CHAPTER II

MATERIALS AND METHODS

Animal and Tissues Preparation

Adult wild-caught giant tiger prawns (*Penaeus monodon*) from natural wild stock, coast of Thailand were used for microsatellite development. Individual pleopods were dissected out from live prawns, kept on ice during transportation and stored individually at -80°C until use. A DNA sample which was prepared from one prawn was used for microsatellite development and 4 samples from each of 2 different locations, Angsila (the Gulf of Thailand) and Kruntrung (the Andaman sea, Thailand), were used for testing the assessment of the microsatellite polymorphism. In the latter part of the study, 19 more samples were added to the test. The new samples were from 4 different locations. Five individual prawns were from Angsila, the Gulf of Thailand, 3 individual prawns from Kruntrung, the Andaman sea, 6 individual prawns from Trat, the Gulf of Thailand and 5 individual prawns from Indonesia.

Giant Tiger Prawn DNA Extraction

Total DNA was extracted from the pleopods using the Proteinase-K phenol-chloroform extraction (Davis et al., 1986) with slight modifications. Individual pleopods were thoroughly homogenized in 400 µl of a chilled extraction buffer (100 mM Tris-HCl; pH 9.0, 100 mM NaCl, 200 mM sucrose, 50 mM EDTA) with a cooled homogenizer. The homogenate was added with 40 µl of 10% (w/v) SDS, mixed briefly and incubated in a water bath at 65°C for at least 30 min. After the homogenate was cooled to room temperature, it was treated with 100 mg/ml Proteinase K and 50 mg/ml DNase-free RNase (pre-boiled for 10 min and cooled on ice), mixed gently and incubated at 37°C for at least 3 hours. Five molar potassium acetate was then added to a 1% final concentration, and the microcentrifuge tube was held on ice for 45 min. The microcentrifuge tube was then centrifuged at 5,000 xg (Kubota 1300 Refrigerator microcentrifuge, Japan) for 10 min and the supernatant was transferred to a new 1.5 ml microcentrifuge tube. Protein was removed by extraction with Tris-saturated phenol (pH 8.0) and chloroform as follows.

An equal volume of Tris-saturated phenol : chloroform : isoamyl alcohol (25:24:1) was added and mixed gently by inversion for 10 min. The tube was centrifuged for 10 min. The liquid layer was transferred to a new tube and a half volume of 7.5 M ammonium acetate was added and mixed. Then 2 volumes of cold absolute ethanol were added to precipitate DNA. After the tube

was inverted several times, it was placed at -20°C overnight. The precipitated DNA formed a clump in the tube. By using a tip-cut microtip, the DNA was removed from the tube, rinsed with 70% cold ethanol and allowed to air-dry. The DNA was finally dissolved in an appropriate volume of TE buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA) and stored at 4°C for further use.

A rapid way to estimate the amount of the extracted DNA was to electrophoresis through 0.8% agarose gel at 100 volt for 2-3 hours with DNA standards (λ/*Hin*dIII). After the gel was stained with ethidium bromide, the intensity of orange-red fluorescence of the DNA bands was observed under ultra-violet light. Because the amount of fluorescence is proportional to DNA length, therefore the quantity of DNA in samples can be estimated by comparing their fluorescence to that of a series of standard DNA (Sambrook et al., 1989). The fluorescent intensity of the standard DNA fragments of 23.1, 9.4, 6.6, 4.3, 2.3, 2.0 and 0.5 kb corresponded to 240, 98, 67, 45, 24, 21 and 6 ng DNA, respectively when 500 ng of λ DNA (48 kb) was digested with *Hin*dIII.

For PCR template, DNA quantity was determined by ultraviolet absorption (Sambrook et al., 1989) using a DU 650 spectrophotometer (Beckman, USA). The DNA concentrations were adjusted to 15 ng/μl by the following formula:

$[15/(OD_{260} \times 50 \times \text{Dilution factor})] \times 100\ \mu l\ DNA + Y\ \mu l\ \text{of sterile}\ H_2O = 100\ \mu l.$

Agarose Gel Electrophoresis

To measure the amount of DNA samples, 0.8% agarose gel was used. A submarine agarose gel was set up in TBE buffer (89 mM Tris HCl, 89 mM boric acid, 2.5 mM EDTA; pH 8.3). One fifth by volume of loading dye (0.1% bromphenol blue, 40% ficoll 400 and 0.5% SDS) was mixed to each sample and the mixture was loaded into wells. The gel was run at 100 volt for 2 hours or until bromphenol blue tracking dye reached the end of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (2.5 μg/ml of ethidium bromide in water) for 5-10 min and then the ethidium bromide fluorescent DNA bands were visualized under UV light from a UV transluminator (UVP) and the gel was photographed through a red filter onto Kodak Tri-X pan400 film. The concentration or molecular weight of DNA sample was compared with the intensity and relative mobility of the standard DNA fragments.

Restriction Endonuclease Digestion of *P. monodon* Genomic DNA

Two micrograms aliquots of *P. monodon* DNA were digested separately with 5-10 units of each of the 4 restriction enzymes (*Alu*I, *Hae*III, *Rsa*I and *Hin*cII) in 20 μl reaction mixtures, each containing 2 μl of 10x restriction enzyme buffer M (10 mM Tris-HCl; pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 50 mM NaCl; Pharmacia) and an appropriate amount of sterile

distilled water. These reaction mixtures were incubated at 37°C for 2 hours before being subjected to agarose gel electrophoresis.

Construction of *P. monodon* Genomic Library

1. Preparation of chromosomal DNA for cloning

Fifty micrograms of genomic DNA from *P. monodon* were co-digested with 25 units each of the following enzymes; *Alu*I, *Hae*III, *Hin*cII and *Rsa*I (Bethesda Research Laboratories) in a total volume of 250 µl for 2 hours at 37°C. Digested DNA was subjected to electrophoresis in 1.5% low melting point agarose (Sea kem) at 70 volt for 2.5 hours with a size standard ladder (100 bp marker, Biolabs) loading along side. Genomic fragments of 300 to 700 bp were recovered from the gel by using a phenol freeze fracture procedure (Qian and Wilkinson, 1991). The gel containing only the marker was excised and stained with ethidium bromide solution (2.5 µg/ml of ethidium bromide in water) for 5-10 min. The marker bands were visualized under UV light. The mobility of the DNA fragments size 300 to 700 bp was measured as compared to those of the DNA marker. Then the gel containing the DNA fragments of 300 to 700 bp was cut off and chopped into small pieces. DNA was recovered from the gel by transferring these pieces to a 50 ml capped tube and freezing the tube at -80°C to solidify the gel pieces. Two volumes of phenol saturated

buffer (Tris 0.1 M; pH 8.0) were added and the tube was vortexed until the frozen gel was completely dissolved. Then 2 - 3 drops of chloroform were added, and mixed vigorously. Centrifugation in a Beckman J2-21 refrigerator at 10,000 x g, 2°C for 20 min was performed to separate an upper aqueous layer. The aqueouse layer was transfered to another tube to extract the remaining phenol in the aqueous layer, an equal volume of chloroform was added and mixed. After centrifugation, the supernate was again transferred to a new tube. The DNA was precipitated by adding 2 µl of 100 mg/ml glycogen, 3 M NaCl to a final concentration of 0.2 M and 2 volumes of absolute ethanol, then mixed well, kept at -80°C for at least 30 min and centrifuged. The DNA pellet was resuspended in 0.5 ml TE and transferred to a new 1.5 ml microfuge tube. The DNA was reprecipitated by adding 3 M NaCl to a final concentration of 0.2 M and 2 volumes of absolute ethanol, kept at -80°C for at least 30 min and centrifuged. The DNA pellet was resuspended in 21 µl TE. The DNA yield was estimated by electrophoresis of a 1 µl aliquot of the sample in 1% agarose gel at 100 volt for 2-3 hours.

2. Ligation

The pUC18, digested with *Sma*I and dephosphorylated (Pharmacia), was used as a vector for blunt-end ligation. The DNA ligation mix contained 50 ng of vector, 100 ng of the digested insert DNA, 1x ligation buffer (10 mM

Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 µl of ATP (10 mM), 1 µl DTT (50 mM) and 1 to 7 U of T_4 DNA ligase (Pharmacia) in a total volume of 20 µl. All ingradients except T_4 DNA ligase were mixed in a 0.5 ml microfuge tube and heated at 65°C for 15 min. The tube was left until it was cooled to room temperature, then T_4 DNA ligase was added. The reaction was incubated at room temperature (20 to 25°C) for 20 hours (Sambrook et al., 1989).

3. Transformation

The portion of the ligation mixture was transformed into host cell *E. coli* strain DH5α genotype; F'φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rel*A1 *end*A1 hsdR17(r_K^-, m_K^+) *pho*A *sup*E44 λ⁻thi-1 *gyr*A96 *rec*A1 with 2 procedures as described below.

3.1. <u>$CaCl_2$ method</u>

Transformation procedure was performed according to the manufacturing instruction of Max Efficiency DH5α competent cell (Bethesda Research Laboratories). The competent cell which had been kept at -80°C was thawed on ice and gently mixed. Thirty microliters of the competent cells were aliquoted into the prechilled tubes. One microliter of the ligation mixture was added into each tube. The mixture was incubated on ice for 30 min. Cells were then heat shocked at 42°C for 45 seconds and chilled on ice immediately. One mililiter of LB medium was added and the cells were incubated with shaking at

37°C for 1 hour. The content was spreaded onto the LB agar plates containing 50 μg/ml ampicillin. The plates were incubated at 37°C overnight.

3.2. Electroporation procedure (Dower, et al. 1988)

3.2.1. Preparation of host cells

A single colony of *E. coli* strain DH5α was cultured as the starter in 3 ml of LB medium by shaking at 37°C overnight. One milliliter of the starter was added into 100 ml of L-broth (1% Bactrotryptone, 0.5% Bacto yeast extract, 0.5% NaCl) and the culture was grown at 37°C with vigorous shaking for 3-4 hours ($O.D._{600}$ ~ 0.5-0.7). The cells were then chilled on ice for 15-30 min, and centrifuged in a precooled rotor (Beckman J2-21, USA) at 4,000 x g for 15 min. After the supernatant was removed, cells were washed by resuspending in 100 ml of cold steriled water, gently mixing and centrifuging. The cells were washed further with different kinds of solutions, first with 50 ml of cold water, followed by 2 ml of ice-cold steriled 10% glycerol and resuspended in a final volume of 200-300 μl ice-cold 10% glycerol. This cell suspension was dispensed in 40 μl aliquots into 0.5 ml microfuge tube and stored at -80°C until use. The cells were good for at least 6 months under these conditions.

3.2.2. Electrotransformation

Forty microliters of the cell suspension were gently thawed on ice, then 1 μl of ligation mixture was added, mixed well and left on ice for

approximately 1 min. The mixture was electroporated in a precooled 0.2 cm cuvette. The setting of the Gene pulser (Bio-Rad) was 25 μF, 200 Ω and 2.5 kV. After electroporation, the cells were immediately resuspended with 2 ml of SOC medium (2% Bactotryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) and transferred to a new tube. The cell suspension was incubated with shaking at 37°C for 1 hour. The content was spreaded on the LB agar plates containing 50 μg/ml ampicillin and incubated at 37°C overnight.

The transformant colonies were counted and screened for microsatellite DNA with colony hybridization as follows.

Screening of the Library for Microsatellite DNA

Colony hybridization, a procedure for the screening of the library for microsatellites, was based on the method devoloped by Grunstein and Hogness (1975).

Colonies were blotted onto Whatman filter paper #42 (Ashless). The filters were pencil-labeled with the numbers of the plates and cut at 3 asymmetric sites on the edge of circles. To lift the colonies, each filter was carefully laid on the agar surface of the plate with the label side up and left until it was wet. Before lifting the filter, the orientation of the filter was marked on the plate with a marking pen. The colonies on the plate were regrown by

incubating at 37°C for 3-4 hours and kept at 4°C. The filters were processed by placing on top of Whatman 3 MM paper presoaked with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 7 min, followed by neutralization with neutralizing solution (1 M Tris-HCl pH 7.6,1.5 M NaCl) 2 times for 3 min each and washing with 2x SSC, 0.2% [w/v] SDS for 30 seconds to 1 min. After air dried for 15 -30 min, DNA was fixed on the filters by baking the filters at 80°C for 2 hours in a vacuum oven. The debris of bacterial colonies cell was washed off from the filters by gently rubbing the filters in 2x SSC, 0.2% [w/v] SDS before prehybridization.

A $(GT)_{15}$ synthetic oligonucleotide synthesized by Bio-Synthesis was used as a probe. One hundred picomole of the oligonucleotide was end-labeled with 6 µl of 3,000 Ci/mmol [γ-^{32}P] ATP (Dupont, NYR), 5 U of T_4 polynucleotide kinase (New England Biolabs) and 1 µl of 10x One Phor All buffer (OPA, Pharmacia : 100 mM tris-acetate (pH7.5), 100 mM magmesium acetate and 500 mM potassium acetate) in a total volume of 10 µl. The reaction was incubated at 37°C for 30 min. One reaction was sufficient to screen six filters.

Filters were prehybridized in a 20 ml of prehybridization solution (Appendix C) containing 2x Denhardt's solution, 5x SSPE, 0.5% (W/V) SDS and 100 µg/ml yeast tRNA at 60°C for at least 1 hour. Radioactive-labeled $(GT)_{15}$ oligonucleotide probe was then added directly to the filters. The

hybridization reaction was performed in a hybridization rotor oven (Hybaid) at 60°C overnight. Hybridized filters were washed 2 times with 2x SSC, 0.2% [w/v] SDS at room temperature for 30 min per wash, followed by 0.2x SSC, 0.2% [w/v] SDS at room temperature for 30 min, then 0.1x SSC, 0.2% [w/v] SDS at 45°C for 30 min and finally 0.1x SSC, 0.2% [w/v] SDS at 60°C for 30 min. Filters were wrapped between two sheets of plastic wrap and subjected to autoradiography (Kodak XAR film or HyperfilmMP, Amersham) at -80°C with intensifying screens for approximately 2 hours.

Individual positive clones which gave strong hybridization signal were identified by aligning the master plate on the autoradiographs and picked up using sterilized toothpicks. The cells were grown in 3 ml Terrific broth (Appendix D) contained 100 μg/ml ampicillin at 37°C with shaking for 24-36 hours. Five hundred microliters of each culture were added with 100 μl of 80% glycerol in a 1.5 ml microfuge tube and kept at -80°C as preserved stock culture. The remaining of the culture was subjected to plasmid DNA preparation for sequencing as described below.

Preparation of Plasmid DNA for Sequencing

A boiling procedure developed by Holmes and Quigley (1981) was used for rapid isolation of plasmid DNA from large numbers of colonies for sequencing.

The culture was poured into a 1.5 ml microfuge tube. The cell pellet was collected by centrifuging at 10,000 x g with microcentrifuge for 10-15 seconds and resuspended completely in 180 µl of STET buffer (8% Sucrose, 5% Triton X-100, 50 mM Tris-HCl , 50 mM EDTA). Then 20 µl of 10 mg/ml lysozyme in STET buffer was added, immediately mixed by vortexing and left the tube at room temperature for 75 seconds. The tube was placed in boiling water for 75 seconds and spinned at 10,000 x g for 10 min. The cell debris at the bottom of tube was removed by using a toothpick. The DNA was precipitated by mixing with an equal volume of cold isopropanol and placed at -80°C for at least 10 min. The DNA was pelleted after centrifugation for 10 min, air dried and resuspended in 80 µl TE.

DNA Sequencing and Primer Design

Sequences of the cloned DNA fragments from the positive hybridization clones were determined by double strand alkaline sequencing (Sanger et al., 1977; Sambrook et al., 1989), with M13-pUC universal primer, and/or reverse primers using T_7- sequencing ™ kit (Pharmacia) as described below.

Ten microliters of extracted plasmid DNA from each positive clone was denatured by adding 2.25 µl of 2N NaOH and incubated for 10 min at room temperature. Then the content was added with 1.25 µl of 3 M Sodium

acetate (pH 4.8) and 1 μl of distilled water. DNA was precipitated with 30 μl of absolute ethanol, mixed well and placed at -80°C for 15 min. The precipitated DNA was collected by centrifuging for 15 min. The pellet was washed with ice-cold 70% ethanol and recentrifuged for 10 min. The pellet was air-dried and redissolved in 11 μl of distilled water and 2 μl of annealing buffer. The resuspended template can be either proceeded immediately to annealing with the primer or stored at 4°C for later use.

The resuspended template was annealed with 1.1 μl of an appropriate primer (5 pmol/μl universal or reverse primer). The tube was incubated at 65°C for 5 min, at 37°C for 10 min and placed at room temperature for at least 5 min. The tube was centrifuged briefly before proceeding to the sequencing reaction.

Labeling reaction was made by mixing the "Labeling Mix-dATP", [α-^{35}S] dATP (>1,000 Ci/mmol, Dupont) with 1:5 diluted "T_7 DNA Polymerase" in the volume proportions of 1.5:1:0.5, respectively. Three point four microliters of the reaction solution was added to each tube containing the annealed template-primer. The tube was incubated at room temperature for 5 min.

After the labeling reaction had been incubated for 5 min, 2.5 μl of the reaction was transferred into each of the four pre-warmed sequencing mixes and incubated at 37°C for 5 min. Four microliters of stop solution (10 mM NaOH, 99% formamide, 0.1% bromphenol blue and 0.1% xylene cyanol) was

added to each tube and mix gently.

The sequencing reaction can be performed using either the "Read Short" or "Read Long" conditions:

The "Read Short" conditions allow sequence to read up to approximately 500 nucleotides from the primer. The four nucleotide mixes with suffix "-Short" as shown in Table 2.1 are designed for such reactions.

The "Read Long" conditions generate fragments covering the sequence up to 1,000 nucleotides or more from the primer. The four nucleotide mixes with suffix "-Long" as shown in Table 2.1 are designed for such reactions.

Sequencing products were separated in a 8% denaturing polyacrylamide gel containing 7.8 M urea (Appendix E) using 1x TBE as running buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA pH 8.3) at 1200 volt for approximately 2.5 hours. Sequencing gels were fixed in fixing solution (10% methanol and 10% acetic acid) for at least 30 min before transferred onto Whatman 3MM paper for vacuum drying with a gel dryer (Model 583, Bio-Rad) for 2 hours. Dried gels were exposed to autoradiography film (Kodak XAR5 or Hyperfilm MP, Amersham) without intensifying screens overnight at room temperature.

The microsatellite-containing regions of the inserts were identified, and the unique sequences flanking the microsatellite were used to design PCR primers. The criteria used for designing primers were: (1) the primer should be

Table 2.1 Components of the sequencing mixes

Type	Components
'A' Mix-short	840 μM each dCTP, dGTP and dTTP; 93.5 μM dATP; 14 μM ddATP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'C' Mix-short	840 μM each dATP, dGTP and dTTP; 93.5 μM dCTP; 14 μM ddCTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'G' Mix-short	840 μM each dATP, dCTP and dTTP; 93.5 μM dGTP; 14 μM ddGTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'T' Mix-short	840 μM each dATP, dCTP and dGTP; 93.5 μM dTTP; 14 μM ddTTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'A' Mix-Long	840 μM each dCTP, dGTP and dTTP; 93.5 μM dATP; 2.1 μM ddATP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'C' Mix-Long	840 μM each dATP, dGTP and dTTP; 93.5 μM dCTP; 2.1 μM ddCTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'G' Mix-Long	840 μM each dATP, dCTP and dTTP; 93.5 μM dGTP; 2.1 μM ddGTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl
'T' Mix-Long	840 μM each dATP, dCTP and dGTP; 93.5 μM dTTP; 2.1 μM ddTTP; 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl

close to the repeat array as much as possible; (2) approximately equivalent GC content was present in both forward and reverse primers close to 50% overall; (3) the primers should contain at least one C or G at their 3' end, where possible; (4) there should be no potential annealing between primers; and (5) the primers should be 19 to 23 bp in length (Kamonrat, 1996). The primers designed for giant tiger prawn microsatellites were synthesized commercially.

PCR Amplification of Microsatellite Loci

Allele length and polymorphism for each microsatellite locus were assessed using a polymerase chain reaction with ^{32}P-labeled primers. It was important to determine whether the reverse or forward labeled primer gave more readily scorable products. The primer was labeled in 10 µl labeling reaction containing 10 pmol of primer, 10 units of T_4 polynucleotide kinase (New England Biolabs), 1 µl of 10x One Phor All (OPA) buffer (Pharmacia) and 3 µl of [γ-^{32}P] ATP (3,300 µCi/mmol, Dupont). Primer labeling was performed at 37°C for 30 min and at 65°C for 15 min to kill the enzyme. Microsatellites were amplified from total genomic DNA samples of 8 individuals of *P. monodon* using a PCR machine (Perkin Elmer Thermal Cycler). The 5 µl standard reaction contained 15 ng template DNA, 0.6 µM of primer 1, 0.575 µM of primer 2, 0.025 µM ^{32}P-labeled primer 2, 1µl of 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 10 mM $MgCl_2$, 0.1%

gelatin), 200 μM dNTPs, 0.25 U of Taq Polymerase (Perkin Elmer) and 0.05% Tween20 (Kamonrat & Cook, in prep). Reactions were overlaid with a drop of mineral oil to prevent evaporation. Amplifications were achieved by running 7 cycles of 1 min at 94°C (denaturation), 30 seconds at a primer specific annealing temperature (T_A), and 1 min at 72°C (elongation) followed by 38 cycles of 30 seconds at 90°C, 30 seconds at annealing temperature, and 1 min at 72°C.

Annealing temperature of each primer (T_a) was determined from the base content by the following equation:

$$T_a\ (°C) = [4(\text{sum of G and C}) + 2(\text{sum of A and T})] - 5$$

Annealing temperature for reaction (T_A) was initially set at the lower value of the primer annealing temperatures (T_a). T_A was adjusted after examining the results of PCR amplification to obtain an optimum condition. For example, when more than one or two stutter bands appeared, annealing temperature was raised by 1°C. Inversely, if no products were visible, temperature was lowered by 1°C until the optimal temperature for each primer pair was attained.

After the amplification was complete, each reaction was added with 5 μl of stop dye (10 mM NaOH, 99% formamide, 0.1% Bromphenol blue, 0.1% Xylene cyanol) and denatured at 94°C for 15 min. Three point five microliters

of the reaction were subjected to gel electrophoresis on a 8% denaturing polyacrylamide sequencing gel using 1x TBE as running buffer at 1200 volts for 2-3 hours (depending on the expected size of the product). The gel was fixed in 10% methanol and 10% acetic acid for 30 min, and transferred onto Whatman 3 MM paper for vacuum drying at 80° for 2 hours before exposing with intensifying screens to an autoradiographic film (Kodak XAR5 or HyperfilmMP, Amersham) at -80°C overnight. Microsatellite alleles were sized relative to a sequencing ladder generated from single-stranded M13 DNA loaded along side the PCR products. A protocol to generate M13 DNA sequence marker (Yanish-Perron et al., 1985) is described below.

To prepare M13 sequence marker, annealing reaction was made using 5 µl of M13mp18 template, 5 µl of dH_2O, 2 µl of annealing buffer and 2 µl of 4 ng/µl of FSP (Forward Sequencing Primers). The reaction was incubated at 65 °C for 15 min and then cooled down to room temperature for 10 min.

Labeling mixture was made by mixing together 1 µl of dH_2O, 3 µl of labeling mix-dATP, 2 µl of diluted T_7 polymerase (0.3µl of T_7 polymerase and 1.7 µl of enzyme dilution buffer) and 1 µl of 800 Ci/mmol [α-^{32}P] dATP. Six microliters of the mixture was added to the annealing reaction. The labeling reaction was incubated at room temperature for 5 min.

The termination process was performed by adding 4.5 µl of labeling reaction to each of the 4 tubes containing 2.5 µl of short mix A, C, G and T,

and then incubated at 37°C for 5 min. Forty microliters of stop solution and 1 drop of mineral oil were added into each tube. The marker was heated at 94°C for 2 min and 3-3.5 μl were loaded onto the gel. The M13 sequence marker can be stored at -20°C for later use.