CHAPTER III
MATERIALS AND METHODS

3.1 Meterials

3.1.1 Shrimp

The white shrimps (*Litopeneaus vannamei*), sized about 2-3 or 6-8 g, were obtained from commercial shrimp farms in the eastern part of Thailand. To test IHHNV-free shrimp, the pleopods were collected to extract genomic DNA to analyze for the IHHNV expression by PCR before performing the experiment. Shrimps were cultured in continuous aerated artificial seawater at 5 or 10 ppt and fed with commercial feed every day.

3.1.2 Virus stock

IHHNV lysate was prepared from IHHNV-infected gill of *P.monodon*, size about 20 g.

3.1.3 Chemicals

The chemicals were molecular grade marketed from commercial companies such as BIO-RAD, Fermentas, Invitrogen, Merck, Molecular Research Center, Promega and Sigma.

3.1.4 Enzymes and buffers

Improm-II ™ reverse transcriptase	Promega
Taq DNA polymerase	Homemade
10X *Taq* DNA polymerase buffer + $(NH_4)_2SO_4$	Fermentas
Ribonuclease A (RNase A)	USBiological
Short cut ribonuclease III (RNase III)	New England Biolab

3.1.5 Kits and miscellaneous

1 kb plus DNA ladder	Invitrogen
100 bp DNA ladder	Invitrogen
λ/*Hin*d III DNA marker	Invitrogen
Deoxyribonucleotide triphosphates (dNTPs)	Promega
TRI®Reagent	Molecular Research Center
Agarose	SeaKem LE
High pure viral nucleic acid kit	Roche

3.1.6 Recombinant plasmids

pET17b-stRab7 containing an inverted repeat of PmRab7 stem. This recombinant plasmid was kindly constructed by Miss Mayuree Chanasakulniyom (Figure 3-1). The stem region of dsRNA-PmRab7 prepared by *in vivo* bacterial expression was at the nucleotide position 246-639 of PmRab7 (GenBank accession number DQ231062).

pET3a-GFP containing an inverted repeat of GFP. This recombinant plasmid was kindly provided by Asst. Prof. Dr. Witoon Tirasophon, Mahidol University.

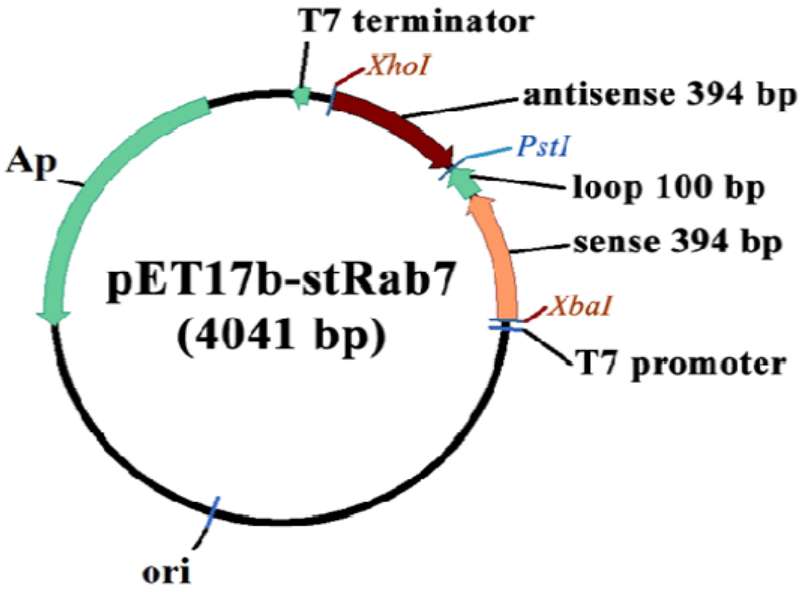


Figure 3-1 Diagram of the recombinant plasmid, pET17b-stRab7.

The sense-loop fragment (494 bp) is at the nucleotide position 246-739. The stem region (394 bp) is at the nucleotide position 246-639 of PmRab7 (GenBank accession number DQ231062).

3.1.7 Bacterial strains

The HT115 genotype is as follows: *(F-, mcrA, mcrB, IN (rrnD-rrnE)1, lambda, rnc14::Tn10 (DE3 lysogen:lacUV5 promoter-T7 polymerase)).* This strain, lacking RNase III activity, contains tetracycline-resistance marker and expressed T7 RNA polymerase by isopropyl-beta-D-thiogalactopyranoside (IPTG) inducible promoter.

3.1.8 Bacterial growth medium

Bacteria were cultured in Luria-Bertani (LB) agar, Luria-Bertani (LB) broth and 2XYT medium. The LB broth contains 1% (W/V) tryptone, 1% (W/V) NaCl and 0.5% (W/V) yeast extract. For LB agar, the medium was added 15% European bacteriological agar. The 2XYT medium contains 1.6% (W/V) tryptone, 1% (W/V) yeast extract and 0.5% (W/V) NaCl. The *E.coli* HT115 strain containing the recombinant plasmid were selected by using 100 μg/ml of ampicillin and 12.5 μg/ml of tetracycline.

3.1.9 Oligonucleotide primers

All of the oligonucleotide primers in all experiments were purchased from PROLIGO Singapore Pty Ltd. The sequences of primers were shown in Table 3-1

Table 3-1 List of oligonucleotide primers

Reaction	Name	Sequence(5′→3′)	Tm (°C)	Product size(bp)
Reverse transcription	PRT	CCGGAATTCAAGCTTCTAGAGGAT CCTTTTTTTTTTTTTTT	70	-
Amplification of LvActin	PmActin-F	GACTCGTACGTCGGGCGACGAGG	62	550 (F/R1) and 350 (F/R2)
	PmActin-R1	AGCAGCGGTGGTCATCACCTGCTC	65	
	PmActin-R2	CGTAGATGGGCACGGTGTGGG	60	
Amplification of LvRab7	GTP-met	ATGGCATCTCGCAAGAAGATT	53	617
	GTP-stop	TTAGCAAGAGCATGCATCCTG	53	
Amplification of IHHNV	IHHNV309F	TCCAACACTTAGTCAAAACCAA	49	309
	IHHNV309R	TGTCTGCTACGATGATTATCCA	50	

3.2. Methods

3.2.1 Shrimp culture

Juvenile shrimps, *L. vannamei*, size about 2-3 g or 6-8 g were obtained from commercial shrimp farms in Thailand. Shrimps were cultured in seawater with continuous aeration. The seawater was renewed every 2 days. Shrimps were fed with commercial shrimp diet every day.

3.2.2 Virus stock

3.2.2.1 Virus stock preparation

The gills of IHHNV-infected shrimp, *Penaeus monodon*, were homogenized in TN buffer containing 20 mM Tris-HCl, 400 mM NaCl at pH 7.4 at concentration of 0.1 g/ml. The mixture was centrifuged at 2,000 g, 4°C for 10 min. The supernatant was filtered through a 0.45 μM filter. The filtrate (IHHNV-lysate) was aliquoted and stored at -80°C.

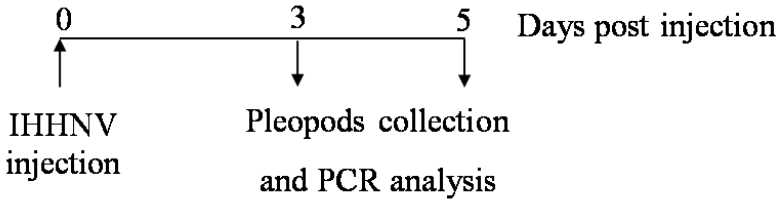
To determine IHHNV titer, the viral nucleic acid was extracted from IHHNV lysate by using High pure viral nucleic acid kit (Roche, USA). The 200 μl IHHNV lysate was added with 200 μl binding buffer supplemented with poly (A) carrier and 50 μl proteinase K and was immediately mixed. The mixture was incubated for 10 min at 72°C and added the 100 μl binding buffer. The sample was transfered to the upper reservoir of the High pure filter tube and centrifuged for 1 min at 8,000 g. The flow through was discarded. The column was added with 500 μl inhibitor removal buffer before centrifugation for 1 min at 8,000 g and the flow through was discarded. The column was washed 2 times with 450 μl wash buffer following to centrifuge at 8,000 g for 1 min. The sample was centrifuged for 10 sec at 13,000 g and discarded the flow through and eluted with 50 μl sterile distilled water.

The 2 μl of purified viral nucleic acid was serially diluted from 10^0 to 10^{-8} and was used to determine the viral titer by PCR. The PCR products (5 μl) were run on 1% agarose gel electrophoresis.

3.2.2.2 IHHNV infectivity

To test the IHHNV infectivity, viral-free shrimps were challenged with 6×10^6 or 6×10^7 IHHNV lysate into hemolymph of about 2 grams shrimps. After 3 and 5 days viral injection, pleopods of each shrimp were collected and extracted DNA in order to analyse IHHNV infectivity. The control group is

shrimps injected with 150 mM NaCl. The plan of shrimp injection followed to this diagram.



3.2.3 Double-stranded RNAs preparation

3.2.3.1 Double-stranded RNAs production by *in vivo* bacterial expression

The recombinant plasmid containing an inverted sequence of dsRNA-PmRab7 (pET17b-stRab7) (kindly constructed by Ms. Mayuree Chanasakulniyom) and dsRNA-GFP were transformed into *E.coli* HT115 which lacked ribonuclease III (RNaseIII) activity. The transformants were spreaded on ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) (amp-tet) LB agar plate for recombinant plasmid and *E.coli* HT115 selection, respectively. A single colony was cultured in 3 ml of ampicillin-tetracyclin LB and incubated at 37°C overnight. The cell culture was inoculated in 2xYT medium at a ratio 1:100 and cultured at 37°C until OD_{600} reached 0.4. The expression of hairpin RNAs of the corresponding genes were induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 and 0.1 mM for dsRNA-GFP and dsRNA-PmRab7, respectively, and incubated at 37°C until OD_{600} reached 1 per 1 ml culture. The 100 OD_{600} of bacterial cells were collected by centrifugation and resuspended in 5 ml phosphate buffer saline (PBS) containing 0.1% SDS. The bacterial cell was boiled for 2 min then quick cooled on ice. Single-stranded RNA in the loop region of the hairpin structure and the endogenous RNA of the bacterial host were eliminated by addition 10 μl (0.1 μl per OD) of RNase A (10 mg/ml) and 1.3 ml (13 μl per OD) of 5x RNase A buffer (1.5 M Sodium acetate, 50 mM Tris-HCl, 25 mM EDTA, pH 8.0). The reaction was incubated at 37°C for 15 min. The TRI reagent (1 ml per 10 OD cells) was used for dsRNA extraction and followed the manufacturer's protocol. The purified dsRNA was dissolved in 1 ml of 150 mM NaCl (10 μl per OD). The dsRNA integrity was

confirmed by RNase A and RNase III digestion assay. DsRNAs concentration was determined by 1% agarose gel electrophoresis.

3.2.3.2 Determination of dsRNAs concentration by gel electrophotoresis

DsRNAs were serially diluted from 1 to 200 fold and their concentrations were determined by running on 1% agarose gel electrophoresis. Densitometric scanning of the band intensities was performed and analyzed by Scion image analysis program.

3.2.3.3 Ribonuclease digestion assay

The dsRNAs integrity was confirmed by RNase A and RNase III digestion assay. The dsRNAs (3 µg) were incubated with RNase A (0.01 µg/µl) or RNase III (1.3 unit/µl) in a total volume of 10 µl reaction following to Table 3-2.

Table 3-2 The components for RNase A and RNase III digestion assay.

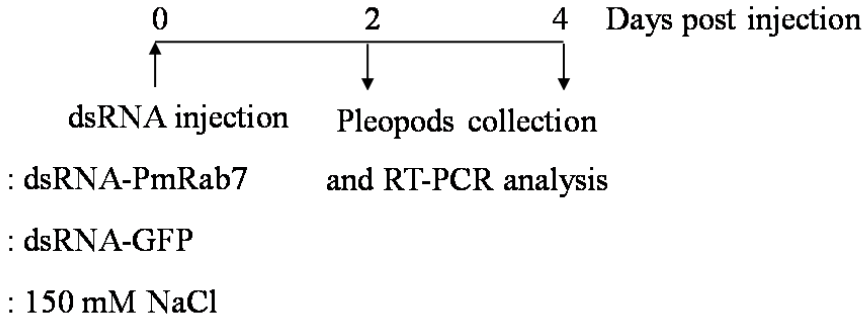
RNase A		**RNase III**	
Components	**Volume**	**Components**	**Volume**
DsRNA	3 µg	DsRNA	3 µg
5X RNase A buffer	2 µl	10X RNase III buffer	1 µl
RNase A	500 ng	RNase III	1.3 units
sterile distilled water	add to 10 µl	10X $MnCl_2$	1 µl
		sterile distilled water	add to 10 µl

The mixtures were incubated at 37°C for 5 min. Then, dsRNAs were run on 1.5% agarose gel electrophoresis.

3.2.3.4 Suppression of LvRab7 by dsRNA-PmRab7

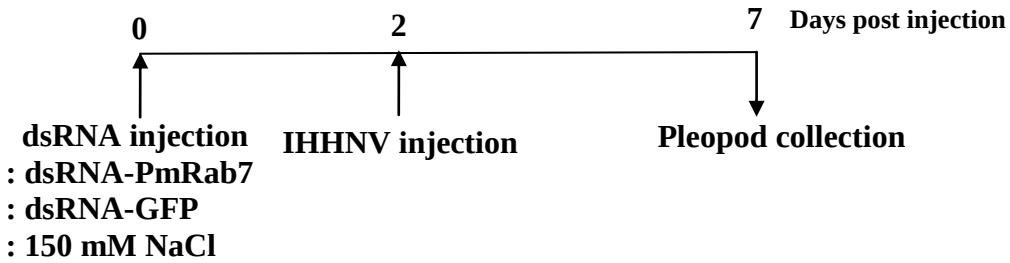
To investigate how long LvRab7 gene was suppressed by dsRNA-PmRab7. *L. vannamei* of size about 2 g was injected with 0.63 µg/g shrimp of dsRNA-PmRab7. Injection of 0.63 µg/g shrimp of dsRNA-GFP was used to investigate the specific effect of dsRNA-PmRab7. The negative control group is shrimps injected with 150 mM NaCl. Two and four days after injection, pleopods were collected and extracted total RNA for RT-PCR analysis to determine LvRab7

expression levels. Actin gene was used as an internal control. The injection plan was shown in the diagram below.



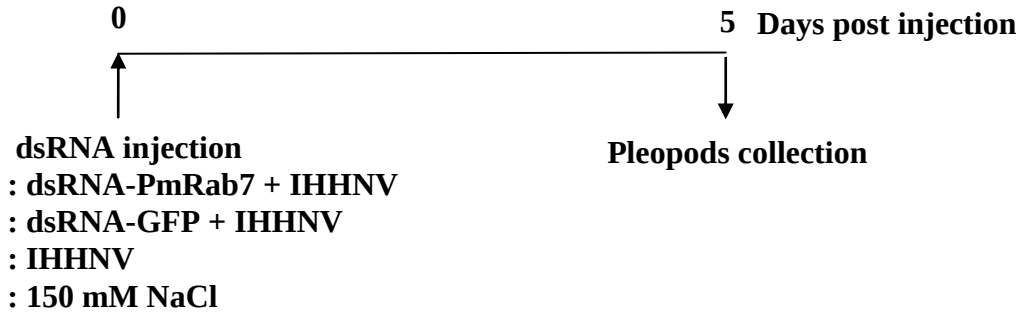
3.2.4 Preventive effect in IHHNV- infected shrimp by dsRNA-PmRab7

To study the preventive effect of LvRab7 knockdown on IHHNV replication in *L. vannamei*, shrimps (size about 2 g) were injected into hemolymph with 0.63 and 2.50 µg/g shrimp dsRNA-PmRab7 two days prior to challenging with 30 µl of IHHNV containing about 6×10^7 particles. Injection of 150 mM NaCl was used as a control group in this study. Furthermore, dsRNA-GFP (0.63 and 2.50 µg/g shrimp) was injected into shrimp in order to use as a non-specific control group. Next, the pleopod of an individual shrimp was collected 7 days post dsRNA injection to extract DNA and RNA. Extraction of genomic DNA and total RNA were performed by using TRI-reagent according to the manufacture's protocol. PCR and RT-PCR analysis were performed to detect expression levels of LvRab7, IHHNV and Actin. The injection plan was shown in the diagram below.



3.2.5 Co-injection of dsRNA-PmRab7 and IHHNV

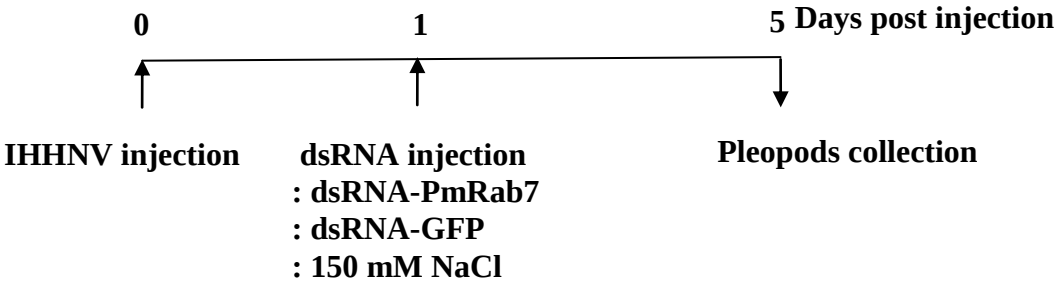
To study the co-injection effect, shrimps (size about 2 g) were co-injected with dsRNA-PmRab7 (0.63 µg/g shrimp) and IHHNV (about 6×10^7 particles) into hemolymph. Injection of 150 mM NaCl or IHHNV alone was used as a control group in this study. Furthermore, co-injection of dsRNA-GFP (0.63 µg/g shrimp) and IHHNV (about 6×10^7 particles) was used as a non-specific control group. The pleopod of an individual shrimp was collected 5 days after IHHNV challenge to extract DNA and RNA. Extraction of genomic DNA and total RNA were performed by using TRI-reagent according to the manufacture's protocol. PCR and RT-PCR analysis were performed to detect expression levels of LvRab7, IHHNV and Actin. The injection plan was shown in the diagram below.



3.2.6 Therapeutic effect in IHHNV- infected shrimp by dsRNA-PmRab7

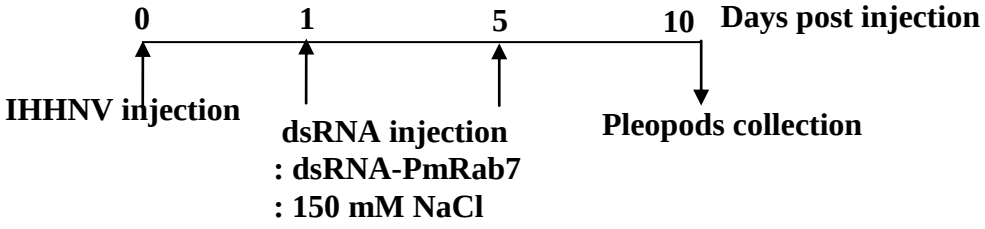
3.2.6.1 Therapeutic effect of dsRNA-PmRab7 on IHHNV replication in a single low dose injection (0.63 µg/g shrimp)

To study the therapeutic effect of dsRNA-PmRab7 on IHHNV replication in *L. vannamei*, shrimp (size about 3 g) were challenged with IHHNV about 6×10^7 particles. One day after IHHNV challenge, shrimp were injected with 0.63 µg/g shrimp of dsRNA-PmRab7 following to the diagram. Injection of 150 mM NaCl or IHHNV alone was used as negative and positive control groups, respectively. In addition, shrimp injected with dsRNA-GFP was used to investigate the specific effect of dsRNA-PmRab7. Pleopods from individual shrimp were collected at 5 days post IHHNV challenge. Genomic DNA and total RNA were extracted and analyzed IHHNV and LvRab7 expression levels by PCR and RT-PCR, respectively**.**



3.2.6.2 Therapeutic effect on IHHNV replication by double injection of dsRNA-PmRab7 (2.5 µg/g shrimp)

For double injection of dsRNA-PmRab7, shrimps were challenged with 6×10^7 particles of IHHNV. One and five day (s) after IHHNV challenge, shrimps were injected with dsRNA-PmRab7 (2.5 µg/g shrimp) following to the diagram. Injection of 150 mM NaCl was used in the control group. Ten days after IHHNV challenge, pleopods from individual shrimp were collected to extract genomic DNA. IHHNV and LvRab7 expression levels were performed by PCR analysis.



3.2.7 Genomic DNA extraction and PCR analysis

3.2.7.1 Genomic DNA extraction

The genomic DNA was isolated from pleopods of individual shrimp by using TRI reagent according to the manufacture's protocol. DNA was isolated from the interphase and phenol phase (lower phase). The residue of aqueous phase (upper phase) was removed. To precipitate DNA, the samples were added with 0.15 ml of 100% ethanol (0.30 ml/ 1 ml TRI reagent) and were inverted. The samples were incubated at room temperature for 3 min and centrifuged at 2,000 g for 5 min at 4°C. The phenol-ethanol supernatant was removed. The DNA pellet was washed twice in 0.5 ml of a solution containing 0.1 mM trisodium citrate in 10% ethanol (1 ml/1 ml TRI reagent) and was incubated at room temperature for 30 min. The samples were vortexed vigorously every 15 min and were centrifuged at 2,000 g for 5 min at 4°C or room temperature. The DNA pellet was resuspended in 1 ml of 75% ethanol (1.5-2.0 ml/ 1 ml TRL reagent) and was incubated at room temperature for 20 min. Then, the mixture was centrifuged at 2,000 g for 5 min at 4°C or 25°C. The supernatant was removed and the pellet was air-dried at room temperature for 10 min. The DNA pellet was dissolved in sterile distilled water and heated at 65°C for 10 min.

To determine the concentration and integrity of the extracted DNA, 1.5 µl of sample was used to measure at wavelength 260 nm (A_{260}) and 280 nm (A_{280}) by using Nano Drop; 1000 UV-Vis. The RNA concentration was shown in ng/µl. The high RNA integrity was presented 1.8-2.0 of the ratio between A_{260}/A_{280}.

3.2.7.2 PCR analysis

The extracted genomic DNA about 200 ng was used to perform PCR by Taq DNA polymerase. To determine the expression level of IHHNV, the primer pairs of IHHNV was used for multiplex PCR. Actin primers were used as an internal control. The PCR products sizes of IHHNV and actin are 309 bp and 550 bp, respectively. The components for PCR reaction in a total volume of 25 µl contained 1x PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 2 mM $MgCl_2$, 0.4 mM dNTP, 0.2 µM each of IHHNV-F and IHHNV-R primers (Table 3-1), 0.2 µM each of PmActin-F and PmActin-R1, 200 ng of the extracted DNA and 0.4 µl of *Taq* DNA polymerase (Homemade). The PCR condition was as followed : 94°C for 1.30 min followed by 25 cycles of PCR under the condition

: a denaturation at 94°C for 30 sec, anannealing at 55°C for 30 sec and extension at 72°C for 45 sec. The final extension step was performed at 72°C for 7 min. The PCR products (5 µl) of IHHNV (309 bp) and actin (550 bp) were analysed on 1.2% agarose gel electrophoresis.

3.2.8 Total RNA extraction and RT-PCR analysis

3.2.8.1 Total RNA extraction

To determine the expression levels of LvRab7 mRNA, the pleopods from individual shrimp were collected to isolate total RNA by using TRI reagent according to the manufacturer's instructions. Pleopods (2-4 pieces) were homogenized in 0.5 ml of TRI® reagent (1 ml/ 50-100 mg tissue) and incubated at room temperature for 5 min. The homogenate was added with 0.1 ml of chloroform (0.2 ml/ 1 ml TRI reagent) and shaked vigorously for 15 sec. The mixture was incubated at room temperature for 15 min and centrifuged at 12,000 g for 15 min at 4°C. To precipitate RNA, approximately 60% of the aqueous phase (upper phase) was transfered to a new 1.5 ml eppendorf tube. To precipitate the RNA, 0.25 ml of isopropanol (0.5 ml/ 1 ml TRI reagent) was added. The mixture was incubated at room temperature for 10 min and centrifuged at 12,000 g for 15 min at 4°C. Then, the supernatant was discarded. The pellet was washed with 0.5 ml of 75% ethanol (1 ml/ 1 ml TRI reagent) and centrifuged at 7,500 g for 5 min at 4°C. The pellet was air dried at room temperature for 10 min and dissolved with sterile distilled water (10-15µl / 2 pleopods). Total RNA was heated at 55-65 °C for completely dissolved of the RNA pellet.

To determine the concentration and integrity of total RNA, total RNA (1.5 µl) was used to measure at wavelength 260 nm (A_{260}) and 280 nm (A_{280}) by using Nano Drop; 1000 UV-Vis. The high RNA integrity was presented 1.8-2.0 of the ratio between A_{260}/A_{280}.

3.2.8.2 RT-PCR analysis

Total RNA (2.5 μg) was used for first-stranded cDNA synthesis using Improm II^{TM} reverse transcriptase (Promega) using 250 ng of oligo dT (PRT) primer (Table 3-1). To anneal the RNA and primer, sterile distilled water was added to 5.5 μl and incubated at 70°C for 5 min before chilling on ice for 5 min. The reaction was added with 4.5 μl of the reverse transcription mixture containing 1X Improm II^{TM} reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTPs, 0.3 μl of sterile distilled water and 100 unit (0.5 μl) of Improm II^{TM} reverse transcriptase (200 U/μl) following to mix and short spin. The reaction was incubated at 25°C for 5 min. To extend the first-stranded cDNA, the reaction was incubated at 42°C for 1 hour 30 min and heated at 70°C for 15 min to stop the Improm II^{TM} reverse transcriptase activity. The first-stranded cDNA was stored at -20°C until used.

3.2.8.3 Polymerase chain reaction (PCR)

The synthesized first-stranded cDNA was used as a template to detect the LvRab7 and actin expression levels by multiplex RT-PCR. The PCR product size of LvRab7 and actin are 617 bp and 350 bp, respectively.

The components for PCR in a total volume of 25 μl contained 1x PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 2 mM $MgCl_2$, 0.4 mM dNTP, 0.4 μM each of GTP-met and GTP-stop primers (Table 3-1), 0.06 μM each of PmActin-F and PmActin-R2 primers (Table 3-1), 1 μl of cDNA and 0.4 μl of *Taq* polymerase. The PCR condition was performed as followed: 94°C for 1.30 min, 5 cycles of touch down PCR consisting of the denaturation at 94°C for 3o sec, reduction the annealing temperature 1 °C per cycle from 58°C to 54°C for 30 sec and extension at 72°C for 45 sec. Next, the reaction was followed by 25 cycles of PCR containing a denaturation at 94°C for 30 sec, an annealing at 53°C for 30 sec and extension at 72°C for 45 sec. The final extension step was performed at 72°C for 7 min. The PCR products (5 μl) of LvRab7 (617 bp) and actin (350 bp) were analysed on 1.2% agarose gel electrophoresis.

3.2.9 Semi- quantitation of PCR and RT-PCR

The PCR products of IHHNV (309 bp), actin (550 bp) and RT-PCR product of LvRab7 (617 bp), actin (350 bp) were run on 1.2% agarose gel electrophoresis. The gel was stained in ethidium bromide for 5 min and destained in

water for 10 min before visualization by UV light. All PCR and RT-PCR products were quantified by using Scion image analysis program. The band intensities were corrected for background. The relative expression levels of LvRab7 and IHHNV were normalized with actin.

3.2.10 Statistical analysis

All semi-quantitative data was expressed as mean ± standard error of mean (SEM). The statistical analysis of mean ± SEM was performed by using ANOVA test. The probability (p) value of < 0.05 was accepted as statistically significant.