# CHAPTER 2

# MATERIALS and METHODS

## 2.1 Equipments

Autoclave LS-2D (Rexallindustries Co. Ltd., Taiwan)

A -20 °C Freezer

A -80 °C Freezer

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

GS Gene Linker™ : UV Chamber (Bio-RAD Laboratories, USA)

Heating block BD 1761G-26 (Sybron Thermerolyne Co., USA)

Hybridization oven (Hybaid, USA)

Incubator 37 °C (Kallenkamp, England)

Microcentrifuge tube 0.5, 1.5 ml (Bio-RAD Laboratories, USA)

PCR Thermal cycler : PCR system 2400 (Perkin Elmer)

PCR Thin wall microcentrifuge tube 0.2 ml (Perkin Elmer)

PCR Workstation Model # P-036 (Scientific Co., USA)

Pipette tips 10, 20, 200 and 1000 µl (Bio-RAD Laboratories, USA)

Power supply: Power PAC 3000 (Bio-RAD Laboratories, USA)

Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)

Spectrophotometer DU 650 (Beckman, USA)

Southern blotter (Hybaid, USA)

White / UV Transilluminator: UVP ImageStore 7500 (Mitshubichi Electric Corporation, Japan)

## 2.2 Chemical Reagents

Absolute ethanol (Merck, Germany)

Agarose (FMC BioProducts, USA)

Ammonium sulfate (Merck, Germany)

Bacto-agar (Difco, USA)

Bacto-yeast extract (Difco, USA)

Bacto-tryptone (Difco, USA)

Boric acid (Merck, Germany)

Bromophenol blue (Merck, Germany)

Chloroform (Merck, Germany)

Ethidium bromide (Sigma Chemical Co.,USA)

Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka,Switzerland)

100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin)

GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)

: 10x PCR buffer (100 mM Tris-HCl pH8.3, 500 mM KCl)

: 25 mM $MgCl_2$

Glucose (Merck, Germany)

Hydrochloric acid (Merck, Germany)

Isoamylalcohol (Merck, Germany)

Nylon membrane filter (Whatman International Ltd., England)

Oligonucleotide primers: 10-mer (Operon Technologies Co. Ltd., University of British Columbia)

Phenol crystal (Fluka, Germany)

pUC18/*Bam*H I/BAP (Pharmacia, USA)

Sodium acetate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide (Merck, Germany)

Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)

Whatman 3 MM paper (Whatman International Ltd., England)

Xylene cyanol (Sigma, USA)

## 2.3 Enzymes

Proteinase K (Gibco BRL life technologies, Inc., USA)

RNase A (Sigma Chemical Co., USA)

Ampli*Taq* DNA polymerase (Perkin - Elmer Cetus, USA)

*Bam*HI (Promega Corporation Medison, Wisconsin)

$T_4$ DNA ligase (Pharmacia, USA)

*Pst* I (Biolabs, New England)

*Eco*R I (Amersham, England)

## 2.4 Bacterial strains

*E. coli* strain DH5α [F' *lendA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* ($Nal^r$)*relA1Δ (lacZYA-argF)U169 deoR (φ80dlacΔ(lacZ)M15)*]

## 2.5 Biological materials

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Samples

The normal black tiger shrimp broodstocks were wild-caught alive from Satun-Trang in the south of Thailand. Pleopods were excised from freshly killed *P. monodon* individual and immediately kept on dry ice for transportation to the laboratory. The samples were transferred to a -70 °C deep freezer until further use.

The viral tolerance black tiger shrimp samples were provided by Shrimp Culture Research Center, Charoen Pokphand group of companies. The viral tolerance samples were placed into 1.5 ml microcentrifuge tubes containing enough amount of absolute ethanol and stored at -20 °C until used.

The term " normal shrimp" refers to shrimps from natural source which died after viral infection while the term " viral tolerance shrimp" refers to shrimps which are PCR positive to white spot virus but still survive and were collected from viral infected farms.

## 2.6 DNA Isolation

Shrimp genomic DNA was isolated from pleopods and hemolymp of *P. monodon*. For the pleopods, proteinase K- phenol- chloform method as described by Davis et al. (1986) was used. For hemolymph, the method was adapted from Cook et al. (summited).

### 2.6.1 DNA Isolation from Pleopod Samples

A pleopod of individual shrimp was homogenized on ice in a 1.5 ml microcentrifuge tube containing 400 µl of cold extraction buffer (100 mM Tris-HCl pH 9.0, 100 mM NaCl, 200 mM sucrose and 50 mM $Na_2EDTA$, pH 8.0). Sodium dodecyl sulfate (SDS) was added to 0.4 % to lyse the nucleated cells and incubated for 1 hour at 60 °C or for 30 minutes at 65 °C. Proteinase K and RNase A were added to the final concentrations of 500 µg/ml and 100 µg/ml, to destroy proteins and RNAs, respectively. The mixture was incubated for 3 hours at 65 °C. Protein precipitate was separated from DNA by adding 5 M potassium acetate to the final concentration of 1 M, chilled on ice for 30 minutes and centrifuged for 10 minutes at 10,000 rpm. The supernatant was removed into a new 1.5 ml microcentrifuge

tube and the pellet was discarded. An equal volume of redistilled phenol was added to the supernatant and mixed very gently. The mixture was spun at 9,000 rpm for 10 minutes. The upper aqueous phase was removed and transferred into a new 1.5 ml microcentrifuge tube. The sample was then added with an equal volume of phenol / chloroform / isoamylalcohol mixture (25:24:1 by volume) and mixed gently. After centrifugation, the upper aqueous phase was removed and extracted with an equal volume of chloroform/isoamylalcohol (24:1 by volume). The sample was mixed gently and spun at 9,000 rpm for 10 minutes. The lower organic phase was then removed and discarded. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.5, mixed gently and added two volume of prechilled -20 °C absolute ethanol. The mixture was mixed and stored at -20 °C overnight. The DNA pellet was hooked out using a pipette tip and washed with 70 % ethanol. The DNA pellet was air-dried and dissolved in TE buffer (10 mM Tris-HCl and 1 mM $Na_2EDTA$, pH 7.4). The DNA was stored overnight at 37 °C for completely solubilization and kept at 4 °C for further experiment.

### 2.6.2 DNA Isolation from Hemolymph Sample

The method, adapted from Cook et al. (summited),is a simple and rapid method which does not requrie organic solvents, e.g. phenol/chloroform/isoamyl-alcohol. This method was suitable for the isolation of DNA from hemolymph. The hemolymph drawn from a viral tolerance shrimp was quickly transferred into a microcentrifuge tube containing 70 % ethanol to prevent coagulation. The sample was spun at 8,000 rpm for 1-2 minutes to remove ethanol. The blood pellet was added 1 ml of high TE buffer (100mM Tris-HCl and 40 mM $Na_2EDTA$,pH 8.0), mixed by gentle vortex and spun at 10,000 rpm for 1-2 min. The blood pellet was added 250 μl of MGPL lysis buffer (10 mM Tris-HCl , 1mM $Na_2EDTA$ , 200 mM LiCl , pH 8.0 and 0.8 % SDS). Proteinase K was added to the final concentration of 200

μg/ml. The mixture was incubated at 45 °C for 15-20 min or until complete lysis. After the solution was clears, 500 μl of TE buffer and 750 μl of cold isopropanol were added. The sample was mixed gently and stored at -80 °C for 30 minutes. DNA was isolated by spinning at 12,000 rpm for 2-3 minutes, washed with 70% ethanol twice and air-dried. DNA was dissolved by adding TE buffer and incubated at 37 °C over night. The DNA was stored at 4 °C for further use.

## 2.7 Primer Screening and Primer Selection

### 2.7.1 Primer Screening and PCR Condition

An arbitrary primers purchased from Operon Technologies were used to amplify genomic DNA from one normal shrimp by polymerase chain reaction adapted from Williams et al. (1990). The optimal RAPD-PCR program parameters for reproducible amplification of *P. monodon* genomic DNA were 35 cycles of 5 sec at 94 °C, 45 sec at 36 °C and 90 sec at 72 °C as described by Pongsomboon (1996). The 25 μl amplified reaction was composed of 50 ng of genomic DNA, 2.5 μl of 100 mM each dNTPs, 2.5 μl of 10x PCR buffer (Perkin Elmer), 2 μl of 25 mM $MgCl_2$, 5 of RAPD primer and 1 unit of Taq DNA polymerase. Distilled water was added to make the final volume 25 μl. The primers that was able to amplify shrimp genome were used for further screening to ensure scorable and reproducible RAPD patterns of other 2-4 shrimp samples per primer. Selected primers were used to examine different RAPD patterns between viral tolerance and normal shrimp. The primers from University of British Columbia were primarily selected according to Pongsomboon (1996).

### 2.7.2 Detection of RAPD Patterns between Viral Tolerance and Normal *P. monodon*

The three samples from each group of viral tolerance and normal shrimps, were amplified using each of the selected primers. The primers that showed different bands between the two groups were further selected to amplify other samples. To confirm the present of RAPD markers which could differentiate between these two groups, a larger number of samples (6-12 individuals) were tested. The DNA band which only appeared on one group was isolated and further characterized.

## 2.8 Isolation and Characterization of RAPD Marker

### 2.8.1 DNA Elutions

The RAPD band from 2.7.2 was eluted from agarose gel using the GENE CLEAN II kit (BIO 101 Inc). The DNA band was excised with a razor blade from the ethidium bromide stained 1.6% agarose gel and transferred into a 1.5 ml microcentrifuge tube. The agarose was weighed to determine approximate volume of the gel slice (1 gram equals approximately to 1 ml). The gel could be sliced into roughly 2 mm cubes to facilitate gel dissolution in the next step. The 4.5 volumes of NaI stock solution and 0.5 volume of TBE modifying buffer were added. The final concentration of NaI was kept above 4 M. The mixture was incubated at 45 °C to 55 °C and mixed every one or two minutes during incubation. After about five minutes, the agarose gel should be completely dissolved. If not, the tube was further incubated until the gel was dissolved completely. The suspended Glassmilk® was added into the solutions (about 10-15 µl for up to 5 µg of DNA).

After mixing, the tube was placed on ice for 5 minutes to allow binding of the DNA to the silica matrix. The mixture was vortexed every 1-2 minutes to ensure that Glassmilk® stayed suspended. The suspension was spun for approximately 30 seconds at 6,400 rpm. The supernatant was removed as much as possible. Ten to 50 volumes (200 to 700 µl) of ice cold New Wash solution was added to wash the white pellet. The supernatant was removed by centrifugation as above and discarded. The wash procedure was repeated twice. After the supernatant from the third wash had been removed, the DNA was eluted by adding 1/10x TE buffer or water or low-salt buffer to the pellet. The tube was incubated at 45 °C to 55 °C for 2 or 3 minutes, mixed by vortexing and centrifuged for about 30 seconds. The elution step was repeated one more time. The supernatants were then combined. The recovery of the DNA was approximately 80 %. Eluted DNA could be use immediately in enzyme reactions or other manipulations.

### 2.8.2 Cloning

#### 2.8.2.1 Preparation of DNA marker flanked by *Bam*H I sites

A primer which showed a different band between normal and viral tolerance *P. monodon* was added at its 5' end with a 10 base sequence containing *Bam*H I site (5' CGGGATCCCG 3'). The resulting 20 base oligonucleotide primer was synthesized by Bioservice Unit (BSU), National Center Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA). This primer was used to amplify the DNA fragment. The amplification profile was identical to that when the primer without the *Bam*H I sequence was used. The amplified product was eluted from the agarose gel and digested with *Bam*H I. The digested DNA was isolated and ligated to the pUC18/*Bam*H I/BAP.

#### 2.8.2.2 Ligation

The mixtures of sticky-end ligation must contained a suitable amount between vector and DNA insert at the ratio of 1:3. The 10 µl ligation reaction was composed of 1 µl of 10x $T_4$ DNA ligase buffer, 5-10 units of $T_4$ DNA ligase, 50 ng of pUC18/*Bam*H I/BAP and 150 ng of DNA insert content was mixed, quick spun for 30 seconds and incubated at 14-16 °C for later use.

#### 2.8.2.3 *E. colli* competent cells preparation

A single colony of *E. coli* DH5α was picked up using a loop and transferred into 3 ml of LB broth. The culture was incubated in a 37 °C shaker for overnight. One percent of the overnight culture was inoculated into 50 ml of SOB medium [0.5 % (w/v) yeast extract, 2 % (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, 20 mM $MgSO_4$] and further incubated at the same temperature for 3-5 hours until the optical density at 600 nm ($O.D._{600}$) of the cell reached 0.3-0.4. The cells were removed into cold falcon tube 2001 and chilled on ice for 10 minutes and harvested by centrifugation at 4,000 rpm for 10 minutes at 4 °C. For the following steps, the cells were not allowed to warm up. The cell pelletes were resuspended by adding half original volume of cold 10 mM $CaCl_2$, placed on ice for 10 minutes and recentrifuged at the same speed. 1/20 original volume of cold 0.1 M $CaCl_2$ were added to resuspend the cell pellets. The competent cell suspension was divided into 50 µl aliquots and stored at -80 °C for long storage (Ausubel et al., 1995).

#### 2.8.2.4 Transformation

The ligated mixture was transformed into *E. coli* DH5α competent cells. One to 3 µl of ligated mixture was added into 50 µl of calcium chloride competent cell

and placed on ice for 30 minutes. The mixture was heat-shocked at 42 °C for 2-3 minutes and placed on ice immediately for 2-3 minutes. One hundred and fifty µl of SOB medium and 1.5 µl of 2 M glucose were then added into the mixture. After incubating at 37 °C with shaking for 1 hour, the mixture was spreaded on LB plate (LB agar per litre: 10 g of Tryptone, 5 g of yeast extract, 5 g of NaCl, 1 ml of 1 N NaOH and 15 g of agar) containing ampicillin (50 µg/ml), x-gal and IPTG (20 µg/ml). The plate was incubated at 37 °C overnight. The white colonies were selected for plasmid DNA isolation.

**2.8.2.5 Plasmid DNA isolation**

A white colony was inoculated into a tube containing 1.5 ml L-broth supplemented with ampicillin and incubated at 37 °C with shaking overnight. The cultures were transferred into 1.5 ml microcentrifuge tube and spun at 10,000 rpm for 30 seconds. The supernatant was discarded. One hundred microlitres of GET buffer (50 mM glucose, 10 mM $Na_2EDTA$ and 25 mM Tris-HCl, pH 8.0) and 2 µl of RNase (10 mg/ml) were added to the pellet. The mixture was thoroughly mixed and placed on ice for 10 minutes. Two hundred microlitres of fresh lysis buffer (0.4 N NaOH and 2% SDS) was added and mixed gently. After incubating on ice for 5 minute, the mixture was added with 150 µl of 3 M sodium acetate, pH 4.8, for renaturation, mixed gently and placed on ice for 5 minutes. The tube was spun at 10,000 rpm for 10 minutes and the white debris was discarded. The supernatant was transferred into the new microcentrifuge tube, an equal volume of cold isopropanol was added, mixed and placed on ice for 5 minutes. After on ice, the mixture was centrifuged at 10,000 rpm for 10 minutes. The plasmid DNA was washed with 70% ethanol and air-dried. The plasmid DNA was dissolved in 20 - 25 µl of TE buffer (Li et al., 1997).

#### 2.8.2.6 Detection of the desired recombinant plasmid

*Bam*H I was used to digest the recombinant plasmid DNA in 20 µl reaction. The reaction contained 2 µl of 10x buffer E (6 mM Tris-HCl, 6 mM $MgCl_2$ and 100 mM NaCl, pH 7.5) for *Bam*H I, 1 µl of restriction enzyme, 0.2 µl of 100x BSA and 200 ng of recombinant plasmid DNA. The mixture was mixed, quick spun and incubated at 37 °C for 3 hours or overnight. After this step, the digested recombinant plasmid DNA was analyzed by agarose gel electrophoresis. The size of DNA insert is estimated by comparing with restriction enzyme digested pUC18.

## 2.9 Dot Blot Hybridization

### 2.9.1 DNA Dot Blotting

The nylon membrane was pre-wetted for 30 min in 0.4 M Tris-HCl, pH 7.4. After soaking membrane, DNA samples were spotted on the membrane. The membrane was then soaked in a denaturing solution (0.5 N NaOH and 1.5 M NaCl) twice for 15 min each and neutralizing solution (1 M Tris-HCl, pH 7.4) twice for 10 min each at room temperature with gently shaking. The membrane was dried at room temperature. After this step, the membrane was ready for hybridization or could be kept at 4 °C.

### 2.9.2 DNA Labeling by the Multiprime Labeling Technique

The labeling reaction mixture contained the DNA fragment of interest (up to 100 ng), 2 µl of hexanucleotide mixture, 2 µl of dNTP labeling mixture (1mM dATP, dCTP, dGTP, 0.65 mM dTTP and 0.35 mM of dig-11dUTP, pH 7.5). Sterile distilled water was added to make the final volume to 19 µl. The mixture was heated at

100 °C for 10 min to denature the DNA and immediately chilled on ice for 5 min. One microlitre of Klenow fragment (2 units/μl) was added and further incubated for 4 hours or overnight at 37 °C. The reaction mixture was stopped by adding 2 μl of 500 mM $Na_2EDTA$, pH 8.0 and the labeled DNA was precipitated by adding 2.5 μl of 4 M LiCl and 75 μl of absolute ethanol. The mixture was placed at least 30 min at -70 °C or 2 hours at -20 °C. The DNA was pelleted by centrifugation at 10,000 rpm for 10 min, then was washed with 70 % cold ethanol, air-dried and dissolved in 50 μl of TE buffer

**2.9.3 Hybridization**

The membrane was prehybridized in a prehybridizaton solution [5x SSC, 1 % (w/v) blocking reagent, 0.1 % (w/v) N-lauryl sarcosine, Na-salt and 0.02 % (w/v) SDS] at the volume 20 ml/100 $cm^2$ of membrane. The membrane was then incubated at 68 °C for at least 1 hr. The labeled DNA was denatured by heating at 100 °C for 10 min and quickly cooled on ice for 5 min. The denatured labeled DNA was added to the prehybridization solution and further overnight incubated at 68 °C with gently shaking. The prehybridization solution was removed and the membrane was then washed twice with 2x SSC, 0.1 % (w/v) SDS at room temperature for 5 min each and further washed twice with 0.1x SSC, 0.1 % (w/v) SDS at 68 °C for 15 min each. The membrane could be air-dried and kept for later use or used directly for the detection of hybridized DNA by immunological method.

**2.9.4 Immunological Detection**

After briefly washing the membrane in buffer I (100 mM Tris-HCl, 150 mM NaCl. pH 7.5) for 1 min, the membrane was then blocked in buffer II [1 % (w/v) blocking reagent in buffer I] for 30 min with shaking. The diluted antibody-

alkalinephosphatase conjugate (150 mU/ml, dilution ratio 1:500 in buffer I) was added. The membrane was then incubated at room temperature for 30 min with gently shaking, washed twice with buffer I for 15 min and then once with buffer III (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min with shaking. After adding 10 ml of colour solution (45 μl NBT solution and 35 μl x-phosphate solution in buffer III), the membrane was kept in the dark without disturbing for 1/2 - 1 hr. The reaction was stopped by soaking in distilled water, then dried and photographed.

## 2.10 Southern Blot Hybridization

### 2.10 .1 DNA Transfer by Vacuum Blotting

Size fractionation of the DNA was carried out by agarose gel electrophoresis. Before transferring to a hybridization membrane, the DNA in the agarose must be treated to ensure efficient transfer and to generate single strand DNA suitable for hybridization. Gentle agitation of the gel was essential to prevent damage to the gel during these steps. The DNA was depurinated by soaking the gel in the depurinating solution (0.25 M HCl) for 10 min at room temperature with gentle shaking. The depurinating solution was replaced twice with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 15 min and gentle shaking. After removing the denaturing solution, twice neutralising solution (1.5 M NaCl, 0.5 M Tris-Hcl, pH 7.4) for 15 min was treated twice with gently shaking. The DNA was transferred to a hybridization membrane cut to the size of the gel. The transfer was carried out by vacuum blotting and completed in 1-1.5 hr.

The white porous supporting screen was placed within the base of the apparatus. Whatman 3 MM™ filter paper was cut larger than the gel and then pre-wet in 10x SSC and placed onto the porous screen. The hybridization membrane

was cut to the same size or slightly larger than the gel, pre-wet in 10x SSC and placed on the filter paper. The rubber mask was soaked with distilled water and placed over the membrane. The rubber mask should have a template cut such that the window was 2-5 mm smaller than the gel or enough to provide for a good seal. The gel was carefully transferred into position over the opening in the rubber mask in contact with the membrane and the lid was placed on the base of the unit. The transfer buffer (10x SSC) was gently poured on to the surface of the gel. The gel should contained sufficient buffer for complete transfer. When completed, the pump was turn off and the remaining transfer was removed. The membrane was then rinsed briefly in 2x SSC and air-dried on a sheet of dry filter paper.

#### 2.10.2 Hybridization and Detection

The DNA was fixed to the membrane by UV crosslinking and was ready for DNA hybridizaton and detection as described in dot blot hybridization using non-radioactive system. The membrane which did not used immediately could be stored between sheets of Whatman 3 MM™ paper in sealed plastic bag at 4 °C.

### 2.11 DNA Sequencing and Analysis

The clone containing desired DNA fragment was sequenced by the ABI-PRISM automated sequencer at Mahidol University, Salaya Campus. The sequence of desired DNA fragment was analysed by sequence alignment to other DNA sequence deposited in the GenBank using BLAST (Basic Local Alignment Search Tool) at the website: http://www.ncbi.nlm.nih.gov (Atschul et al., 1997). For other analysis, open reading frame (ORF), start and stop codon and restriction site were detected by DNA striders1.2.

## 2.12 PCR Amplification of DNA Fragment Specific to Normal Shrimp.

### 2.12.1 Primer Designation

Upper and lower primers for the amplification of specific fragment were designed from partial nucleotide sequences of the 800 bp inserted nucleotide sequences using Oligo4.0s program.

### 2.12.2 PCR Amplification and Detection of PCR Specific Fragment in *P. monodon*

The parameters, to effect reveal amplification, e.g. activity of Ampli*Taq* DNA polymerase, $Mg^{2+}$ concentration, primer concentration, template concentration and annealing temperature for the primers, were optimized. The performance of PCR, the number of cycles, the choice of temperature and time at temperature for each step in the cycle, were also determined. Basically, the PCR profile were 35 cycles of 15 sec at 94 °C, 30 sec at 52 °C and 30 sec at 72 °C. The 20 µl PCR reaction contained 25 ng of genomic DNA, 2 µM of each primers, 200 µM of each dNTPs , 2.5 mM of $MgCl_2$, and 0.5 unit of *Taq* DNA polymerase.

After optimization, twenty individuals in each group of geographically separated *P. monodon* in Thailand were examined for the presence of the 173 bp DNA fragment.