy cells during inflammation (Doumas, 2005). The potential targets for Lva-miR-79 were mediator of RNA polymerase II transcription subunit, glutamate dehydrogenase, prophenoloxidase, toll protein and farnesoic acid O-methyltransferase. Mediator of RNA polymerase II transcription subunit is annotated to regulate transcription where it helps to pass on information from gene-regulatory proteins to RNA polymerase II transcription machinery (deduced from UniProtKB, 2008). Glutamate dehydrogenase is found to be involved in amino acid metabolic processes where its functions include binding, oxidoreductase and catalytic activity (narrated from EMBL-EBI Database, 2009). Farnesoic acid O-methyltransferase is categorized as an enzyme that transfers methyl groups from one compound to others where its function relates to methyltransferase activity (deduced from UniProtKB, 2008). The 3' UTR of a chitinase

gene was predicted to be regulated by lva-miR-261 with imperfect complementary. Therefore, lva-miR-261 can be possibly involved in chitin catabolic processes.

The candidate lva-miR-281 has been predicted to target mRNA of tumor protein, splicing factor and penaeidin (as shown in Table 4.4). Tumor protein is participated in B cell differentiation and it can interact with identical protein to form homodimer or non-identical protein to form heterodimer (UniProtKB, 2008). Splicing factor is annotated as functioning in mRNA splice site selection where it can be bound to DNA, RNA or protein complex for subsequent splicing event (UniProtKB, 2008). Lva-miR-279 was predicted to bind imperfectly to the 3' UTR of beta-D-glucan-binding protein, secretory leukocyte proteinase inhibitor, lysozyme and ecdysteroid-regulated protein. UniProtKB (2008) described that beta-D-glucan-binding protein participates in recognizing the pathogens and binds specifically to beta-1,3-glucan to activate prophenoloxidase. Ecdysteroid-regulated protein is a kind of hormone that regulates larval development (Voet and Voet, 2004). In crustaceans, the molting process is regulated by ecdysteroid (Covi *et al*., 2009). Ecdysteroid hormone was found to bind temporally to *Drosophila* chromosomal loci during larval-prepupal puffing cycle (Möritz *et al*., 1984). Lva-miR-752 was predicted to target chaperonin (TCP1 subunit beta), proteasome, eukaryotic translation elongation factor 1 epsilon-1 and calreticulin 9as shown in Table 4.4). Chaperonin is known to take part in assisting proper protein folding and is often associated with heat shock proteins (Voet and Voet, 2004). The proteasome is a multicatalytic proteinase complex which is able to cleave the Arg, Phe, Glu, Leu and Tyr residues at existing peptides bonds and usually regulates protesomal ubiquitin-dependent protein catabolic processes (UniProtKB, 2008). Eukaryotic translation elongation factor 1 epsilon-1is annotated to regulate apoptosis, DNA damage response, translation and negative regulation of cell proliferation (UniProtKB, 2008).

Calreticulin takes part in the endoplasmic reticulum to facilitate glycoprotein folding and protect glycoprotein from degradation and subsequent transfer to the Golgi apparatus (Voet and Voet, 2004). Lva-miR-36* was predicted to bind partially to ATP synthase (refer to Table 4.4). ATP synthase is involved in transporting protons across membranes for ATP synthesis (UniProtKB, 2008). Last but not least, the function of miRNA basically depends on their targets. The identified 11 miRNA candidates are most probably involved in cellular, developmental, cell death, metabolic and immune-response process, as summarized in Table 4.4).

**Table 4.4**
**Potential miRNA Targets**

| Candidate miRNA | Potential miRNA Target (GenBank Accession No) | Potential Targeted Protein | miRNA: mRNA Target Binding (candidate miRNA is indicated in red and target is shown below miRNA) |
|---|---|---|---|
| lva-miR-256 | FE148324 (EST) FE154839 (EST) | Nucleoporin | 3′-ATGTCAGAAAATAAGTAAGGA-5′<br>  \| :  \|\|\|\|\|\|\|\|\|\|\|\|\|<br>5′-TCTTTTCTTTTATTCATTCAA-3′ |
| | FE176050 (EST) | Troponin C | 3′-ATGTCAGAAAATAAGTAAGGA-5′<br>  \| :\|  \| \|\|\|\|\|\|\|\|\|\|\|\|<br>5′-TTTATACATTTATTCATTCCT-3′ |
| | EU373096 | Prophenoloxidase | 3’UTR:<br>3′-ATGTCAGAAAATAAGTAAGGA-5′<br>  \|  \| \|\|\|\|\|\|\|\|\|\|\| :<br>5′-TTGTATATTTTATTCATTATA-3′ |
| | EF156402 | Crustacean hyperglycemic hormone- Ion transport peptide | 3’UTR:<br>3′-ATGTCAGAAAATAAGTAAGGA-5′<br>  :\|\|\| :  \|\|  \|\|\|\|\|\|\|<br>5′-GGCAGCTCCTTTATCATTCCA-3′ |

| | | | |
|---|---|---|---|
| | AY466196<br><br>AF360105 | Microsatellite sequence | Repeat region:<br>3′-ATGTCAGAAAATAAGTAAGGA-5′<br>5′-TCCTCTCTTTTACTCATTCCT-3′<br><br>3′-ATGTCAGAAAATAAGTAAGGA-5′<br>5′-CTCTCTCTTTTTTTCATTCCA-3′ |
| lva-miR-272 | FE191662 (EST)<br>FE191568 (EST)<br>FE079172 (EST)<br>FE084442 (EST)<br>FE087839 (EST)<br>FE079172 (EST) | T-cell receptor beta chain ANA 11 | 3′-GTTTGTGTGTACGTATGT-5′<br>5′-CAAACACACACGCATACA-3′ |
| | EF073268 | Alpha-2-macroglobulin | 3’ UTR:<br>3′-GTTTGTGTGTACGTATGT-5′<br>5′-TACACACACATGCATACA-3′ |
| | X82502 | Hemocyanin | 3’ UTR:<br>3′-GTTTGTGTGTACGTATGT-5′<br>5′-AAAGAACATATGCATACA-3′ |

| | | | |
|---|---|---|---|
| lva-miR-1476-3p | FE135112 (EST) FE146431 (EST) FE135201 (EST) FE128941 (EST) FE135848 (EST) FE139993 (EST) FE135848 (EST) BF024268 (EST) | Gamma-interferon-inducible-lysosomal thiol reductase | 3′-TCGAGACTCCTTGTCATGTACAG-5′<br>\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|<br>5′-AGCTCTGAGGAACAGTACATGTC-3′ |
| | DQ923424 | Toll protein | 3’UTR:<br>3′-TCGAGACTCCTTGTCATGTACAG-5′<br>\| :\|:\| \|\|\|\|\|\|<br>5′-ACTTTTTTTTCCGTGTACATGAG-3′ |
| | AY170126 | Lysozyme | 3’UTR:<br>3′-TCGAGACTCCTTGTCATGTACAG-5′<br>\|: : \|\| : \|\|\|\|\|\|\|<br>5′-CGTCTAGATATTAGATACATGTC-3′ |
| | FJ228728 | Cyclin B | 3’UTR:<br>3′-TCGAGACTCCTTGTCATGTACAG-5′<br>\| \| \|: \|\|\|\|\|\|\|:<br>5′-ATAAAGTAAATATTGTACATGTT-3′ |
| lva-miR-33 | AF390145 | Antimicrobial peptide (penaeidin) 3i | 3’ UTR:<br>3′-TTTACGTTAATGTTACGTG-5′<br>:\| \| : \|\|\|\|\|\|\|<br>5′-GATTTTTCCATGAATGCAC-3′ |

| | | | |
|---|---|---|---|
| | EF467169 | Secretory leukocyte proteinase inhibitor | 3’ UTR:<br>3′-TTTACGTTAATGTTACGTG-5′<br>|| |||||||<br>5′-CTTCGCCTAACCAATGCAA-3′ |
| lva-miR-79 | FE057636 (EST) | Mediator of RNA polymerase II transcription subunit | 3′-AAGAAACCATTATATAGAAAT-5′<br>: ||||||||||||<br>5′-CCTCCCACTAATATATCTTTA-3′ |
| | EU496492<br>AM076955 | Glutamate dehydrogenase (gdh) | 3’ UTR:<br>3′-AAGAAACCATTATATAGAAAT-5′<br>||: | | | |||||||<br>5′-TTTGGTCATCCCACATCTTTA-3′ |
| | EU373096<br>EF115296 | Prophenoloxidase | 3’ UTR:<br>3′-AAGAAACCATTATATAGAAAT-5′<br>||:| | | |||||||||<br>5′-TTTTCTTTGATGATATCTTTA-3′<br><br>3′-AAGAAACCATTATATAGAAAT-5′<br>||: | | |||||||||<br>5′-TTTATGTTAAATATATCTGTT-3′ |
| | DQ923424 | Toll protein | 3’ UTR:<br>3′-AAGAAACCATTATATAGAAAT-5′<br>|||||||<br>5′-AGACAGATGAATATATATTTA-3′ |
| | AY823408<br>DQ067631 | Farnesoic acid O-methyltransferase | 3’ UTR:<br>3′-AAGAAACCATTATATAGAAAT-5′<br>: || |:: ||||||||<br>5′-CATGTTTTTGGAATATCTTTT-3′ |
| lva-miR-261 | AF315689 | Chitinase | 3’UTR:<br>3′-CGACTTTTGTTTTTTCGAT-5′<br>|: | :::: |||||||<br>5′-GTCGCGGGTTAAAAAGCAG-3′ |
| lva-miR-281 | FE103907 (EST) | Tumor protein D54-like / similar to CG5174 CG5174-PA | 3′-AGACTCTGGTTAAGGTACTGT-5′<br>| | ||||||||||<br>5′-GATTTCAACAATTCCATGAAA-3′ |

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| | FE172531 (EST) FE049236 (EST) | Splicing factor | 3′-AGACTCTGGTTAAGGTACTGT-5′<br>:\| \| \|\|\|\|\|\|\|\|\|\|<br>5′-GTTTCATCAAATTCCATGACC-3′ |
| | DQ206401 | Antimicrobial peptide (penaeidin) PEN2-1 | 3'UTR:<br>3′-AGACTCTGGTTAAGGTACTGT-5′<br>\|\| \| : : \|\|\|\|\|\|\|\|<br>5′-GCTAAAGATTCTTCCATGAAT-3′ |
| | DQ206403 | Antimicrobial peptide (penaeidin) PEN3-1 | 3'UTR:<br>3′-AGACTCTGGTTAAGGTACTGT-5′<br>\|:\| \| : : \|\|\|\|\|\|\|\|<br>5′-TTTAAAGATTTTTCCATGAAT-3′ |
| | DQ206402 | Antimicrobial peptide (penaeidin) PEN4-1 | 3'UTR:<br>3′-AGACTCTGGTTAAGGTACTGT-5′<br>\|\| \| : : :\|\|\|\|\|\|\|\|<br>5′-GCTAAAGATTGTTCCATGAAT-3′ |
| | DQ211701 | Antimicrobial peptide (penaeidin) PEN4-3 | 3'UTR:<br>3′-AGACTCTGGTTAAGGTACTGT-5′<br>\|:\| \| : : \|\|\|\|\|\|\|\|<br>5′-TTTTAAGATTTTTCCATGAAT-3′ |
| lva-miR-279 | AY249858 | 1,3-beta-D-glucan-binding protein precursor | 3' UTR:<br>3′-TACTAACACCTAGATCACG-5′<br>\| \|\|\|\|\|\|\|<br>5′-CATTCACGGCATCTAGTAA-3′ |
| | EF467169 | Secretory leukocyte proteinase inhibitor | 3' UTR:<br>3′-TACTAACACCTAGATCACG-5′<br>: \|: \|\|\|\|\|\|\|:<br>5′-GGGGACTGAATTCTAGTGT-3′ |
| lva-miR-315 | AY170126 | Lysozyme | 3' UTR:<br>3′-AGAAGACTCGTTGGTAGTTTT-5′<br>\| :\| \| \|\| \|\|\|\|\|\|\|<br>5′-AGTGTTTTGGAAACATCAAAT-3′ |

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| | DQ398569 | Ecdysteroid-regulated protein | 3’ UTR:<br>3′-AGAAGACTCGTTGGTAGTTTT-5′<br>\|:\| \|\|::\|\|\|\|\|\|\|\|<br>5′-TTTAACCTGCGGCCATCAAAC-3′ |
| lva-miR-752 | FE066748 (EST) CK591280 (EST) | Chaperonin containing TCP1, subunit 2 (beta) | 3′-GTTGGTGGTAACGACTGT-5′<br>\|\|\|\|\|\|:\|\|\|\|\|\|\|\|\|\|<br>5′-CAACCATCATTGCTGACA-3′ |
| | FE065535 (EST) | Proteasome subunit Y/ beta type 6-like | 3′-GTTGGTGGTAACGACTGT-5′<br>\| :\|\|\|\|\|\|\|\|\|\|\|\|<br>5′-CCCAGGCCATTGCTGACA-3′ |
| | FE142966 (EST) | Eukaryotic translation elongation factor 1 epsilon-1 | 3′-GTTGGTGGTAACGACTGT-5′<br>: : \|\|\|\|\|\|\|\|\|\|\|\|:<br>5′-TACTAACCATTGCTGACG-3′ |
| | FE135873 (EST) | Calreticulin | 3′-GTTGGTGGTAACGACTGT-5′<br>\| \|\|\|\|\|\|\|\|\|\|\|\|\|\|<br>5′-CCCCCACCATTGCTGACC-3′ |
| lva-miR-36* | CD526679 (EST) | ATP synthase subunit 6 | 3′-TTTGGTTCAATGGTATGTGA-5′<br>\|\|\|\|\|\|\|\|\| \|\|\|\|\|\|<br>5′-TTTACAAGTACCAGACATCT-3′ |