# CHAPTER II
# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Equipment

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Thermo Forma)

24 well Crystallization plate (VDX Plate) (Hampton Research)

96 and 24 well cell culture cluster, flat bottom with lid (Costar)

Acid washed glass beads, 500 µm (Sigma)

Amersham™ Hyperfilm ECL (18 × 24 cm) (GE Healthcare)

Amersham™ Hypercassette™ Autoradiography Cassette (GE Healthcare)

Amicon Ultra-4 concentrators (Millipore).

Avanti J-30I high performance centrifuge (Beckman Coulter)

Autoclave Model # LS-2D (Rex all industries Co. Ltd.)

Automatic micropipette (Gilson Medical Electrical S.A.)

Automatic micropipette P10, P20, P100, P200, and P1000 (Gilson Medical Electrical S.A.)

Centrifuge 5804R (Eppendorf), Centrifuge Avanti™ J-301 (Beckman Coulter)

CFX96™ Real-Time PCR Detection System (Bio-Rad)

Confocal Laser Scanning Microscopy (OLYMPUS FV1000)

CX31 Biological Microscope (Olympus)

French press cell disrupter (Thermo Scientific)

Gel documentation system (GeneCam FLEX1, Syngene)

Gene Pulser (Bio-RAD)

Hematocytometer (Cole-Parmer)

Hybridization oven (Hybrid)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Innova® 4080 incubator shaker (New Brunswick Scientific)

Innova® 43 incubator shakers (Eppendorf)

LABO Autoclave (SANYO)

Laminar flow: Dwyer Mark II Model # 25 (Dwyer instruments)

Laminar Airflow Biological Safety Cabinets Class II (NuAire, Inc., USA)

Leica® microscope

Microcentrifuge tube 0.5 and 1.5 ml (Axygen)

Microscope coverglasses (D.A.T. Scientific)

MRC 2 Well Crystallization Plate (Swissci)

Nipro disposable syringe (Nissho)

Orbital Shaker (Gallenkamp)

Oven series 8000 (Contherm)

PCR Mastercycler (Eppendorf AG, Germany)

PCR thin wall microcentrifuge tube 0.2 ml (Axygen)

Peristaltic pump P-1 (GE Healthcare)

pH meter Model # SA720 (Orion)

Pipette tips 10, 100 and 1000 µl (Axygen)

Polylysine slides (Thermo Scientific)

Power supply: Power PAC 300 (Bio-RAD Laboratories)

Refrigerated centrifuge Model # J2-21 (Beckman)

SpectraMax® M-series Multi-Mode Microplate Readers (Molecular Devices)

Sterring hot plate (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

Ultrafree Centifugal Filter Concentrator with Biomax-30 Ultrafiltration Polyethersulfone Membrane (Millipore)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

### 2.1.2 Chemicals, Reagents and Biological substance

0.22 μm millipore membrane filter (Millipore)

100 mM dATP, dCTP, dGTP, and dTTP (Thermo Scientific)

2-Mercaptoethanol (Fluka)

Absolute ethanol (BDH)

Acrylamide (Merck)

Agarose (Sekem)

Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen)

Alexa Fluor 568 goat anti-mouse IgG antibody (Invitrogen)

Alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.)

Alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.)

Ammonium persulfate (USB)

Ampicillin (BioBasic)

Anti-actin antibody, clone C4 (Millipore)

Anti-His tag antibody (GE Healthcare)

Anti-NPC antibody (Abcam)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

*Bam*H I (NEB)

BCIP (5-bromo-4-chloro-indolyl phosphate) (Roche, Germany)

BenchMark™ Unstained Protein Standard (10747-012)

Boric acid (Merck)

Bovine serum albumin (Fluka)

Bromophenol blue (MERCK)

Calcium chloride (MERCK)

Chloramphenicol (Sigma)

Chloroform (MERCK)

Complete protease inhibitor cocktail tablets (Roche)

Coomassie brilliant blue G-250, R-250 (Fluka)

D-(+)-Glucose (Sigma)

D-(+)-Galactose (Sigma)

Developing reagent (Kodak)

Diethyl pyrocarbonate (DEPC) (Sigma)

Dimethyl sulfoxide (DMSO), $C_6H_6SO$ (Amresco)

di-Sodium hydrogen orthophosphate anhydrous (Carlo Erba)

Ethanol (MERCK)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA) (Fluka)

FBS (Fetal Bovine Serum, Gibco®/BRL)

Fixing reagent (Kodak)

Formaldehyde (BDH)

GeneRuler™ 1 Kb DNA Ladder (Thermo Scientific)

GeneRuler™ 100 bp DNA Ladder (Thermo Scientific)

Glacial acetic acid (J.T. Baker)

Glucose (Ajax chemicals)

Glycerol (Scharlau)

Glycine (Scharlau)

Horseradishperoxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.)

Hydrochloric acid (MERCK)

Imidazole (Fluka) (Sigma)

Isopropanol (MERCK)

Isopropyl-ß-D-thiogalactoside (IPTG) (USBiological)

Lithium acetate (Sigma)

Magnesium chloride (MERCK)

Methanol (MERCK)

Minimum Essential Medium (Invitrogen)

n-Decyl-ß-D-maltopyranoside (Affymetrix)

N, N, N', N'-Tetramethylethylenediamine (TEMED) (BDH)

N, N', methylenebisacrylamide (Fluka)

*Not* I (NEB)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Paraformaldehyde (Sigma)

pDDGFP2 vector

PEG 3350 (Sigma)

pEGFP-1 (Clontech)

pET22b(+) vector (Novagen)

pGEX4T-3 vector (GE Healthcare)

Phenol, saturated (MERCK)

Porcine Trypsin (Sigma)

Prestained protein molecular weight marker (Thermo Scientific)

Prolong Gold Antifade Reagent (Invitrogen)

RNase-free DNase I (Promega)

Single-stranded carrier DNA, salmon sperm (Sigma)

Skim milk powder (Mission)

*Sma* I (NEB)

Sodium acetate (Carlo Erba)

Sodium azide (Sigma)

Sodium chloride (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dihydrogen orthophosphate (Carlo Erba)

Sodium dodecyl sulfate (Sigma)

di-Sodium hydrogen orthophosphate anhydrous (Carlo Erba)

Sodium hydroxide (Eka Nobel)

TO-PRO-3 iodide (Invitrogen)

Tris-(hydroxy methyl)-aminomethane (USB)

TRI Reagent® (Molecular Research Center)

Triton™ X-100 (MERCK)

Tween™ -20 (Fluka)

Urea (Fluka)

Vivaspin® Centrifugal Concentrator (GE Healthcare)

Whatman 3 MM™ filter paper (Whatman)

*Xba* I (NEB)

Yeast nitrogen base without amino acids (BD)

Yeast synthetic drop-out medium supplement without Ura (Sigma)

Yeast peptone dextrose media (Sigma)

### 2.1.3 Enzymes and Kits

Advantage® 2 PCR kit (Clontech)

Bicinchoninic acid (BCA) protein assay kit (Pierce)

Crystal Screen I & II (Hampton Research)

First Strand cDNA Synthesis Kit (Thermo Scientific)

Index™ (Hampton Research)

Membfac™ (Hampton Research)

NucleoSpin® Extract II kit (Macherey-Nagel)

PEG/Ion 1 & 2 Screen (Hampton Research)

PEG Rx™ 1 & 2 (Hampton Research)

Pierce® Fast Western Blot Kit, SuperSignal® West Femto Substrate, Mouse

Pierce® Fast Western Blot Kit, SuperSignal® West Femto Substrate, Rabbit

Pre-crystallization test by PCT™ (Hampton Research)

Presto™ Mini Plasmid Kit (Geneaid)

RBC *Taq* DNA polymerase (RBC Bioscience)

RQ1 RNase-free DNase (Promega)

Salt Rx™ (Hamton Research)

SsoFast™ EvaGreen® Supermix (Bio-Rad)

Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific)

T4 DNA ligase (New England Biolabs)

T7 RiboMAX™ Express Large Scale RNA Production System (Promega)

### 2.1.4 Microorganisms

*Escherichia coli* strain BL21-CodonPlus® (DE3), C41 (DE3), C43 (DE3)

Escherichia coli strain TOP10

*Saccharomyces cerevisiae* strain FGY217 (MATa, ura3-52, lys2D201,pep4D) (Kota *et al.* 2007)

White spot syndrome virus (WSSV)

### 2.1.5 Softwares

BlastX (http://www.ncbi.nlm.nih.gov/BLAST/)

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2)

Compute pI/Mw (http://web.expasy.org/compute_pi)

DBS-Pred (http://www.netasa.org/dbs-pred/)

DiANNA 1.1 web server (http://clavius.bc.edu/~clotelab/DiANNA/)

EMBOSS Pairwise Alignment (http://www.ebi.ac.uk/Tools/emboss/align/)

GENETYX (Software Development Inc.)

ImageJ (ver 1.47)

Olympus Fluoview FV10-ASW Software (ver *1.7b*)

SECentral (Scientific & Educational Software)

SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP)

SMART (http://smart.embl-heidelberg.de/)

SPSS statistics 17.0 (Chicago, USA)

TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/)

### 2.2 Animal cultivation

Juveniles black tiger shrimp, *P. monodon*, of about 10-15 g bodyweight were obtained from shrimp farm in Nakhon Si Thammarat Province, Thailand. The animals were acclimated in laboratory tanks at ambient temperature (28 ± 4 °C), and maintained in aerated water with a salinity of 20 ppt for at least 7 days before use.

### 2.3 Purification of WSSV

The WSSV stock was diluted in TN buffer (20 mM Tris-HCl pH 7.4 and 400 mM NaCl). White shrimp, *Litopenaeus vannamei*, were intramuscularly injected with 100 µl WSSV solution and reared to the moribund stage. The gill tissue was collected for the purification of viral particles. The purification method was slightly modified from (Xie *et al.* 2005). Gill tissue was homogenized in TNE buffer (50 mM Tris-HCl pH 8.5, 400 mM NaCl and 5 mM EDTA) and, then, centrifuged at 3,500× g for 15 min at 4 °C. The supernatant was filtrated with MILLEX®-HP Filter Unit 0.45 µm (Merck Millipore) and centrifuged at 30,000 xg for 30 min at 4 °C to pellet the virions. The pellet was rinsed with TM buffer (50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$) and centrifuged at 3,500 xg for 10 min at 4 °C. Subsequently, the pellet was resuspended in TM buffer and, then, supernatant was subjected to centrifugation at 30,000× g for 30 min at 4 °C. The pellet was then suspended in TM buffer and divided into aliquots and stored at -80 °C until use.

### 2.4 Shrimp hemocyte primary cell cultures

Hemolymph was drawn from each shrimp (~10-15 g body weight) using a sterile 1 ml syringe with 500 µl of anticoagulant, pH 5.6 (0.82% (w/v) sodium chloride, 0.55% (w/v) citric acid, 1.98% (w/v) glucose and 0.88% (w/v) sodium citrate; adjusted to pH 5.6 with sodium hydroxide solution). The hemolymph-anticoagulant mixture was immediately centrifuged at 800 x g for 10 min at 4 °C to separate the hemocytes from the plasma. The hemocyte pellet was resuspended in 1 ml of L-15 culture medium (1.6x Leibovitz L-15 medium (Gibco) supplemented with 20% (v/v) fetal bovine serum (FBS), 1% (w/v) glucose, 0.4% (w/v) sodium chloride, 100 IU/ml penicillin and 100 µg/ml streptomycin; pH 7.6; adjusting the osmotic pressure to 750 ± 15 mOsm/kg with sodium chloride solution).

The number of hemocyte in the cell suspension was counted by a hematocytometer. Cell concentration was adjusted to $10^6$ cells per ml per well by L-15 fresh medium to obtain a final volume of 400 µl per well. The 24-well plate was then incubated at 28 °C for 24 h, prior to be used in the experiments.

### 2.5 Total RNA extraction by TRI reagent, and DNase treatment

Hemocyte cells were homogenized in 1 ml of TRI Reagent® (Molecular Research Center). Subsequently, 200 µl per 1 ml of TRI Reagent® of chloroform was added and vortexed vigorously for 15 sec. After incubation at room temperature for 5 min, the mixtures were separated by centrifugation at 12,000 x g for 15 min at 4 °C, and then transfer of the RNA-containing aqueous (colorless upper aqueous) was then transferred into a fresh tube. Total RNA was precipitated by adding a volume of absolute isopropanol, followed by incubating at - 20 °C for 15 min. The supernatant was removed by centrifugation at 12,000 x g for 15 min at 4 °C. The RNA pellet was washed with 1 ml of 75% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water. The ethanol supernatant was completely removed by centrifugation at 12,000 x g for 15 min at 4 °C. The RNA pellet was air-dried at room temperature for 5-10 min, and dissolved in an appropriate amount of DEPC-treated water. The obtained total RNA was further treated with RQ1 RNase-free DNase I (Promega). One µg of total RNA was treated with 1 µl of RQ1 RNase-free DNase I at 37 °C for 30 min to remove the contaminating chromosomal DNA. Then, the RNA pellet was purified using TRI Reagent as described above.

### 2.6 Single-stranded cDNA synthesis using a First Strand cDNA Synthesis Kit (Thermo Scientific)

The DNase-treated RNA (up to 1 µg) was combined with 0.5 µg of Oligo $(dT)_{18}$ primer in nuclease-free water to obtain a final volume of 10 µl per a reverse transcription reaction. The sample was heated at 70 °C for 5 min, and immediately chilled in ice-water for at least 5 min. Total RNA concentration was determined using spectrophotometer (Beckman). One µg of DNase-treated total RNA was used to synthesize the first stand cDNA. The solution was incubated at temperature in a series of 25 °C for 5 min, 37 °C for 60 min and 70 °C for 5 min, respectively and 1 µl of 1: 5 dilution of the cDNA was used as template in a 25 µl PCR reaction volume.

## 2.7 Determination of *Pm*VRP15 mRNA expression in response to WSSV infection in shrimp hemocyte primary cell culture by RT-PCR and Real-time PCR

After pre-treatment of primary hemocyte cultures as described in Material and Method section 2.4, 400 µl of L-15 culture medium was removed from each well and replaced with 400 µl of fresh L-15 culture medium with or without 50 µl of the diluted WSSV solution (~15,000 viral copies/µl). Then, hemocyte cultures were re-incubated at 28 °C. Total RNA was extracted from the hemocytes at 0, 6, 12, 24, 48 and 72 h post-WSSV infection using the TRI Reagent® (Molecular Research Center) followed by DNase I, RNase-free (Thermo scientific) treatment, and used for single-stranded cDNA synthesis by the First Strand cDNA Synthesis Kit (Thermo Scientific).

The transcription level of target genes was identified by RT-PCR using an equal amount of cDNA template with gene-specific primers (Table 2.1). The Elongation factor-1 alpha (EF-1α) was used as an internal control. The PCR conditions consisted of 94 °C for 3 min, followed by 33 cycles (for *Pm*VRP15) or 28 cycles (for EF-1α) cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis and visualized using Gel Documentation System (Syngene). The differential expression level of VRP15 was reported as relative to EF-1α.

Table 2.1 Nucleotide sequences of primers for RT-PCR amplification

| Primer | Sequence (5 -3') | Usage |
|---|---|---|
| *ie1*-FRT | GACTCTACAAATCTCTTTGCCA | RT-PCR |
| *ie1*-RRT | CTACCTTTGCACCAATTGCTAG | RT-PCR |
| VP28-FRT | TCACTCTTTCGGTCGTGTCG | RT-PCR |
| VP28-RRT | CCACACACAAAGGTGCCAAC | RT-PCR |
| *Pm*VRP15-RTF | CGATCACCACTCTCGTTCTT | RT-PCR |
| *Pm*VRP15-RTR | ACAGCGACTCCAAGGTCTACGA | RT-PCR |

The expression level of *Pm*VRP15 and EF-1α gene was further analyzed by quantitative real time RT-PCR (qRT-PCR). VRP15 and EF-1α specific primers for qRT-PCR were shown in Table 2.2. The reaction volume of 10 µl contained 5 µl of 2X SsoFast™ EvaGreen® Supermix (Bio-Rad), 0.5 µM of each forward and reverse primers, and 1 µl of 1:3 diluted cDNA template. A negative control reaction contained no cDNA template. The qRT-PCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad). The PCR condition was performed as follows: 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec, 58 °C for 5 sec and 60 °C for 5 sec. The experiment was carried out in triplicate. The threshold cycle ($C_T$) for each sample was analyzed by a mathematical model described by (Pfaffl 2001). The data were shown as means ± standard deviations (SD). Statistical analysis was done using the one-way ANOVA followed by post hoc test (Duncan's new multiple range test). Data differences were considered significant at $P < 0.05$ (*), $P < 0.01$ (**).



Table 2.2 Nucleotide sequences of primers for qRT-PCR amplification

| Primer | Sequence (5'-3') | Usage |
|---|---|---|
| EF1-α QF | GGTGCTGGACAAGCTGAAGGC | qRT-PCR |
| EF1-α QR | CGTTCCGGTGATCATGTTCTTGATG | qRT-PCR |
| VP28QF | GGGAACATTCAAGGTGTGGA | qRT-PCR |
| VP28QR | GGTGAAGGAGGAGGTGTTGG | qRT-PCR |
| *Pm*VRP15-QF | CGTCCTTCAGTGCGCTTCCATA | qRT-PCR |
| *Pm*VRP15-QR | ACAGCGACTCCAAGGTCTACGA | qRT-PCR |

### 2.8 Production of *Pm*VRP15 and GFP dsRNA

*Pm*VRP15 DNA templates with a single T7 promoter at the 5' ends were PCR amplified from the *Pm*VRP15-recombinant plasmid by two separate PCR reactions using two primer sets (Table 2.3). PCR was performed as followed : 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. Subsequently, *Pm*VRP15 dsRNA was produced using T7 RiboMAX™ Express Large Scale RNA Production System (Promega) to produce two

complementary ssRNAs according to the manufacturer's instruction. Then, equal amounts of each of the complementary ssRNAs were mixed together and incubated at 70 °C for 10 min, and slowly cooled down at room temperature to allow a formation of dsRNA. The *Pm*VRP15 dsRNA solution was treated with 2 units of RQ1 RNase-free DNase (Promega) at 37 °C for 15 min to remove DNA template, and then purified by standard phenol-chloroform extraction.

Total RNA was isolated using Trizol reagent by mixing 0.2 ml of chloroform per 1 ml of Trizol reagent. Samples were vortexed vigorously and incubate at RT for 5 min and centrifuged at 13,500 rpm at 4 °C for 15 min. The upper aqueous phase was transferred into fresh tube. Samples were add 0.1 volume of 3 M Sodium Acetate (pH 5.2) and 2.5 volumes of 95% ethanol. Samples were mixed and placed at -20 °C for 30 min. Then, samples were then centrifuged at 13,500 rpm at 4 °C for 15 min and supernatant was discarded. The pellet was washed with 70% cold ethanol and centrifuged 13,500 rpm at 4 °C for 15 min. RNA Pellet was air-dried and dissolved in nuclease-free water. Store at -80 °C.



Green fluorescent protein (GFP) DNA templates with a single T7 promoter at the 5' ends were PCR amplified from the pEGFP-1 vector (Clontech) using the GFP-F and GFP-R primers (Table 2.3). PCR was performed as followed : 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. Accordingly, GFP dsRNA was produced described as above.

### 2.9 Gene silencing of *Pm*VRP15 in WSSV-infected hemocyte cell culture

RNA interference (RNAi) is a powerful strategies to silence the expression of specific genes using dsRNA. This technique helps identify gene function. In this work, gene silencing was used to study the important role of *Pm*VRP15 in the WSSV-infection in primary hemocyte cell culture. The dsRNA *Pm*VRP15 was used to knockdown *Pm*VRP15 transcript prior to WSSV infection. WSSV propagation in hemocyte cell culture ,was then investigated by measuring VP28 viral mRNA expression. The *Pm*VRP15 dsRNA and GFP dsRNA (control group) were synthesized using T7 RiboMAX™ Express Large Scale RNA Production System (Promega) following the manufacturer protocol (see section 2.8).

Table 2.3 Nucleotide sequences of primers for dsRNA production

| Primer | Sequence (5'-3') | Usage |
|---|---|---|
| GFP-F | ATGGTGAGCAAGGGCGAGGA | dsRNA synthesis |
| GFP-R | AGAAGGAAGGGCGCTGAC | dsRNA synthesis |
| *Pm*VRP15-1F RNAi | GGATCCTAATACGACTCACTATAGGCGCGA CCGAGCCAAGAGAACAT | dsRNA synthesis |
| *Pm*VRP15-1R RNAi | TGAGCTGACGGAAGGCCACAGA | dsRNA synthesis |
| *Pm*VRP15-2F RNAi | CGCGACCGAGCCAAGAGAACAT | dsRNA synthesis |
| *Pm*VRP15-2R RNAi | GGATCCTAATACGACTCAC TATAGGTGAGCTGACGGAAGGCCACAGA | dsRNA synthesis |



**2.10 Investigation of VP28 transcript level after *Pm*VRP15 silencing in WSSV-infected hemocytes by RT-PCR and quantitative Real-time PCR**

To prepare the hemocyte primary cell culture, the shrimp hemocytes were collected, separated by centrifugation and resuspended in L-15 medium supplemented with 20% fetal bovine serum (GIBCO). The total hemocyte number was counted with a hemocytometer under a light microscope. The hemocyte cell suspension was aliquot into each well of the 24-well plate ($10^6$ cells/ml per well) and incubated at 27 °C overnight. Fresh L-15 medium supplemented with 20% fetal bovine serum was replenished. The dsRNA was pre-incubated with histone H2A (calf thymus, type II-A; Sigma) for 10 min at RT (Liu *et al.* 2006) before used.

The hemocyte cell cultures, divided into 3 groups, were incubated with 50 µl of L-15 medium with or without, 20 µg/well of GFP dsRNA or *Pm*VRP15 dsRNA. After 12 h, 50 µl of L-15 medium with or without, GFP dsRNA (10 µg/well) and *Pm*VRP15 dsRNA (10 µg/well) were added along with 50 µl of the diluted WSSV solution (~15,000 viral copies/µl) and incubated at 27 °C. At 24 h post-WSSV infection, hemocyte cells were harvested and total RNA was extracted from hemocytes using the TRI Reagent® (Molecular Research Center) followed by DNase I, RNase-free (Thermo scientific) treatment, and then single-stranded cDNA was synthesized with

the First Strand cDNA Synthesis Kit (Thermo Scientific) and cDNA was used to analyze VP28 mRNA expression by RT-PCR with EF-1α as an internal reference.

The expression of VP28, VRP15 and EF-1α genes were analyzed by RT-PCR using gene specific primers (Table 2.1). RT-PCR reaction of 25 µl total volume contained 1x PCR buffer, 200 µM dNTP, 0.4 µM of each specific primer, 1 unit of *Taq* DNA polymerase (RBC) and 1 µl of 1:10 dilution of the cDNA preparation. The reaction were carried out using the following conditions: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and the final extension at 72 °C for 10 min. The PCR reactions were analyzed by agarose gel electrophoresis and visualized by gel documentation system (SynGene).

The expression level of VP28 and EF-1α gene was also analyzed by quantitative real time RT-PCR (qRT-PCR). VP28 and EF-1α specific primers were used for qRT-PCR are shown in Table 2.2. The reaction volume was 10 µl, containing 5 µl of 2X SsoFast™ EvaGreen® Supermix (Bio-Rad), 0.5 µM each forward and reverse primers, and 1 µl of 1:3 diluted cDNA template. The qRT-PCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad). The PCR profile was as follows: 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec, 58 °C for 5 sec and 60 °C for 5 sec. Each sample was done in triplicate. The threshold cycle ($C_t$) for each sample was analyzed by a mathematical model described by (Pfaffl 2001). The data were shown as means ± standard deviations (SD). Statistical analysis was done using the one-way ANOVA followed by post hoc test (Duncan's new multiple range test). Data differences were considered significant at $P < 0.05$.

Comparative $C_T$ method was used to compare the gene expression in two different samples. The fold change of gene expression was calculated using following formula.

$$\text{Fold change} = 2^{-\Delta\Delta C_T}$$

$\Delta\Delta C_T$ = [($C_T$ gene of interest - $C_T$ internal control) sample A - ($C_T$ gene of interest - $C_T$ internal control) sample B)]

### 2.11 Purification of primary antibody

To purify the IgG antibody form anti-serum standard protocol, protein A Sepharose CL-4B (GE Healthcare) was used according to the manufacturer. The protein A Sepharose CL-4B bead was applied into the column and washed with 10 volumes of PBS. Anti-serum was loaded into the column and incubated at RT for 10 min. After washing with PBS to remove non-specific proteins, IgG antibody was eluted with elution buffer (100 mM glycine pH 2.5) and immediately neutralized with 1 M Tris pH 9.5. Then, eluted primary antibody was added with 0.02% Sodium azide as preservative to prevent microbial contamination.

### 2.12 Visualization of normal and *Pm*VRP15-knockdown hemocyte-infected by WSSV using confocal immunofluorescence microscopy



Twenty-five µl of 150 mM sodium chloride containing GFP dsRNA (5 µg/g shrimp) or *Pm*VRP15 dsRNA (10 µg/g shrimp) was injected into each shrimp (~10 g body weight). After 24 h, the shrimp were injected with 25 µl of 150 mM sodium chloride containing GFP dsRNA (5 µg/g shrimp) or *Pm*VRP15 dsRNA (5 µg/g shrimp), along with 30 µl of the diluted WSSV solution (~ 4 $x10^3$ viral copies). At 48 h post-injection, hemolymph was immediately fixed with of 4% (w/v) paraformaldehyde in PBS, pH 7.4 (ratio 1:1) and incubate at RT for 10 min. Hemocytes were collected by contrifugating 800 x g for 10 min and washed three times with PBS, pH 7.4, and then immersed in PBS, pH 7.4, and kept at 4 °C until the next treatment. A hemocytometer was used to count the number of hemocytes and to make $1x10^6$ cells/ml of hemocytes.

Hemocytes were attached onto a polylysine coated microscope slide (Polysine slides, Thermo Scientific) using cytospin centrifugation. The slide was washed by PBS for 5 min and later covered by 200 µl of 0.1% (v/v) Triton-X 100 in PBS for 5 min at RT to permeabilize the hemocyte membrane. After rinsing the slide in PBS for 5 min, it was immersed in 200 µl of blocking solution (10% (v/v) FBS in PBS) for 1 h at RT followed by washing in PBS. The fixed hemocytes were incubated with a 1:500 dilution of purified rabbit anti-*Pm*VRP15 polyclonal antibody and 1:100 dilution of mouse anti-VP28 polyclonal antibody in PBS, pH 7.4, containing 1% (v/v) FBS at RT for 3 h. Then, washed three times with PBS, pH 7.4, and then incubated with a 1:500 dilution of Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen) and Alexa Fluor® 568 goat anti-mouse IgG antibody (Invitrogen) in PBS, pH 7.4 at RT for

1 h in the dark, and washed three times with PBS, pH 7.4. Subsequently, the nucleus was stained with a 1:1500 dilution of TO-PRO-3 iodide (Invitrogen) in PBS, pH 7.4, at RT for 10 min in the dark, and washed three times with PBS, pH 7.4. The cover slips contained the stained and fixed hemocyte samples were then coated with Prolong Gold Antifade Reagent (Invitrogen). Next, the slide was kept in dark at 4 °C until it was visualized under a confocal laser scanning microscope (Olympus).

### 2.13 Confirmation of *Pm*VRP15 and β-actin in shrimp hemocyte of normal and *Pm*VRP15-silenced by Western blotting analysis

#### 2.13.1 Hemocyte lysate preparation

Twenty-five µl of 150 mM sodium chloride containing 5 µg/g shrimp of GFP dsRNA (control) or 5 µg/g shrimp of *Pm*VRP15 dsRNA was injected into each shrimp (~5 g body weight). After 24 h, hemolymph was drawn from normal and *Pm*VRP15-silenced shrimp with 10% (v/v) sodium citrate as an anticoagulant. Hemocyte was separated by centrifugation at 800 xg at 4 °C for 10 min. Hemocyte was washed twice with 10 mM Sodium carcodylate (CAC) buffer. After centrifuge at 800 xg 4 °C for 10 min, supernatant was discarded and hemocyte was homogenized in 10 mM CAC buffer with BD Insulin Syringe ultra-Fine Needle 29G. Then, hemocyte lysate was separated by centrifugation at 13,000 rpm at 4 °C for 10 min.



#### 2.13.2 Protein expression of *Pm*VRP15 and β-actin in shrimp hemocyte.

To detect the protein expression of *Pm*VRP15 and β-actin in shrimp hemocyte. The concentration of normal and *Pm*VRP15-silenced HLS were measured by BCA assay (Pierce). The total 20 µg of normal and *Pm*VRP15-silenced HLS were loaded on 15% SDS-PAGE and 12.5% SDS-PAGE to detect the protein expression of β-actin and *Pm*VRP15, respectively. The total protein of HLS was subjected to Western blot analysis. The *Pm*VRP15 and actin protein expression was observed using purified rabbit polyclonal anti-VRP15 (1:1,000) and mouse monoclonal anti-actin (Millipore) (1:5,000), respectively. The colors were developed using secondary antibodies conjugated with alkaline phosphatase (purple color).

### 2.14 Protein localization of *Pm*VRP15 by subcellular protein fractionation

To prepare the hemocyte primary cell culture, the shrimp hemocytes were collected, separated by centrifugation and resuspended in L-15 medium supplemented with 20% fetal bovine serum (GIBCO). The total hemocyte number was counted with a hemocytometer under a light microscope. The hemocyte cell suspension was aliquot into each well of the 24-well plate ($1x10^5$ cells/ml per well) and incubated at 27 °C overnight. Fresh L-15 medium supplemented with 20% fetal bovine serum was replenished.

The hemocyte cell cultures were incubated with 50 µl of the diluted WSSV solution (~15,000 viral copies/µl) and incubated at 27 °C. For 48 h post-WSSV infection, total protein was extracted from hemocytes using Subcellular fractionation kit for cultured cells (Thermo Scientific). Cytoplasmic, membrane, soluble nuclear, chromatin-bound and cytoskeletal fractions were extracted. The total protein concentration of each fraction was determined using BCA protein assay and 10 µg of proteins were loaded in each lane. The result was analyzed by SDS-PAGE and Western blotting. Each fraction was probed with anti-*Pm*VRP15 as primary antibody (1:1000) at 37 °C for 3 h. After washing with PBST, membrane was probed with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000). The *Pm*VRP15 protein expression was observed compare to positive control (r*Pm*VRP15).



### 2.15 Quantification of WSSV copy number in normal and *Pm*VRP15 silencing hemocytes after WSSV infection

*Pm*VRP15 was located near or at nuclear membrane and it may involve in viral entry or viral shedding to or from the nucleus. Thus, there are two main hypotheses when it comes to explaining. First, if *Pm*VRP15 involves in viral entry (Figure 2.1A), after silencing *Pm*VRP15 gene, a lot of WSSV will be found in cytoplasmic fraction as WSSV cannot enter into nucleus. Thus, the ratio of DNA virus (WSSV) in nucleus to cytoplasmic fraction of *Pm*VRP15 silencing will be lower than that of control (GFP silencing).

However, if *Pm*VRP15 involves in viral exit (Figure 2.1B), after silencing *Pm*VRP15 gene, a lot of WSSV will be found in nuclear fraction since WSSV can enter into nucleus to replicate but cannot leave the nucleus. Thus, the ratio of DNA virus (WSSV) in nucleus to cytoplasmic fraction of *Pm*VRP15 silencing will be higher than that of control (GFP silencing).

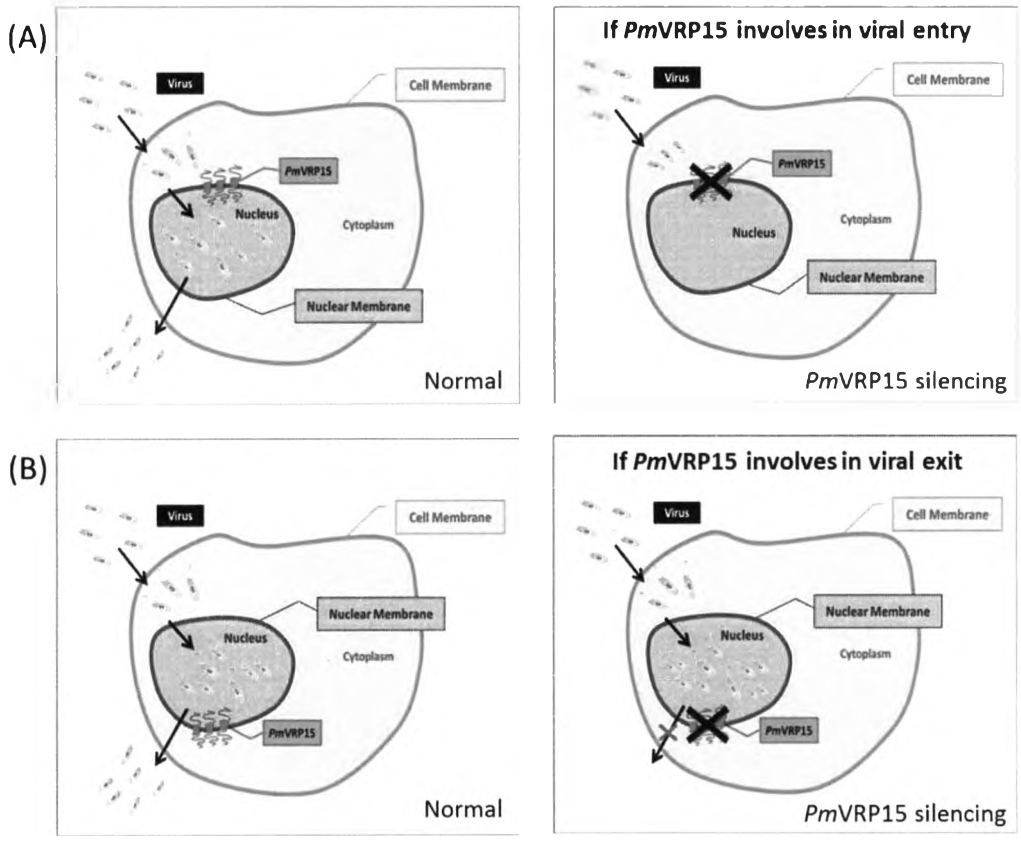


Figure 2.1 Experiment hypotheses : (A) *Pm*VRP15 involves in viral entry. (B) *Pm*VRP15 involves in viral exit.

To prepare the hemocyte primary cell culture, the shrimp hemocytes were collected, separated by centrifugation and resuspended in L-15 medium supplemented with 20% fetal bovine serum (GIBCO). The total hemocyte number was counted with a hemocytometer under a light microscope. The hemocyte cell suspension was aliquot into each well of the 24-well plate ($1x10^6$ cells/ml per well) and incubated at 27 °C overnight. Fresh L-15 medium supplemented with 20% fetal bovine serum was replenished. The dsRNA was pre-incubated with histone H2A (calf thymus, type II-A; Sigma) for 10 min at RT (Liu et al., 2006) before used.

The hemocyte cell cultures, divided into 2 groups, were incubated with 50 µl of GFP dsRNA (20 µg/well) or VRP15 dsRNA (20 µg/well). After 12 h, 50 µl of GFP dsRNA (10 µg/well) and VRP15 dsRNA (10 µg/well) were added, along with 50 µl of the diluted WSSV solution (~15,000 viral copies/µl) and incubated at 27 °C. For 24 h post-WSSV infection, total DNA was extracted from hemocytes using Subcellular

fractionation kit for cultured cells (Thermo Scientific). The cytoplasmic and nuclear fractions were separated and sent to Charoen Pokphand Foods PCL for the determination of the WSSV copy number in each fraction by Real-time PCR with the WSSV1011F/WSSV1079R primers, as described by (Durand 2002), using an ABI7000 Sequence Detection System. The data was carried out in triplicate and WSSV recombinant plasmid (known copy number) was used as the standard for quantification.

**2.15.1 Confirmation of cross contamination in cytoplasmic and nuclear fractions of *Pm*VRP15 silencing and control (GFP silencing) using Western blotting analysis**

To confirm cytoplasmic and nuclear fractions are no cross-contaminated, anti-Nuclear Pore Complex (NPC) antibody was used for Western blotting. NPC was found in nuclear fraction only. Cytoplasmic and nuclear fractions were extracted from cultured cells and total protein concentration of each fraction was determined using BCA protein assay. Ten µg of proteins were loaded in each lane. The result was analyzed by Western blotting (Pierce® Fast Western Blot Kit, SuperSignal® West Femto Substrate). The Pierce Fast Western Blot Kit is optimized for blots that are not pre-blocked. Pre-blocking the membrane can cause a decrease in assay sensitivity. After wash membrane with 1X wash buffer, each fraction was probed with anti-NPC as primary antibody (1:1000) at 37 °C for 2 h. After washing with washing buffer, membrane was probed with Fast Western Mouse optimized HRP-labeled secondary antibody, Femto. Membrane was soaked and shaked for 1 hour at RT. Wash 3 times with wash buffer for 10 min/time, working solution (Luminol/Enhancer solution 1 : 1 Peroxide reagent) was added to membrane for 15 min at RT. The excess buffer was removed and membrane was placed on a plastic wrap. Then, signal was detected using X-ray film. Membrane was set on a film cassette and X-ray film was placed above blot surface and close the cassette for 10 min at RT. X-ray film was developed to observed the signals. First, X-ray film was exposed to the developer solution. Developing reagent was act on the film by reducing the exposed silver halide crystals to black metallic silver. Next, development process was stopped by washing with water. Then, unexposed silver halide crystals was removed using fixing reagent. Film was washed with water to remove all the processing chemicals and dried for viewing.

## 2.16 Expression of r*Pm*VRP15 gene in *Saccharomyces cerevisiae*

### 2.16.1 Cloning of *Pm*VRP15 gene into pDDGFP2 vector by homologous recombination

*Pm*VRP15 gene was integrated into pDDGFP-2 vector by homologous recombination in one step via a mixture of linearized plasmid and PCR products.

The pDDGFP-2 plasmid, which has a GAL1 promoter, URA selection marker, and *S. cerevisiae* strain FGY217 were used (Newstead *et al.* 2007) (Figure 2.2). In addition, introduce a *Sma* I site preceding the TEVp-GFP-8His sequence (Figure 2.3a). *Pm*VRP15 gene was amplified with *Pm*VRP15-F and *Pm*VRP15-R primers (Table 2.4) that include 5' overhangs complementing the upstream and downstream sequences to either side of the *Sma* I site in pDDGFP-2 vector (Figure 2.2b). PCR product of 5 µl and *Sma* I-linearized vector solution of 3 µl were ligated by homologous recombination and transformed into *S. cerevisiae* competent cells as described below.



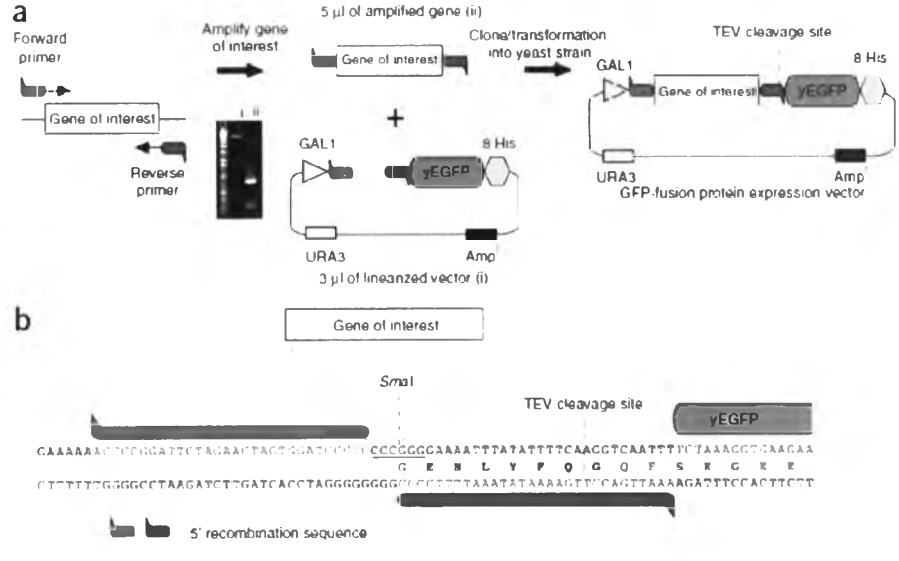


Figure 2.2 Cloning by homologous recombination into *Saccharomyces cerevisiae* GFP-fusion vector. (a) The amount of PCR and vector used in cloning is depicted in the UV-exposed agarose-gel inset: i, *Sma* I linearilized vector; ii, amplified gene with overhangs required for homologous recombination. (b) Cloning site used in GFP-fusion vector pDDGFP-2. TEV, tobacco etch virus; yEGFP, yeast-enhanced green fluorescent protein (Drew *et al.* 2008).

Table 2.4 Nucleotide sequences of primers for PCR amplification

| Primer | Sequence (5'-3') | Usage |
|---|---|---|
| *Pm*VRP15-F | TCGACGGATTCTAGAACTAGTGGATCCCCC | Cloning of *Pm*VRP15 into pDDGFP-2 vector |
| *Pm*VRP15-R | AAATTGACCTTGAAAATATAAATTTTCCCC | Cloning of *Pm*VRP15 into pDDGFP-2 vector |

### 2.16.2 Amplification of *Pm*VRP15 gene

Reaction of 50 µl total volume contained 10X Advantage® PCR buffer, 0.2 mM dNTP, 0.5 µM of each specific primer, 1 unit of 50X Advantage® 2 polymerase Mix and 1 µl of *Pm*VRP15 template. The reaction were carried out using the following conditions: an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 68 °C for 1 min, and the final extension at 68°C for 10 min. The PCR reactions were analyzed by 1.5% agarose gel electrophoresis and visualized by gel documentation system (SynGene).



### 2.16.3 Digestion of pDDGFP-2 vector with *Sma* I

The pDDGFP-2 vector was digested by *Sma* I to ligate with *Pm*VRP15 gene which contained the overhangs complementing the upstream and downstream sequences to *Sma* I site in pDDGFP-2 vector by homologous recombination.

The reaction of 20 µl total volume contained 1 unit of *Sma* I, 10X buffer 4 and 10 µl of pDDGFP-2 vector (50 ng/µl). The reaction were incubated at 37 °C overnight, followed by heat inactivated at 65 °C for 20 min. The reactions were analyzed, in comparison to non-digested pDDGFP-2 vector by 1% agarose gel electrophoresis and visualized by gel documentation system (SynGene).

### 2.16.4 Transformation of gene-vector construct in *S. cerevisiae*

#### 2.16.4.1 Preparation of competent cells

First, 5 ml of yeast peptone dextrose (YPD) medium was inoculated with *S. cerevisiae* (FGY217) in a 50-ml capped tube and shaked at 280 rpm overnight at 30 °C. Overnight culture was diluted into 50 ml of YPD in a 250-ml shaker flask to an $OD_{600}$ of 0.1 and cells were grown in an orbital shaker at 280 rpm at 30 °C until an $OD_{600}$ of 0.5-0.6.

Then, cells were harvested by centrifugation at 3,000 xg for 5 min at 4 °C, supernatant was discarded and pellet was resuspended in 25 ml of sterile $dH_2O$. Cells were centrifuged at 3,000 xg for 5 min at 4 °C, supernatant was discarded and cell pellet was resuspended in 1 ml of 100 mM lithium acetate (LiAc). Cell suspension was transferred into a 1.5-ml tube and centrifuged at 8,000 xg for 15 sec. Supernatant was discussed and cell pellet was resuspended in 400 µl of 100 mM LiAc.



#### 2.16.4.2 Transformation

Prepare new 1.5-ml tubes containing 240 µl of 50% (w/v) PEG 3350 for each transformation and put tubes on ice. Competent cells of 50 µl were added into each tube and vortexed for 5 sec, followed by addition of 25 µl of 2 mg/ml single-stranded carrier DNA into each tube and vortexed for 5 sec. After that, 50 µl of DNA mix (3 µl of 25 ng/ml *Sma* I-digested vector, 5 µl of 150 ng/ml PCR product and 42 µl of sterile $dH_2O$) were added into each tube and vortexed for 5 sec. Cells were incubated for 30 min at 30 °C, and heated cells for 25 min at 42 °C, subsequently, cells were centrifuged at 8,000 xg for 15 sec at room temperature. Supernatant was discarded and cell pellet was resuspended with 1 ml of sterile $dH_2O$ and plated cell onto a -URA selective and ampicillin plate (final concentration 100 µg/ml) and incubated for 2-3 d at 30 °C.

### 2.16.5 Production of recombinant *Pm*VRP15 (r*Pm*VRP15) in *S. cerevisiae* expression system

A colony of the yeast transformant was inoculated in an aerated 50-ml tube containing 10-ml URA medium with 2% glucose and incubated overnight in an orbital

shaker at 280 rpm at 30 °C. The overnight culture was diluted to an $OD_{600}$ of 0.12 into 50-ml aerated tubes, containing 10 ml-URA medium with 0.1% glucose (Make sure that 0.1% glucose is used and not 2% glucose in the expression media because high levels of glucose repress the GAL1 promoter, while the former helps to maintain cell growth). The cultures was incubated at 280 rpm at 30 °C. At an $OD_{600}$ of 0.6, expression of r*Pm*VRP15 was induced by adding 20% (w/v) galactose (final concentration of 2%). At 0 h and 22 h after induction, cells was centrifuged at 3,000 xg for 5 min, supernatant was removed and cell pellets were resuspended in 200 µl of YSB buffer (50 mM Tris–HCl pH 7.6, 5 mM EDTA, 10% glycerol and 1X complete protease inhibitor cocktail tablets).

After that, glass beads were added to break yeast cells using a mixer-mill disruptor set at 30 Hz for 10 min. Supernatant (soluble protein) and pellet (inclusion bodies protein) were separated by centrifugation at 8,000 xg for 20 min. Protein concentration was measured by BCA Protein assay and 10 µg of proteins were loaded in each lane, in order to compare amount of protein in soluble and inclusion bodies fractions and the result was analyzed by SDS-PAGE. Protein expression of crude r*Pm*VRP15, fraction of soluble and inclusion bodies protein were analyzed by 15% SDS-PAGE.



## 2.17 Cloning and Expression of full-length and truncated r*Pm*VRP15 into pGEX4T-3 vector

To express *Pm*VRP15 protein in the soluble form, full-length and truncated *Pm*VRP15 were cloned into pGEX4T-3 vector and expressed in *E. coli* system. The pGEX4T-3 vector has glutathione S-transferase tag which helps induce, high level expression of genes fused with *Schistosoma japonicum* GST. Fusion proteins contain the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. This may also help express genes of interested in soluble form.

### 2.17.1 Amplification of full-length and truncated r*Pm*VRP15

The open reading frame of *Pm*VRP15 gene was amplified by PCR from cDNA library using a specific primer (Table 2.5). The PCR conditions were 94 °C for 3 min,

30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. PCR products were analyze by 1.5% agarose gel.

Table 2.5 Specific primers used for cloning full-length and truncated *Pm*VRP15 into pGEX4T-3 vector

| Primer | Sequence (5'-3') | Usage |
|---|---|---|
| Full-length of *Pm*VRP15-F | GCGGGCCGGATCCATGTTAACAGAGGACTTAGTAAA CCTGGTGTAC | Cloning into pGEX4T-3 |
| Full-length of *Pm*VRP15-R | CTCGAGGCGTTTATAGGAGGCGATTCT | Cloning into pGEX4T-3 |
| N-terminal part of *Pm*VRP15-F (residue 1-112) | GCGGGCCGGATCCATGTTAACAGAGGACTTAGTAAA CCTGGTGTAC | Cloning into pGEX4T-3 |
| N-terminal part of *Pm*VRP15-R | GCGGCCGCTTATGTTGAGACGAATGGTATGGAAG | Cloning into pGEX4T-3 |
| C-terminal part of *Pm*VRP15-F (residue 186-414) | GGATCCTATGCTAGGGGGAAGTTCAAAAGC | Cloning into pGEX4T-3 |
| C-terminal part of *Pm*VRP15-R | CTCGAGGCGTTTATAGGAGGCGATTCT | Cloning into pGEX4T-3 |

*Pm*VRP15 fragments were ligated into pGEX4T-3 using *Bam*H I and *Not* I cloning sites. The ligation reaction was carried out at 22 °C 1 h. The recombinant plasmid was transformed into *E. coli* TOP10 competent cells by heat shock method. Transformants were grown on Luria-Bertani (LB) agar containing 100 µg/ml ampicillin at 37 °C overnight. The recombinant plasmid containing *Pm*VRP15 was subjected to nucleotide sequencing (Macrogen INC., South Korea). Full-length and truncated *Pm*VRP15 were then expressed in various *E. coli* strains.

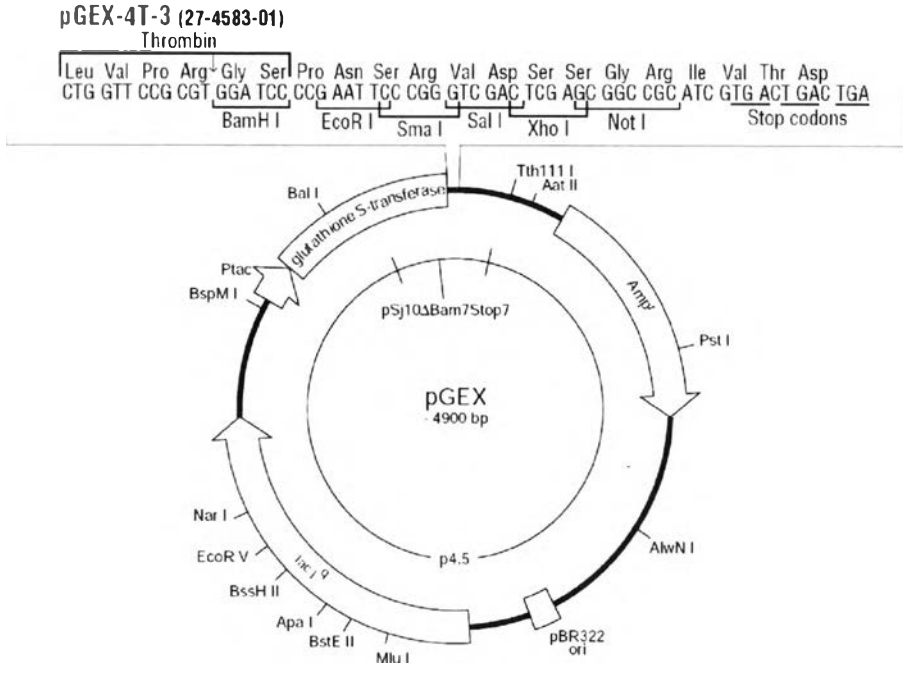


Figure 2.3 Map of the pGEX4T-3 expression vector (GE Healthcare)

### 2.17.2 Optimization of full-length and truncated r*Pm*VRP15 expression

The *Pm*VRP15-recombinant pGEX4T-3 was transformed into *E. coli* C41 (DE3), C43 (DE3) or BL21-CodonPlus® (DE3) competent cells by heat shock method and transformants were grown on LB agar containing 100 µg/ml ampicillin at 37 °C overnight. A single colony of transformed *E. coli* was grown in LB medium containing 100 µg/ml ampicillin at 37 °C overnight with agitation at 250 rpm. The overnight culture was diluted 1:100 in fresh LB medium supplemented with 100 µg/ml ampicillin and grown until an $OD_{600}$ of the cultures reached 0.6. Protein expression was induced by addition of IPTG to final concentration of 1 mM. The cells were harvested at 1, 2, 3, 4 and 16 hour post-induction by centrifugation at 8,000 x g for 10 min at 4 °C, then the cells were sonicated 10 rounds of at 35% amplitude, pulse on 2 sec, pulse off 3 sec for 40 sec/round. Soluble and inclusion bodies protein fractions were separated by centrifugation at 12,000 rpm for 20 min at 4 °C. Expression of r*Pm*VRP15 was analyzed by 15% SDS-PAGE and western blot analysis.

Soluble proteins and inclusion bodies *Pm*VRP15 protein were run on 15% SDS-PAGE and transferred onto nitrocellulose membrane (Whatman) by a semi-dry

blotter. The membrane was immersed in 1X phosphate buffered saline (PBS), pH 7.4 containing 5% (w/v) skim milk and 0.05% (v/v) Tween 20 at room temperature overnight, and then incubated with a 1:1,000 dilution of mouse anti-GST antibody in PBS, pH 7.4 containing 1% (w/v) skim milk and 0.05% (v/v) Tween 20 at 37 °C for 3 h. The membrane was washed three times with washing buffer (PBS, pH 7.4, containing 0.05% (v/v) Tween 20), and incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG antibody in blocking buffer at room temperature for 1 h. Then, the membrane was washed three times with washing buffer, and the color was developed with NBT/BCIP solution according to the manufacturer's protocol.

### 2.18 Production of r*Pm*VRP15 in *E. coli* expression system

*Pm*VRP15 open reading frame was successfully cloned into the pET22b(+) expression vector between *Nde* I and *Xho* I cloning sites as described in (Vatanavicharn *et al.* 2014). Recombinant plasmid *Pm*VRP15 was transformed into TOP10 competent *E. coli* which provided stable replication of high-copy number plasmids and enhanced genomic DNA cloning capabilities by heat shock method. Recombinant plasmid was then transformed into *E. coli* C43 (DE3) which are effective in overexpressing toxic and membrane proteins from all classes of organisms, including viruses, eubacteria, archaea, yeasts, plants, insects, and mammals (Dumon-Seignovert *et al.* 2004).

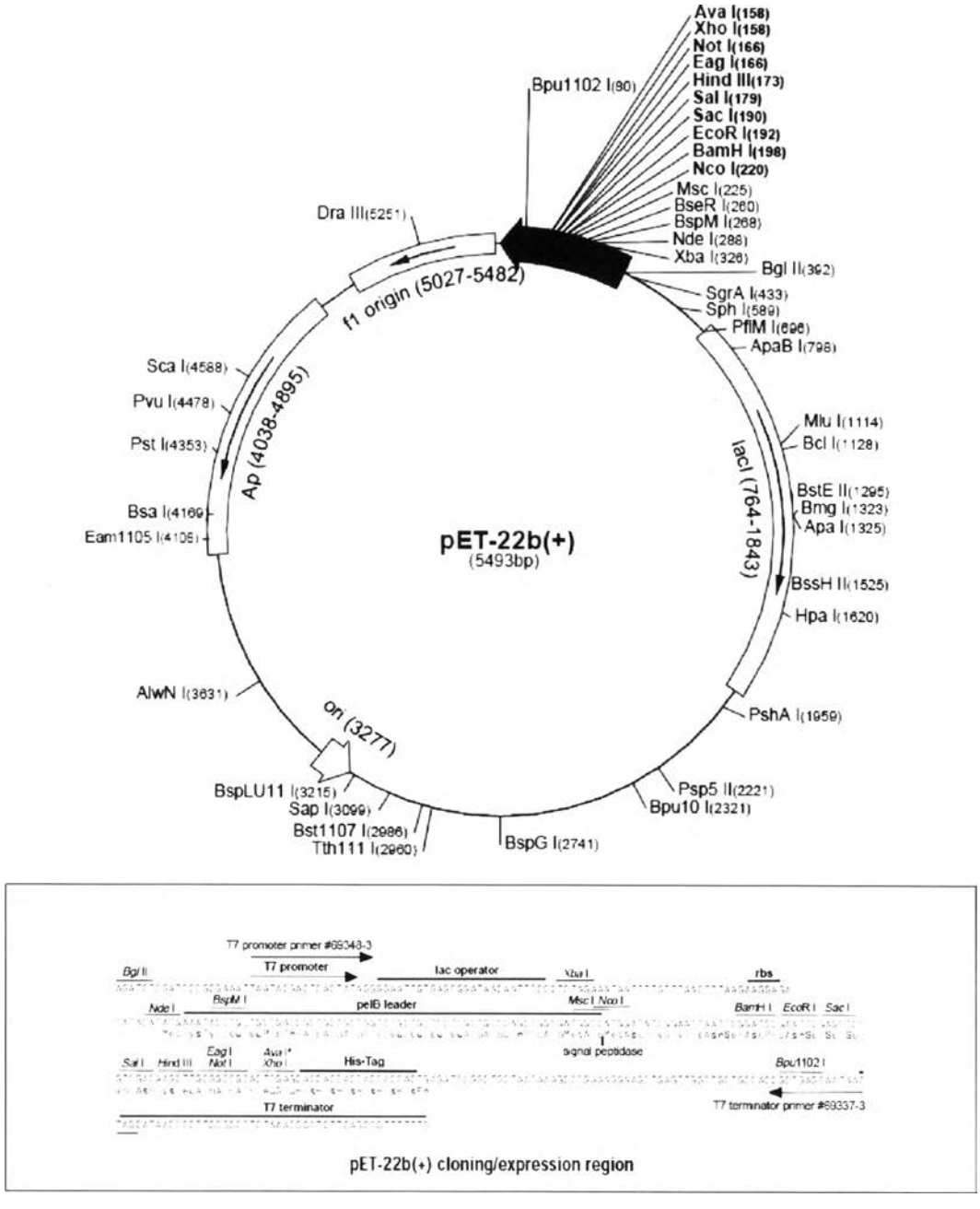


Figure 2.4 Map of the pET-22b (+) expression vector (Novagen)

### 2.18.1 Optimization of the r*Pm*VRP15 expressions

The *Pm*VRP15-recombinant pET22b(+) was transformed into *E. coli* C43 (DE3) competent cells by heat shock method and transformants were grown on LB agar containing 100 µg/ml ampicillin at 37 °C overnight. A single colony of transformed *E. coli* was grown in LB medium containing 100 µg/ml ampicillin at 37 °C overnight with agitation at 250 rpm. The overnight culture was diluted 1:100 in fresh LB medium supplemented with 100 µg/ml ampicillin and grown until an $OD_{600}$ of the cultures reached 0.6. Protein expression was induced by addition of IPTG to final concentration of 1 mM. The cells were harvested at 0, 1, 2, 3, 4, 5 and 6 hour post-induction by centrifugation at 8,000 xg for 10 min at 4 °C. The overexpression of r*Pm*VRP15 was analyzed by SDS-PAGE and Western blotting.



### 2.18.2 Membrane preparation of the r*Pm*VRP15

The *Pm*VRP15-recombinant pET22b(+) *E. coli* was grown in LB medium containing 100 µg/ml ampicillin at 37 °C overnight with agitation at 250 rpm. The culture was diluted 1:100 in fresh LB medium supplemented with 100 µg/ml ampicillin and grown until an $OD_{600}$ of the cultures reached 0.6. Cells were harvested at 1 h post-induction by centrifugation at 8,000 xg for 10 min at 4 °C, then the cells were resuspended in 50 mM Tris-HCl buffer, pH 7.0 containing complete Protease inhibitor cocktail tablets (Roche). The cell suspension was disrupted by three rounds of freeze/thaw followed by sonication. Cells were sonicated 10 rounds of at 35% amplitude, pulse on 2 sec, pulse off 3 sec for 40 sec/round. Supernatant was collected after centrifugation at 8,000 xg 4 °C for 20 min and membrane r*Pm*VRP15 protein was obtained by centrifugation at 100,000 xg 4 °C for 1 h. Membrane r*Pm*VRP15 protein was homogenized in 50 mM Tris-HCl buffer, pH 7 containing protease inhibitor tablet by homogenizer.

### 2.18.3 Purification of the r*Pm*VRP15 by Nikel-nitrilotriacetic acid (Ni-NTA) Sepharose™ 6 Fast Flow (GE Healthcare)

Membrane r*Pm*VRP15 protein was solubilized in solubilization buffer (50 mM Tris-HCl, pH 7, 20 mM Imidazole, 300 mM NaCl, 20% glycerol and 1% n-decyl-β-d-maltopyranoside, DM) at 4 °C overnight. The r*Pm*VRP15 solubilized protein was purified using the affinity chromatography by Ni-NTA Sepharose™ 6 Fast

Flow (GE Healthcare) according to the manufacturer's suggestion. First, r*Pm*VRP15 was incubated with Ni-NTA Sepharose™ 6 FF bead in binding buffer (50 mM Tris-HCl, pH 7, 20 mM Imidazole, 10% glycerol and 0.1% DM) and then, non-specific protein was eluted with wash buffer (50 mM Tris-HCl, pH 7, 50 mM Imidazole, 5% glycerol and 0.1% DM). r*Pm*VRP15 protein was eluted with elution buffer (50 mM Tris-HCl, pH 7, 300 mM Imidazole, 5% glycerol and 0.1% DM).

The purified r*Pm*VRP15 was dialyzed against dilution buffer (10 mM Tris-HCl, pH 7, 2.5% glycerol and 0.07% DM). The purity of the recombinant protein was analyzed by 15% SDS-PAGE.

### 2.18.4 Purification of the r*Pm*VRP15 by HiTrap DEAE Fast Flow (GE Healthcare)

The partial r*Pm*VRP15 protein was further purified by weak anion exchanger chromatography, HiTrap DEAE Fast Flow. First, r*Pm*VRP15 in binding buffer (20 mM Tris-HCl, pH 7, 20 mM NaCl, 10% glycerol and 0.1% DM) was loaded onto Hitrap DEAE FF column. Then, non-specific protein was eluted with wash buffer (20 mM Tris-HCl, pH 7, 20 mM NaCl, 5% glycerol and 0.1% DM) and r*Pm*VRP15 protein was eluted with elution buffer (20 mM Tris-HCl, pH 7, 150 mM NaCl, 5% glycerol and 0.1% DM). The purified r*Pm*VRP15 was analyzed by 15% SDS-PAGE. The purified r*Pm*VRP15 was confirmed by Western blot analysis.



### 2.19 Western blot analysis

After running the SDS-PAGE, the protein gel for electroblotting was soaked in transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 30 min together with Whatman® 3MM chromatography paper and nitrocellulose membrane which were cut to the size of the gel. The Whatman® 3MM paper was placed onto the Trans-Blot® SD (Bio-Rad). This is then wet with transfer buffer. The prewetted nitrocellulose membrane was put onto the filter paper and a gel was put on top of the membrane followed by filter paper again. Great care should be taken to ensure that there are no air bubbles. Proteins in transfer buffer are negative in charge and move from negative to positive poles. Western blotting was performed for 60-120 min at approximately constant 90 mA (Figure 2.5).

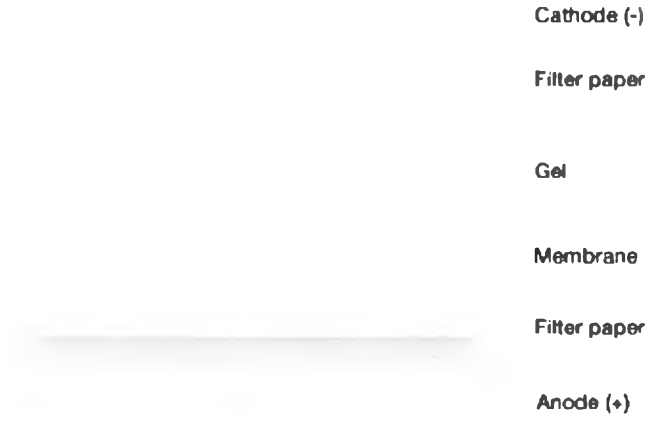


Figure 2.5 Gel and membrane set up for electrophoretic transfer (Bio-Rad).

After finishing transfer protein from gel to membrane, the membrane was incubated in blocking buffer (1X phosphate buffered saline (PBS), pH 7.4 containing 5% (w/v) skim milk and 0.05% (v/v) Tween™-20) at room temperature overnight with gently shaking. It was then washed 3 times for 10 minutes each in washing buffer, PBS-Tween buffer (1X PBS buffer containing 0.025% (v/v) Tween™20). The nitro cellulose membrane was incubated with a 1:3,000 dilution of mouse anti-His antibody in PBS, pH 7.4 containing 1% (w/v) skim milk and 0.05% (v/v) Tween™-20 at 37 °C for 3 h. with volume enough to cover it. After gently mixing at ambient temperature for 3 hours, it was washed 3 times for 10 minutes each in washing buffer and subsequently incubated in a secondary antibody solution, the alkaline phosphatase-conjugated rabbit anti-mouse IgG, 1:10,000 dilution in washing buffer with 1% (w/v) skim milk, with agitation at room temperature for 1 hour. Then, the membrane was washed 3 times with wash buffer as above and detected in darkness by color development with 10 mL of detection buffer (100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5) containing 44 µl of 4-nitroblue tetrazolium (NBT) and 33 µl of 5-bromo-4-chloro-indolyl phosphate (BCIP) as substrate. The BCIP was prepared 50 mg/mL in dimethyl formamide and NBT was also 50 mg/mL in dimethyl formamide. Reaction product is purple and appears in a few minutes. Finally, the development was stopped by washing the membrane with deionized water.

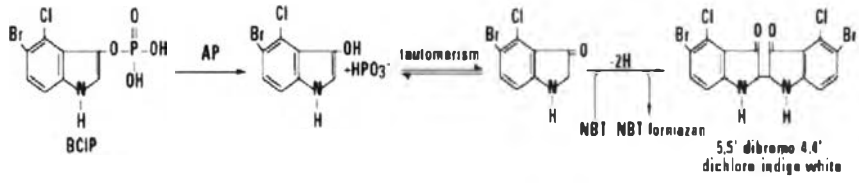


Figure 2.6 Chemical reaction of NBT and BCIP substrates with alkaline phosphatase. BCIP is hydrolyzed by alkaline phosphatase to form an intermediate that undergoes dimerization to produce an indigo dye. The NBT is reduced to the NBT-formazan by the two reducing equivalents generated by the dimerization. (http://www.piercenet.com/browse.cfm?fldID=01041003)

## 2.20 Determination of protein concentration by Pierce® BCA Protein Assay Kit (Thermo scientific)

The purified protein was concentrated using centricon columns (Millipore) with a 10 kDa cut-off membrane. Protein concentration of r*Pm*VRP15 was determined by Pierce® BCA Protein Assay Kit (Thermo scientific). Twenty- five of sample solution was mixed with 200 µl of BCA working buffer and left for 30 minutes before measuring the absorbance at 562 nm and bovine serum albumin (BSA) was used as a standard protein. The BCA working reagent was a mixture of solution A: solution B (50:1).

## 2.21 Molecular mass analysis of r*Pm*VRP15 using MALDI-TOF Mass spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to detect large molecules such as those present in synthetic and natural macromolecules. MALDI-TOF MS is a high sensitivity technique for detect low femtomole to attomole levels of proteins and peptides in order to study biochemical processes. Mass spectrometer is used to determine the molecular weights of the molecules in the surface layers of the sample by generating ion images of samples in mass-to-charge (m/z) values. It provides the capability of mapping specific molecules to two-dimensional coordinates of the original sample.

To determine the molecular weights of r*Pm*VRP15, purified r*Pm*VRP15 was sent to Mahidol university for MALDI-TOF MS analysis.

## 2.22 Determination of secondary structure of r*Pm*VRP15 using Circular Dichroism (CD) spectroscopy

To determine investigate the secondary structure of r*Pm*VRP15, circular dichroism (CD) spectroscopy was used. It is based on the dependence of the optical activity of the protein in the 170-240 nm wavelength with the backbone orientation of the peptide bonds with minor influences from the side chains. Different types of secondary structure producing characteristic spectra, the spectrum of a given protein can be used to estimate its percentage content on the major secondary structure types. Circular dichroism studies were carried out with a JASCO J-715 CD Spectropolarimeter with temperature controller using a 1 mm optical cuvette. The (+)-10-camphorsulfonic acid (CSA) was used as calibration standard before measuring CD spectrum. Spectra were recorded between 180-320 nm using a bandwidth of 2 nm and a response time of 2 sec with 50 mm/min scanning speed. Each spectrum was the average of three scans and background was subtracted with the spectrum of 10 mM Phosphate buffer pH 7.0 (blank). The CD spectra of purified r*Pm*VRP15 (0.42 mg/ml) proteins in 10 mM phosphate buffer pH 7.0. was measured. CD spectra of r*Pm*VRP15 revealed that the major secondary structure type of r*Pm*VRP15 was alpha-helix compared to standard protein which known secondary structure (http://www.ogic.ca/projects/k2d3/).



## 2.23 Crystallization of r*Pm*VRP15 protein

### 2.23.1 Pre-crystallization test by PCT™ (Hampton Research)

The PCT™ (Pre-Crystallization Test) is used to determine the appropriate protein concentration for crystallization screening. It was used to optimize protein concentration prior to initial screens, provide insight to sample homogeneity and select the appropriate protein concentration for crystallization screens.

To observed the appropriate precipitate, 0.5 to 1.0 ml of PCT Reagent A1 (0.1 M TRIS-HCl pH 8.5, 2 M Ammonium sulfate) was added into reservoir A1 of the VDX Plate with sealant and 0.5 to 1.0 ml of PCT Reagent A2 (0.1 M TRIS-HCl pH 8.5, 0.2 M Magnesium chloride hexahydrate, 30% w/v Polyethylene glycol 4,000) was

added into reservoir A2 of the VDX Plate. Protein sample (0.05 to 1 µl) was added onto the center of a single glass cover slide. A1 reagent from reservoir A1 was added into the sample drop on the siliconized cover slide (Do not mix the drop). Cover slide was inverted with the drop over reservoir A1 and sealed. A2 reagent was also added into the sample drop and inverted with the drop over reservoir A2. The reactions were incubated for 30 min and visualized under a light microscope. The result was compared to those in Figures 2.8. Compare results to Table 2.6 and proceed as suggested under Action. The suitable protein concentration should have a microcrystalline or light granular precipitate throughout the drop.

Table 2.6 PCT results and recommended action

| PCT Reagent A1/B1 Results | PCT Reagent A2/B2 Results | Recommended Action |
|---|---|---|
| Heavy Amorphous Precipitate | Heavy Amorphous Precipitate | Dilute sample 1:1, repeat step 1-7 |
| Clear | Clear | Concentrate sample to half the original volume, repeat steps 1-7 |
| Light granular precipitate | Clear | Perform Screen |
| Clear | Light granular precipitate | Perform Screen |
| Heavy Amorphous Precipitate | Light granular precipitate | Perform Screen |
| Heavy Amorphous Precipitate | Clear | Perform PCT with B1 & B2 / perform diagnostic testing |
| Clear | Heavy Amorphous Precipitate | Perform PCT with B1 & B2 / perform diagnostic testing |

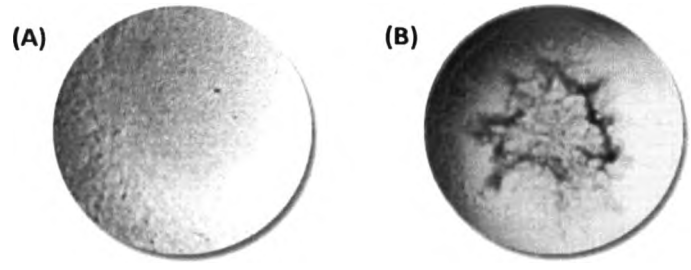


Figure 2.7 Pre-crystallization test results (A) light precipitate and (B) heavy amorphous precipitate

### 2.23.2 Crystallization screening of r*Pm*VRP15 protein

In this study, Index™, MembFac™ and Crystal Screen 2™ were used. Index™ screening is a primary reagent system crystallization screen for proteins which is effective in producing crystals or limiting sample solubility. Results from Index can be used to design optimization experiments and to identify follow on screens by reagent class. MembFac™ screening is a highly effective sparse matrix screen specifically designed as a preliminary screen for the crystallization of membrane proteins. MembFac™ is based upon the highly effective biased sparse matrix methodology. Crystal Screen 2TM reagent kits are designed to provide a highly effective and rapid screening method for the crystallization of macromolecules. The formulation utilized in Crystal Screen 2 evaluates 48 unique mixtures of pH, salts, polymers and organics, and their ability to promote crystal growth.

The purified rPmVRP15 was used for crystallization screening using crystal screening kits (Hampton Research). Drops of protein and reservoir solution were set up at the ratio of 1:1 in 96-well crystallization plate using sitting drop vapor diffusion method (Figure 2.8A). Plate was sealed off and incubated at 18 °C. Crystallization plates were observed every week up to three months.

After preliminary screening, the conditions that gave crystals were optimized in 24-well crystallization plate using hanging drop vapor diffusion method (Figure 2.8B). One µl of reservoir solution and 1 µl of purified r*Pm*VRP15 were pipetted onto a 0.22 mm siliconized glass cover slide. Next, cover slide was turned over and held on the grease plate and well. Crystallization plates were incubated at 18 °C and observed under a light microscope every week.

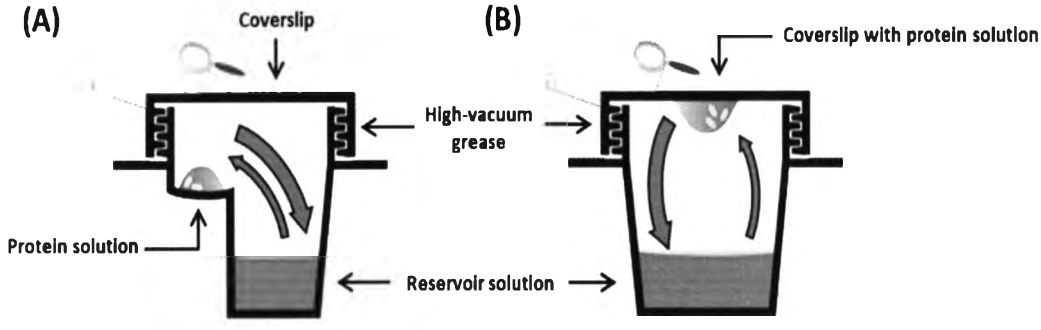


Figure 2.8 Two main techniques used in protein crystallization. (A) Sitting drop vapor diffusion method and (B) Hanging drop vapor diffusion method.