การโคลนและลักษณะสมบัติของตัวรับโทลล์ในกุ้งกุลาดำ *Penaeus monodon*

นายปรเมศวร์ เจียรนัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CLONING AND CHARACTERIZATION OF TOLL RECEPTOR IN BLACK TIGER SHRIMP *PENAEUS MONODON*

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กุ้งกุลาดำ (*Penaeus monodon*) เคยเป็นสัตว์น้ำเศรษฐกิจที่สำคัญทางของประเทศไทย อย่างไรก็ตามปัจจุบันผลผลิตของกุ้งชนิดนี้มีแนวโน้มลดลง เนื่องจากสาเหตุหลักจากการติดเชื้อไวรัสในอุตสาหกรรมการเพาะเลี้ยงกุ้ง ความเข้าใจในระบบการต้านโรคของกุ้งถือเป็นปัจจัยหนึ่งที่จะช่วยหาแก้ปัญหาการเกิดการติดเชื้อไวรัสในกุ้งได้ ระบบภูมิคุ้มกันกุ้งเป็นภูมิคุ้มกันที่มีมาแต่กำเนิดตัวรับโทลล์ และตัวรับเหมือนโทลล์ได้ถูกพบในแมลงหวี่ หนอนตัวกลม และมนุษย์ ปัจจุบันได้มีการค้นพบตัวรับโทลล์ในกุ้งตระกูลพีเนียสหลายชนิด และศึกษาหน้าที่ของตัวรับโทลล์ ยกเว้นกุ้งกุลาดำวัตถุประสงค์ของการศึกษาเพื่อโคลน cDNA ที่มีความยาวเต็มสายของตัวรับ PmToll และศึกษาการทำงานของตัวรับนี้ cDNA ที่มีความยาวเต็มสายของ PmToll ประกอบด้วยสำดับเบสจำนวน 4,129 นิวคลีโอไทด์ถอดรหัสเป็นกรดอะมิโนจำนวน 931 ตัว ส่วนตัวรับ PmToll ประกอบด้วย โมทีฟโครงสร้าง/หน้าที่ของตัวรับโทลล์ มีส่วนที่อยู่ภายนอกเซลล์ซึ่งประกอบด้วย leucine-rich repeats (LRRs) ที่มีส่วน cysteine-rich motifs ติดอยู่ ถัดมาเป็นส่วนที่ฝังอยู่ในเยื้อหุ้มเซลล์และส่วน Toll/Interleukin-1 receptor (TIR) ซึ่งอยู่ภายในเซลล์ การแสดงออกของ PmTollในเนื้อเยื่อต่างๆ สามารถตรวจพบได้หลายแห่ง ได้แก่ เหงือก หัวใจ ต่อมน้ำเหลือง ตับ กล้ามเนื้อ เส้นประสาท ขาว่ายน้ำ และกระเพาะอาหารแต่พบการแสดงออกน้อยในตับ การวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการของกรดอะมิโนระหว่าง PmToll กับสิ่งมีชีวิตต่าง ๆ บ่งชี้ว่า PmToll มีความสัมพันธ์ใกล้เคียงกับตัวรับโทลล์ของกุ้งอื่นๆ โดยเฉพาะ FcToll ส่วนในการศึกษาหน้าที่ของ PmToll ในกุ้งกุลาดำ โดยการยับยั้ง PmToll ด้วย dsRNA-PmToll พบว่า PmToll ไม่สามารถถูกยับยั้งการแสดงออกในกุ้งกุลาดำได้สำหรับในการศึกษาครั้งนี้

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : RNA INTERFERENCE / TOLL RECEPTOR / PENAEUS MONODON

PORAMATE JIARANAI : CLONING AND CHARACTERIZATION OF TOLL RECEPTOR IN BLACK TIGER SHRIMP *PENAEUS MONODON*. ADVISOR : ASST. PROF. WANCHAI ASSAVALAPSAKUL, Ph.D., CO-ADVISOR : PAKORN WINAYANUWATTIKUN, Ph.D., 85 pp.

Black tiger shrimp (*Penaeus monodon*) was the most important economic aquatic animals in Thailand. However, the production loss of shrimps caused by viral diseases are still remained a major problem of shrimp farming industries. One of the key to overcome this loss is to understand shrimp immunity. Shrimps immunity relied on innate immune response. Toll or Toll-like receptors have been identified in fruit fly, nematode, and human. Recently, Toll receptors in penaeid shrimps have been discovered and studied their functions, except in black tiger shrimp. The objectives of this study are to clone the full length cDNA of PmToll receptor and characterize its function. The full-length cDNA of PmToll receptor consists of 4,129 nucleotides, which can be encoded to 931 amino acids. PmToll receptor contains the distinct structure/functional motif of the Toll receptor family including an extracellular domain, which consists of leucine-rich repeats (LRRs) flanked by cysteine-rich motifs, a single-pass transmembrane portion, and a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain. The expression of PmToll can be found in several tissues such as gill, heart, lymphoid, muscle, nerve, pleopod, stomach and but less expression can also be found in the hepatopancreas. The analysis of phylogenetic relationship between the deduced amino acid of PmToll and other organisms' Toll suggests that PmToll is closely related to other shrimp Tolls, especially FcToll. The function of PmToll in black tiger shrimp was studied, by knocking down PmToll using dsRNA-PmToll. The result suggested that the PmToll cannot be suppressed in black tiger shrimp.

Department	Microbiology	Student's Signature	
Field of Study	Industrial Microbiology	Advisor'sSignature	
Academic Year	2011	Co-Advisor's Signature	

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CHAPTER I

INTRODUCTION

Fruit fly (*Drosophila*) is an invertebrate which used to be a model to study in evolution, mechanism of developments and innate immune system (Kimbrell and Beutler, 2001). Like other invertebrates, fruit fly has only innate immune system. The innate immune system could be classified into two groups; humoral and cellular response. The Toll pathway, which is a one of the innate immune systems in *Drosophila*, has been discovered and studied in *Drosophila* (Lemaitre et al., 1996). Toll pathway is activated by interaction between leucine-rich repeated (LRR) N-terminal region of Toll receptor and 106 amino acids active C-terminal of Spätzle. The pro-Spätzle is activated by Spätzle processing enzyme (SPE) into active Spätzle. The interaction between two proteins lead to intracellular signaling cascade, resulting in secretion of immune genes such as *Drosomycins*, *Metchnikowin* (antifungal) and *Defensin* (antibacterial) (Hoffman, 2003).

Moreover, toll pathway has been discovered and studied in mammalian. The mammalian and *Drosophila* toll pathways are share homology and mechanism among them (Rock et al., 1998). Since, the mammalian Toll-like receptors (TLRs) have been identified and characterized, the TLR functions are recognition the foreign component, follow by innate immune activation (Janssens and Beyaert, 2003). Human TLR3 (hTLR3) is the first identified antiviral TLR, it recognize double-stranded RNA (dsRNA) as its ligand. So, hTLR3 is assumed to have a central role in the host response to RNA viruses because dsRNA is a universal viral molecular pattern (Schröder and Bowie, 2005).

RNA interference (RNAi) known as post-transcriptional silencing, was found in Nematodes, insects and mammalians (Robalino et al., 2008). The mechanism is happened by when dsRNA was in cells, the dsRNA was processed to 21 to 25 bp longs by dicer enzyme resulting as short interfering RNA (siRNA). Then, siRNA is unwound while associated in proteins complex (RNA induce silencing complex; RISC). This single-stranded siRNA in RISC will locate mRNA targets, resulting in gene silencing by nucleolytic degradation of targeted mRNA (Hammond, 2005).

Recently, many penaeid tolls from *Penaeus monodon* (Arts et al., 2007), *Litopenaeus vannamei* (Yang L-S. et al., 2007; Wang et al., 2012), *Marsupenaeus japonicus* (Mekata et al., 2008) and *Fenneropenaeus chinensis* (Yang C. et al., 2008)

have been discovered and characterized of their function. However, the full-length cDNA of Toll receptor from black tiger shrimp has not been reported. Therefore, the objectives of this project are to clone a full-length toll receptor from *P. monodon* and to characterize its function.

CHAPTER II

LITERATURE REVIEW

2.1 Black Tiger Shrimp (*Penaeus monodon*)

Thailand, Vietnam, Indonesia, and China are the main shrimp export producers. Among these four countries, Thailand is the largest shrimp exporter which accounts for approximately 23 percent worldwide. The value of exports in 2010 is 98,245.1 million baht (Office of Agricultural Economics, Thailand). In the past, the black tiger shrimp, *Penaeus monodon*, is a main species of shrimp exported product in Thailand. However, the lacking development and scanty of breeders of the black tiger shrimp is resulting in small size, slow growth, high costs of culture and liable to infect by pathogen. These leads to only 1% of black tiger shrimp culturing in present. So, if researcher can find way to success in breed development and increase immune to pathogens in black tiger shrimp, farmers will turn to produce black tiger shrimp more than present. The one of the success keys is studying and understanding in black tiger shrimp immune response.

2.2 RNA interference (RNAi)

Since, the RNAi has been discovery in *Caenorhabditis elegans* in 1998 (Fire et al., 1998). Many reports showed RNAi pathway in various organisms such as plant (Gazzani et al., 2004), insects (Ober et al., 2006) including penaeid shrimp (Robalino et al., 2004). The RNAi pathway has known as post transcriptional silencing but showed the different mechanism in various organisms (Meister and Tuschl, 2004).The purposed of RNAi mechanism in marine invertebrate was based on *D. melanogaster*. The mechanism was started with when dsRNA was transferred into cell, long dsRNA were processed into short interfering RNA (siRNA) by dicer enzyme and siRNA will be assembled to protein into RNA induce silencing complex (RISC). Then, siRNA will be unbound and single-stranded siRNA will be complement to targeted mRNA. These resulted in mRNA degradation by RNase activity in RISC. However, the complete mechanism for RNAi has not been elucidated (Kao and Megraw, 2004).

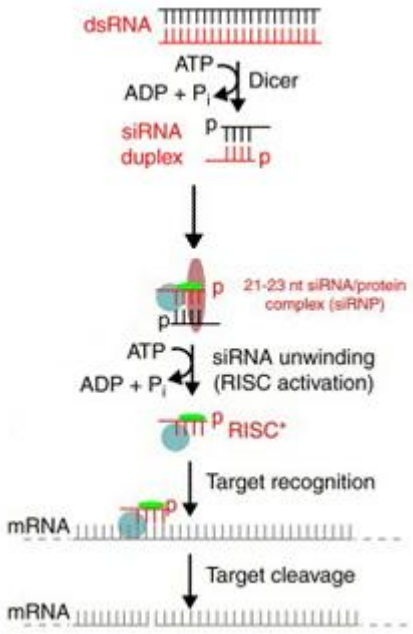


Figure 2.1 Mechanism of RNAi in *D. melanogaster* (modified from Hutvágner and Zamore, 2002).

2.3 Toll receptor

The gene encoding Toll was first discovered in *Drosophila* in 1980s (Rock et al., 1998). Toll receptor is a transmembrane receptor which composes of 3 domains; extracellular domain, transmembrane domain and the intracellular (Toll /interleukin I) domain. The extracellular domain has a horseshoe shape of Leucine-rich repeated series where the ligand was interacted while the intracellular (Toll /interleukin I) domain has roles in intracellular signaling. To activate Toll, Spätzle which is recognized by Toll receptor as a ligand was enzymatically cleaved to active form. Then, the interaction between extracellular of toll receptor and Spätzle are lead to intracellular signaling cascade. In intracellular, the Toll/interleukin (Toll/IL) receptor domain will be bound to 3 proteins, Myeloid differentiation factor 88 (MyD88), Tube and Pelle. The result of intracellular signaling cascade by toll receptor is activation of two proteins, which related to NF-κB proteins in immune responsive tissue [Dorsal and Dorsal-related immunity factor (DIF)] (Hoffmann, 2003). The effect of signal transduction is the phosphorylation and degrading of Cactus, resulting in dissociation of Dorsal/DIF from Cactus. Then, Cactus will be traslocated to nucleus and lead to activation of several sets of target genes (Hoffmann, 2003) see in **Figure 2.2**.

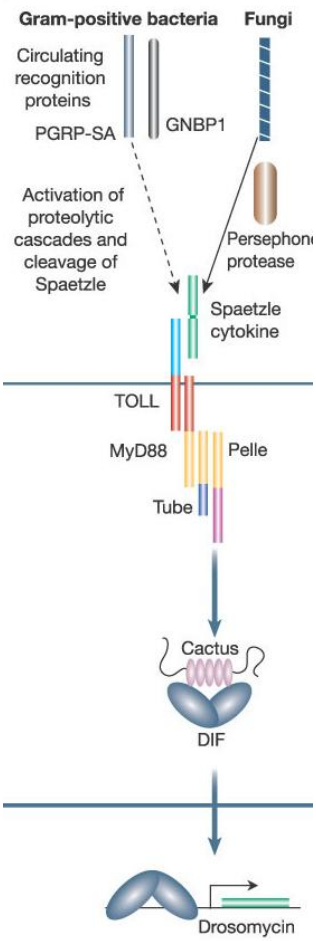


Figure 2.2 The signal transduction of Toll Pathway in *D. melanogaster* (Hoffmann, 2003).

Recently, many penaeid tolls from *Penaeus monodon* (Arts et al., 2007), *Litopenaeus vannamei* (Yang L-S. et al., 2007; Wang et al., 2012), *Marsupenaeus japonicus* (Mekata et al., 2008) and *Fenneropenaeus chinensis* (Yang C. et al., 2008) have been discovered and characterized of their function. However, the full-length cDNA of Toll receptor from black tiger shrimp has not been reported. Therefore, the objectives of this project are to clone a full-length toll receptor from *P. monodon* and to characterize its function.

CHAPTER III

MATERIALS AND METHODS

3.1 Microorganisms

Table 3.1 List of microoraganisms which use in research

Bacteria	Genotype
Escherichia coli DH5α	*sup*E44 ΔlacU169 (Φ80 ΔM15 *lac*Z) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1
Escherichia coli HT115	F⁻, *mcr*A, *mcr*B, IN*(rrn*D-*rrn*E*)1, lambda-, rnc14::Tn10(DE3 lysogen:lacUV5 promoter-T7 polymerase)*

3.2 Plasmid

Plasmid which use in research was showed in **Figure 3.1** and **Figure 3.2**

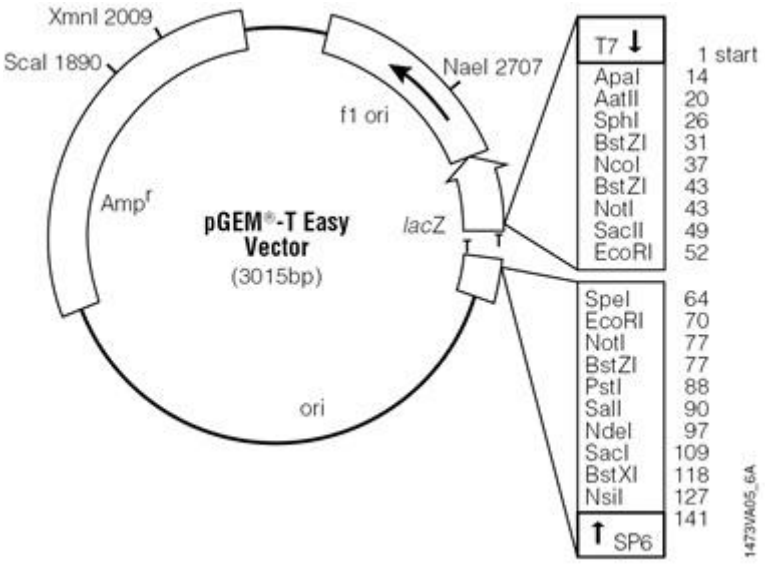


Figure 3.1 Plasmid pGEM®-T Easy vector (Promega)

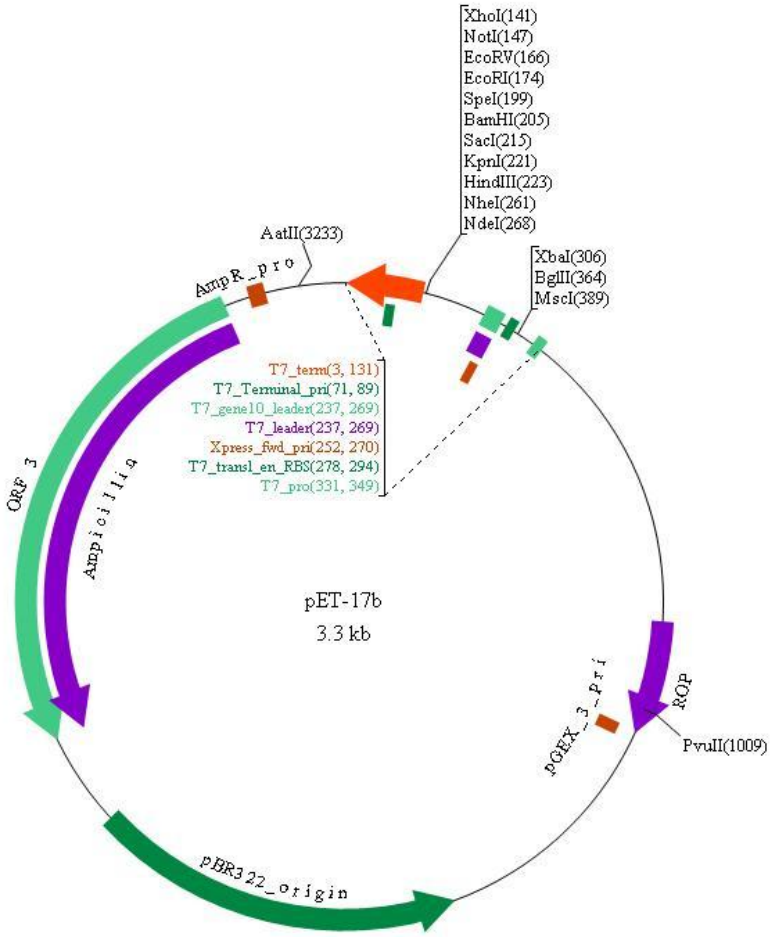


Figure 3.2 pET17b vector (Stratagene)

3.3 Restriction enzymes

Table 3.2 Restriction enzymes

Restriction Enzyme	Restriction site (5'-3')	Buffer	Optimal temperature (°C)
Eco RI	G^AATTC	*Eco* RI	37
Xba I	T^CTAGA	Tango	37
Xho I	C^TCGAG	R	37

Eco RI Buffer : 50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl, 0.02 % Triton X-100, 0.1 mg/ml BSA

Tango Buffer : 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM Magnesium acetate, 66 mM Potassium acetate, 0.1 mg/ml BSA

R Buffer : 10 mM Tris HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA

3.4 RNA extraction

Several shrimp organs were collected and then were extracted total RNA by using Tri-reagent® (Molecular Research Center) according to the manufacturer's instructions. First, 1 ml of Tri-reagent® (Molecular Research Center) was added to 50-100 mg of tissue in a nuclease-free microcentrifuge tube and homogenized. Then, standed for 5 min at room temperature and 200 µl of chloroform was added. The mixture was mixed vigorously by vortex mixer for 10 sec. The mixture was stored at room temperature for 5 min and phase separation was performed by centrifugation at 4 °C 13,000 rpm for 20 min. The aqueous phase was transferred to a new nuclease-free microcentrifuge tube and then, an equal-volume of isopropanol was added. The tube was inverted the tube several time and incubated at -20 °C for 20 min. To recover RNA pellet, the solution was centrifuged 4 °C 13,000 rpm for 15 min. RNA pellet was kept and washed with 1 ml of 75% ethanol, centrifuged 4 °C 8,000 rpm for 5 min and then, removed the ethanol wash and briefly air-dry the RNA pellet for 3 - 5 min. RNA pellet was solubilized with DEPC-treated water and kept at -80 °C until use.

3.5 Rapid Amplification of cDNA End (RACE)

3.5.1 3'- Rapid Amplification of cDNA End (3'-RACE)

3.5.1.1 Reverse transcription

Total RNA from heart was used as template to generate first strand cDNA. First, 2 µg of total RNA was mixed with 0.5 µg PRT [oligo (dT) plus PM1 linker] (Table 3.3) for final volume of 10 µl. The reaction mixture was heated at 70 °C for 5 min, and then rapidly chilled on ice for 3 min. Then, the following components were add to the mixture to a final concentration of 1X revertaid® M-MuLV reverse transcriptase buffer (Fermentas), 0.5 mM dNTP and DEPC-treated water were added to the mixture to a final volume of 19 µl, then incubated at 37 °C for 5 min. Then, 200 unit of revertaid® M-MuLV reverse transcriptase (Fermentas) was added and gently mixed. The reaction mixture was incubated at 42 °C for 90 min. To inactivate the enzyme, the reaction mixture was incubated at 70 °C for 10 min.

3.5.1.2 Amplification of 3'-RACE Toll DNA fragments by polymerase chain reaction (PCR)

The first strand cDNA from 3.5.1.1 was use as template for PCR amplification. PCR was subsequently performed by PM1 adaptor primer and Toll_RDW primer (Table 3.3). The reaction mixture composed of 1 µl of cDNA, 1X *Taq* polymerase buffer plus ammonium sulfate (Fermentas), 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTP, 2.5 unit of recombinant *Taq* DNA polymerase (Fermentas). The first cycle for amplification is denaturation at 94 °C for 4 min, annealing at 55 °C for 30 sec and extension at 72 °C for 1.5 min. After the initial cycle, the amplification was performed with 35 cycles each of denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1.5 min. The last PCR cycle was follow by final extension at 72 °C for 10 min. PCR product was analyzed by agarose gel electrophoresis.

Table 3.3 List of primers use in 3'-RACE

Primer Name	**sequence 5'-3'**
PRT	*Eco* RI *Xba* I *Bam* HI 5'-CCG **GAA TTC** AAG CT**T CTA GAG GAT CC**T TTT TTT TTT TTT TTT TT-3'
PM1	*Eco* RI *Xba* I *Bam* HI 5'-CCG **GAA TTC** AAG CT**T CTA GAG GAT CC**T T-3'
Toll_RDW	5'-TGC CTT CAC TAC CGC GAC TGG-3'

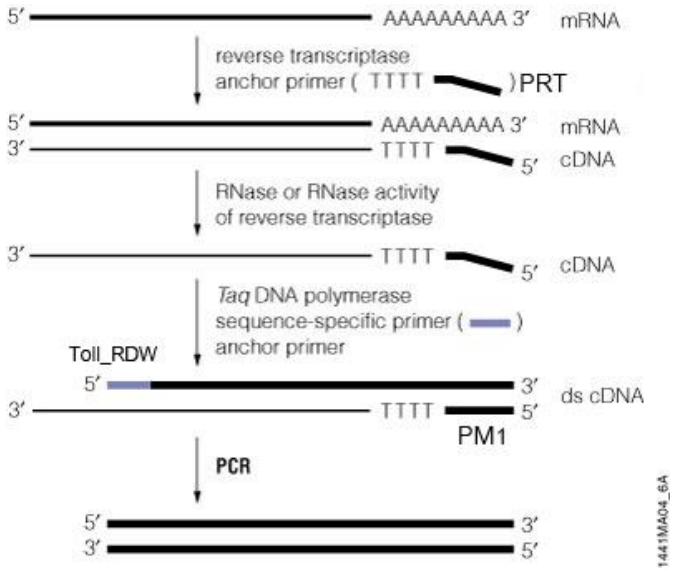


Figure 3.3 3'-Rapid amplification of cDNA end. A Schematic of 3' end amplification of Toll receptor cDNA by 3'-RACE. PRT primer was used to synthesize cDNA. PM1 Toll_RDW primers were used to amplify DNA fragment. (http://www.promega.com/ resources/product-guides-and-selectors/protocols-and-applications-guide/pcr amplification/).

3.5.1.3 Purification of DNA fragment using Geneaid® gel/PCR extraction kit

DNA fragment was extracted using Geneaid® gel/PCR extraction kit followed the instruction's manual. First, the desired DNA fragment was fractionated by agarose gel electrophoresis and excised from the gel with a clean blade. The gel slice was weighted and 500 µl of DF buffer per 300 mg of gel weight was added in a microcentrifuge tube. The tube was incubated at 55 °C until gel slice has completely dissolved, inverted the tube every 2 or 3 min during incubation. The mixture was applied to a DF column placed

in 2 ml collection tube and centrifuged at 13,000 rpm for 1 min at room temperature. The flow-through solution was discarded and the DF column was replaced in the same collection tube. Then, 400 µl of W1 buffer was added and followed by centrifugation at the same speed for 1 min. After that, the column was washed with 600 µl of wash buffer and centrifuged at 13,000 rpm for 1 min. After discarding the flow-through, the column was centrifuged for an additional 3 min to dry the column matrix. The column was placed into a clean 1.5- microcentrifuge tube. Finally, the DNA was eluted by addition of 30-50 µl of elution buffer or sterile milli-Q water. The column was left stand at room temperature for 5 min, and then centrifuged at 13,000 rpm for 3 min to collect the DNA solution.

3.5.1.4 Ligation of DNA fragment to pGEM-T® easy vector

PCR product, which was amplified by *Taq* DNA polymerase, frequently resulted in the addition of extra deoxyadenines at the 3'-end was direct cloned to pGEM-T® easy vector (Promega), which was contain a single compatible deoxythymidine overhang. The ligation reaction was carried out in 10 µl reaction mixture containing purified DNA fragment and pGEM-T® easy vector in the insert: vector molar ratio of 3:1, 400 unit of T4 DNA ligase (New England BioLabs), 1X T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP) and nuclease-free water was added to final volume. The reaction was mixed gently, centrifuged briefly and then, incubated at 16 °C for overnight.

3.5.1.5 Preparation of competent cells by simple and efficient method (SEM) (Inoue *et al*, 1990).

A single colony of *E. coli* DH5α was inoculated into 250 ml of SOB medium, pH 7.0 [2% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$] in a 1 L flask. Cells were incubated at 16 °C with vigorous shaking until the OD_{600} reached 0.4-0.6. The culture was placed on ice for 10 min before being transferred to a 250 ml centrifuge bottle and centrifuged at 4,000 rpm, 4 °C for 10 min. The cell pellet was resuspended in 80 ml of ice-cold transformation buffer (TB) [10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$ and 250 mM KCl], incubated on ice for 10 min and centrifuged at 4,000 rpm, 4 °C for 10 min. The cells were resuspended in 10 mL ice-cold TB. Then, dimethyl sulfoxide (DMSO) was slowly added with gently swirling to give a 7% (v/v) final concentration and the culture was incubated on ice for another 10 min. Finally, the cell suspension was immediately dispensed into 150 µl aliquoted into 1.5-ml microcentrifuge tubes, snap-frozen in liquid nitrogen and stored at -80 °C.

3.5.1.6 Transformation to *E. coli* competent cells

A 150 µl of competent *E. coli* was thawed and then, mixed with 10 µl of ligation reaction. The mixture was incubated on ice for 30 min. After that, the mixture was incubated at 42 °C for exactly for 90 sec and rapidly chilled on ice for 3 min. Then, 850 µl of SOC (SOB plus 20 mM glucose) medium or LB medium was added and cultured at 37 °C for 1 h. The transformed cells were then spread on selectable LB agar plate.

In case of blue-white color selection, 40 µl of 20 mg/ml X-gal solution and 10 µl of 0.4 M IPTG were spread on selectable marker LB agar plates prior to spread the transformed cells.

3.5.1.7 Screening recombinant clones using simplified rapid size screening

A single colony of each recombinant clone was picked and lysed in 25 µl of pre-warmed lysis buffer [100mM NaOH, 60 mM KCl, 5 mM EDTA, 10% (w/v) sucrose and 0.05% bromphenol blue]. The reaction was incubated at 37 °C for 5 min, followed by chilling on ice for 5 min and centrifugation at 13,000 rpm for 5 min at room temperature. After that, 20 µl of upper phase was examined on agarose gel electrophoresis.

3.5.1.8 Plasmid DNA extraction using Geneaid® High-Speed Plasmid mini kit

The overnight bacterial culture was collected by centrifugation at 13,000 rpm for 1 min at room temperature, and the supernatant was discarded. The cell pellet was resuspended in 200 µl of PD1 buffer and then added with 200 µl of PD2 buffer. The mixture was gently mixed by inverting the tube for 10 times and stored at room temperature until the lysate is clear. A volume of 300 µl of PD3 buffer was added and mixed immediately by inverting the tube for 10 times, then centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant was transferred in to PD column in a 2 ml collection tube and centrifuge at 13,000 rpm for 1 min. The flow-through was discarded, and then 400 µl of W1 buffer was added and washed with 600 µl of wash buffer, respectively. The flow-through of each solution was discarded. The PD column was centrifuged for additional at 13,000 rpm for 3 min to dry column matrix. After that, PD column was transferred to a nuclease-free microcentrifuge tube, the plasmid DNA was eluted by adding 35-50 µl of elution buffer or sterile milli-Q water, left standing for 5 min and followed by centrifugation at 13,000 rpm for 3 min to collect DNA solution.

3.5.1.9 Automated DNA sequencing and sequences analysis

Recombinant plasmids were sent to 1st BASE Sequencing Unit (Malysia). The sequencing data from all selected clones were compared against the nucleotide and protein sequences in all databases using BLAST program Multiple sequence alignment and construction of phylogenetic tree were performed using the Vector NTI 9.0.0, ClustalX2, Genedoc and MEGA4 program.

3.5.2 5'- Rapid amplification of cDNA end (5'-RACE)

3.5.2.1 First 5'-RACE

First, 2 µg of total RNA and 2 µl of 10 µM GSP1 primer were added into sterile, nuclease-free tube, then, reverse transcription was performed same as 3'-RACE method. cDNA was purified using Geneaid® gel/PCR extraction kit. cDNA was used as template for PCR reaction by using GSP1 and TollPMF1 as primers [Table 3.4]. The reaction mixture composed of 1 µl of cDNA, 1X *Taq* polymerase (Fermentas) buffer plus ammonium sulfate, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTP, 2.5 unit of recombinant *Taq* DNA polymerase (Fermentas). The first cycle for amplification is denaturation at 94 °C for 4 min, annealing at 50 °C for 30 sec and extension at 72 °C for 1.5 min. After the initial cycle, the amplification was performed with 35 cycles each of denaturation at 94 °C for 45 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1.5 min. The last PCR cycle was follow by final extension at 72 °C for 10 min. Then, 1st PCR product was use as template to confirm the expected fragment using semi-nested PCR, GSP2 and PMF1 were used as primers. The thermal cycling was used same as 1st amplification but change annealing step to temperature 55 °C. The expected fragment was extracted using Geneaid® gel/PCR extraction kit and cloned into pGEM-T easy® vector (Promega).

Table 3.4 List of primers use in 5'-RACE (second fragment)

Primer Name	sequence 5'-3'
TollPMF1	5'-AGT GTA CCT GAA GAC CTC TT-3'
GPS1	5'-AGC CTG GGA GTG AGC TGC C-3'
GSP2	5'-CGG CTG TCC TCT ACA CTC TGC-3'

3.5.2.2 Second 5'-RACE

To find 5'-end of Toll receptor sequence, Toll_gen_cDNA [Table 3.5] was used as specific primer for generation cDNA and then, cDNA was purified using Geneaid® gel/PCR extraction kit using PCR clean up protocol. Purified cDNA was added oligo-dC using terminal nucleotidyl transferase (TdT). The reaction composed of 250 ng of purified cDNA, 1x reaction buffer (500 mM Potassium cacodylate, 25 mM Tris-HCl and 0.25 mg/ml of BSA), 2.5 mM $CoCl_2$, 5 µM dCTP and 400 unit of terminal deoxynucleotidyl transferase, TdTase (Roche applied science), the mixture was incubated at 37 °C for 1 h. The reaction was stop by placed on heat block at 80 °C for 10 min.

The 1st PCR amplification was performed, GSP3 and PRC [oligo (dG) plus PM1 primer] [Table 3.5] was used as primer. The reaction mixture composed of 1 µl of modified cDNA, 1X *Taq* polymerase (Fermentas) buffer plus ammonium sulfate, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTP, 2.5 unit of recombinant *Taq* DNA polymerase (Fermentas). The first cycle for amplification is denaturation at 94 °C for 4 min, annealing at 50 °C for 30 sec and extension at 72 °C for 1.5 min. After the initial cycle, the amplification was performed with 35 cycles each of denaturation at 94 °C for 45 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1.5 min. The last PCR cycle was follow by final extension at 72 °C for 10 min. Then, the 1st PCR product was diluted and used as template to perform 2nd PCR amplification for confermation expected fragment by using GSP4 and PM1 as primers (Figure 3.2) [Table 3.5]. The expected fragment was extracted using Geneaid® gel/PCR extraction kit and cloned into pGEM-T easy® (Promega) vector.

Table 3.5 List of primers use in 5'-RACE (third fragment)

Primer Name	**sequence 5'-3'**
PRC	*Eco* RI *Xba* I *Bam* HI 5'-CCG **GAA TTC** AAG CT**T CTA GAG GAT CC**T TGG GGG GGG GGG GGG GG-3'
Toll_gen_cDNA	5'-GAG TTC TTC CAA GCT CCT GAG ATC-3'
GSP3	5'-GCC TAT TTG TGA TGT CAC TC-3'
GSP4	5'-GCG AAG AGG TCT TCA GGT ACA CT-3'

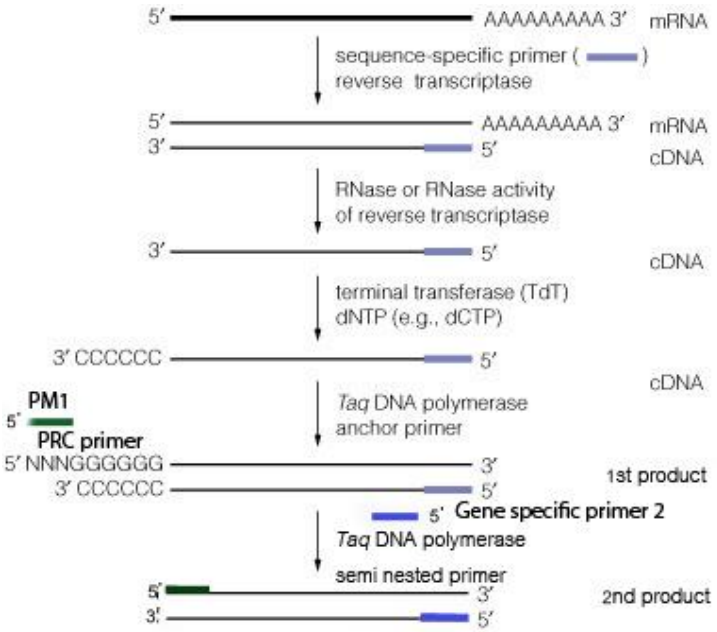


Figure 3.4 5'- Rapid amplification of cDNA end. Oligo-dG plus adaptor primer and GSP1 primers were used for first amplification. Then, PM1 and GSP2 primers were used for nested amplification.

3.6 Construction of Toll double-stranded RNA (dsRNA) plasmid

DNA fragment was amplified using dsToll-F1 and dsToll-R1 (Table 3.6) for sense strand and dsToll-F2 and dsToll-R2 (Table 3.6) for anti-sense strand each of reaction compose of 1X ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ and 0.1% Triton X-100), 0.2 µM of each primer, 0.2 mM dNTP and 1 unit of *Vent*$_R$® DNA polymerase (New England Biolabs). The first cycle for amplification is denaturation at 94 °C for 4 min, annealing at 55 °C for 30 sec and extension at 72 °C for 45 sec. After the initial cycle, the amplification was performed with 35 cycles each of denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 45 sec. The last PCR cycle was follow by final extension at 72 °C for 10 min. Then, PCR products were analyzed on agarose gel electrophoresis, expected fragment was sliced from gel and extracted using Geneaid® gel/PCR extraction kit.

Each 2 µg sense fragment was digested with *Xba* I and *Xho* I (Fermentas), same as pET17b vector (Figure 3.4). The *Xba* I digestion reaction composed of 1X Tango™ buffer [33 mM Tris-acetate (pH 7.5 at 37°C), 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA] and 10 units of enzyme and the *Xho* I digestion reaction composed of 1X Buffer R [10 mM Tris HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA] and 10 units of enzyme. The reaction mixture was incubated at 37 °C for 16 hours. Then, DNA fragment was purified, ligated into pET17b vector (Stratagene) and transformed into *E. coli* DH5α. Recombinant plasmids were extracted and sequencing.

To integrate the anti-sense, the anti-sense fragment and recombinant plasmid were digested with *Xho* I and *Eco* RI. The *Xho* I digestion reaction composed of 1X Buffer R [10 mM Tris HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA] and 10 units of enzyme and The *Eco* RI digestion reaction composed of 1X *Eco*RI buffer [50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl, 0.02% Triton X-100 and 0.1 mg/ml BSA] and 10 units of enzyme. The reaction mixture was incubated at 37 °C for 16 hours. Then, DNA fragment was purified and ligated into pET17b vector. The recombinant plasmid was transformed into *E. coli* DH5α plasmids were extracted, linearized using *Eco* RI and sequencing.

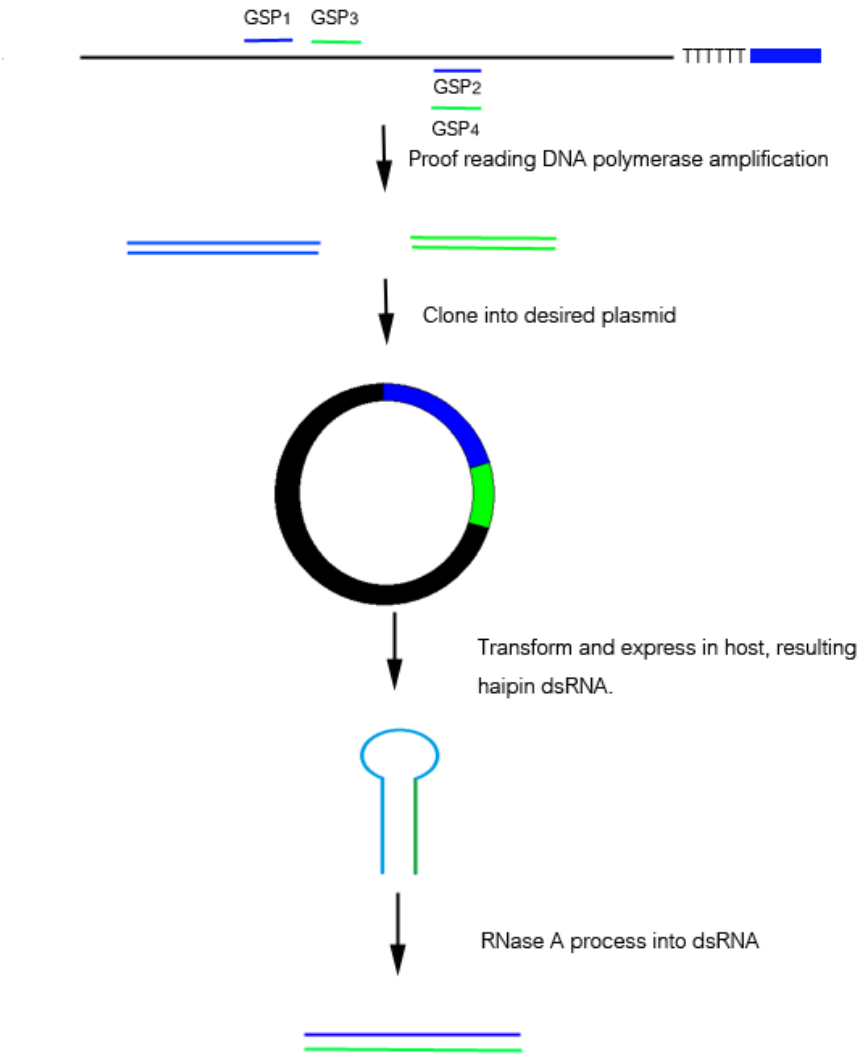


Figure 3.5 Schematic of construction dsRNA. Gene specific primers including restriction site were used to amplify sense and anti-sense DNA fragment and clone into vector for production of dsRNA.

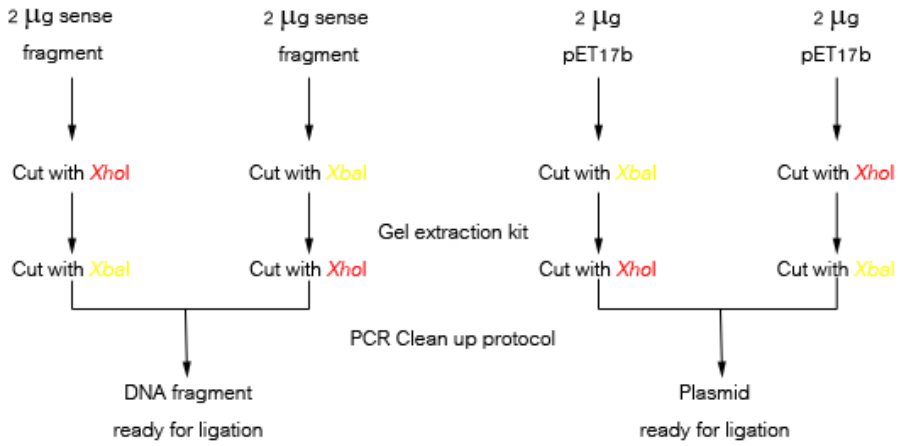


Figure 3.6 Restriction digestion step before ligation. This scheme showed how to construct plasmid which expressed dsRNA.

Table 3.6 List of primers use in construction of dsRNA

Primer Name	**sequence 5'-3'**
dsToll-F1	*Xba* I 5'-GGG G**TC TAG A**GA TCT GAA AAC TGA C-3'
dsToll-R1	*Xho* I *Eco* RI 5'-GGG G**CT CGA G**GG GG**G AAT TC**C TTT TCC TGA ACA ATC TTT GC-3'
dsToll-F2	*Xho* I 5'-GGG G**CT CGA G**GA TCT GAA AAC CAA TGA C-3'
dsToll-R2	*Eco* RI 5'-GGG G**GA ATT C**TC CCT CAA GTG ACA ATG-3'

3.7 Expression of dsRNA

The dedicated plasmid (dsToll and dsGFP) was transformed into *E. coli* HT115. For expression of dsRNA, the starter was prepared by inoculated single colony in Luria-Bertani broth, supplemented with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline cultured for 16 h. The starter (0.1 OD_{600}/15ml) was added to new culture medium at 0.1 OD_{600}, culture until OD_{600} reached to 0.4 then isopropyl β-D-1 thiogalactopyranoside (IPTG) was added to final concentration of 0.4 mM, additional cultured for 3 hr, then cells were harvested by centrifugation. dsRNA was extracted using Tri-reagent® (Molecular Research Center).

In extraction step, 100 µl of 0.1% SDS-PBS were added to 10 OD_{600} of cultured, then boiled for 2 min. After that, 26 µl of 5X RNaseA buffer (300 mM sodium acetate, 10 mM Tris–HCl, pH 8.0) and 10 µg of RNaseA were added and incubated at 37 °C for 1.5 hours, followed by RNA extraction using 1 ml of Tri-reagent® (Molecular Research Center). RNA pellets were solubilized in 150 mM NaCl.

3.8 Injection of dsRNA for knock-down expression of Toll receptor.

The different sized (1-5 grams) of shrimps (*Penaeus monodon*) were injected with (2.5 or 5 µg/g shrimp) dsRNA (Toll and GFP) and 150 mM NaCl and then, shrimps were collected in various intervals (24 hours interval for 5 days; 12 hours intervals for 3 days and 6 hours interval for 1.5 days).

Gills were isolated to extract total RNA using Tri-reagent® (Molecular Research Center) (Method 3.1). One microgram of total RNA was used as template, and then converted to cDNA by using Revertaid® M-MuLV reverse transcriptase (Fermentas) and oligo-dT as primer (Table 3.7). Then, 1 µl of cDNA was use as template for PCR amplification. The reaction mixture composed of 1X *Taq* polymerase buffer plus ammonium sulfate (Fermentas), 2 mM $MgCl_2$, 0.1 µM of Toll-F and Toll-R, 0.02 µM of Actin-F and Actin-R, 0.2 mM dNTP, 2.5 unit of recombinant *Taq* DNA polymerase (Fermentas). The first cycle for amplification is denaturation at 94 °C for 4 min, annealing at 55 °C for 30 sec and extension at 72 °C for 45 sec. After the initial cycle, the amplification was performed with 30 cycles each of denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 45 sec. The last PCR cycle was

follow by final extension at 72 °C for 7 min. PCR reactions were analyzed on 1.8% agarose gel electrophoresis.

Table 3.7 List of primer which used in density analysis

Primer Name	sequence 5'-3'
Oligo-dT	5'-TT TTT TTT TTT TTT TT-3'
Toll-F	5'-GTC CAA TCA GTT GGA GCT GC-3'
Toll-R	5'-GAA ATC GAG CGT CTT CAC ATG C-3'
Actin-F	5'-GAC TCG TAC GTG GGC GAC GAG G-3'
Actin-R	5'-AGC AGC GGT GGT CAT CTC CTG CT-3'

CHAPTER IV

RESULTS

4.1 Total RNA extraction

Total RNA extracted from gill and several organs were analyzed on agarose gel electrophoresis. The results in **Figure 4.1** showed intact band at approximately 1.8 kb and 1.2 kb. The absorbance ratio (A_{260}/A_{280}) of the RNA sample was in the range of 1.8-2.0, indicating the purity of the RNA samples.

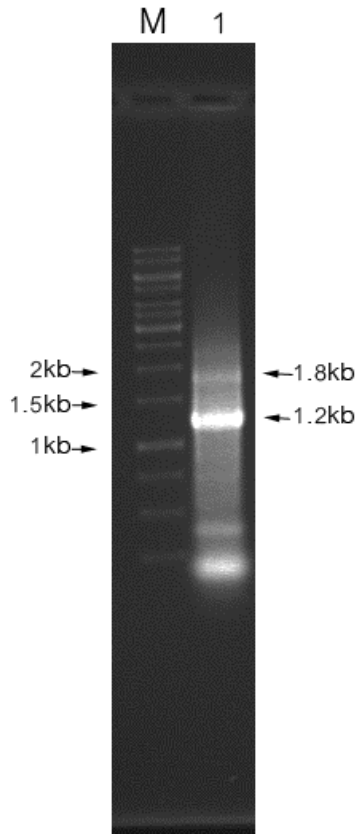


Figure 4.1 Total RNA from gill of *P. monodon*. Total RNA was extracted from gills of *P. monodon* and analyzed on 1% agarose in 1X TAE buffer.

Lane M : 1 kb ladder marker.

Lane 1 : total RNA from gill.

4.2 Rapid amplification of cDNA ends (RACE)

4.2.1 Amplification of the 3' end cDNA by 3'-RACE

The 3' end fragment of *P. monodon* Toll receptor (PmToll) cDNA was amplified. First, total RNA from heart was used for first strand cDNA synthesis with PRT primer (oligo-dT plus PM1 linker). The cDNA was used as the template for amplify with PM1 primer and degenerated (Toll_RDW) primer. The PCR product of 3'-RACE was analyzed on 0.8% agarose gel electrophoresis and revealed the band approximately 1,400 bp (data not showed). This fragment was purified using Geneaid® gel/PCR extraction kit and cloned into pGEM-T® easy vector. The recombinant clones were verified with *Eco* RI digestion and the desired clones were chosen for DNA sequencing. Sequence analysis show in **Figure 4.2**, the fragment about 1,410 bp (included PM1 primer) and found some variation nucleotides among them. The sequences were compared to Genbank database using tBLASTx program, the result showed translate nucleotide shared homology to *Litopenaeus vannamei* Toll receptor (LvToll). So, specific primer (GSP1) was designed from 5' upstream of sequence to performed 5'-RACE experiments.

Figure 4.2 Alignment of 3' end fragment sequence. Alignment of 3' end fragment of partial PmToll cDNA from clone 12, clone 21, clone 24, clone 44 and clone 45 were aligned using ClustalX2 software. Black highlight indicates identical nucleotides among the four sequences. Nucleotides primers are boxed.

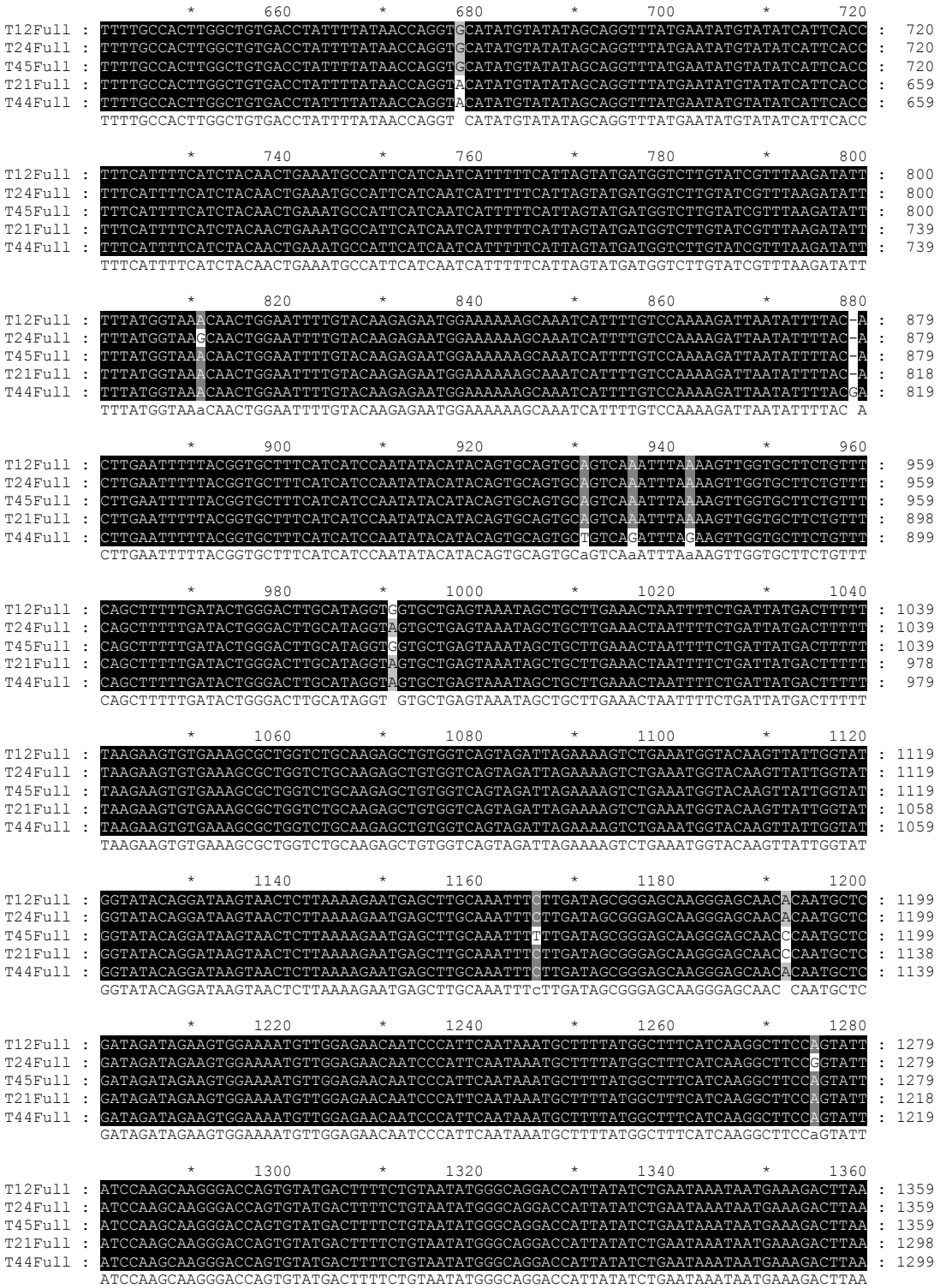


Figure 4.2 Alignment of 3' end fragment sequence (cont.).

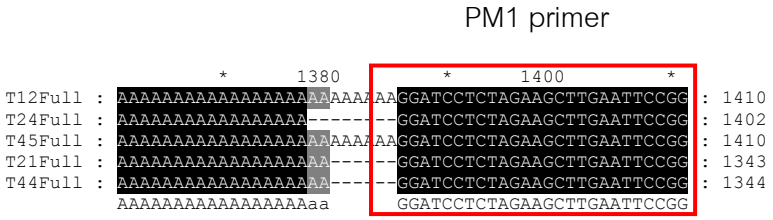
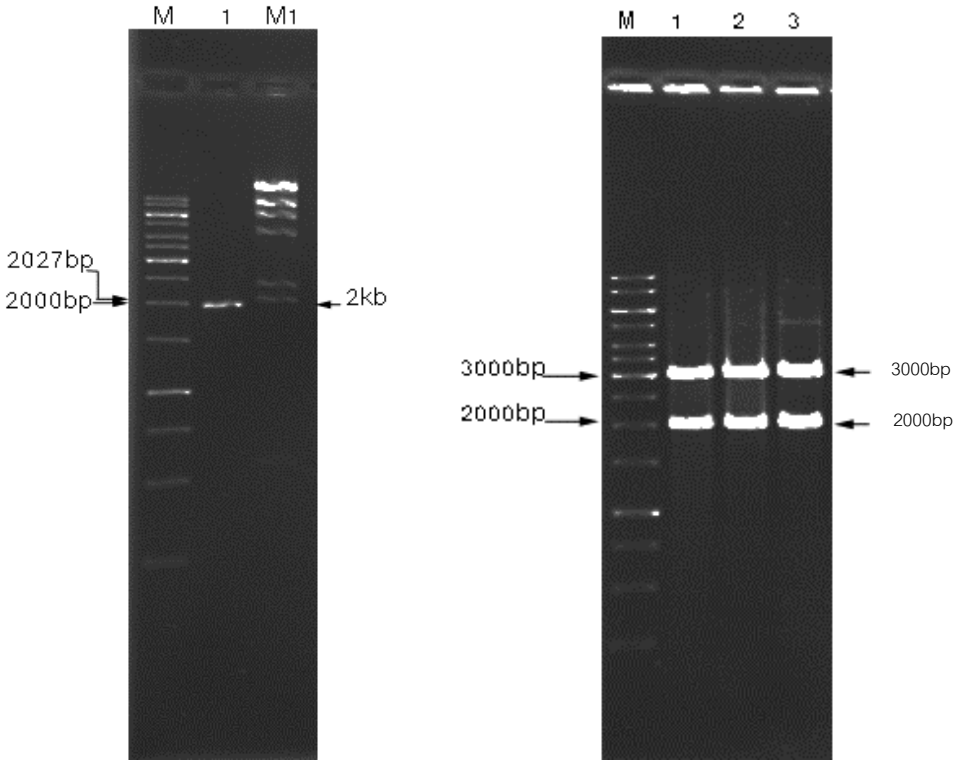


Figure 4.2 Alignment of 3' end fragment sequence (cont.).

4.2.2 Amplification and analysis 5'-RACE product

The second fragment of PmToll gene was obtained PCR amplification by using 2 specific primers. First, the cDNA was synthesized by using GSP1 primer, which designed from 3'-RACE nucleotide sequence. Then, PCR was performed by TollPMF1 and GSP1 primers. The result revealed band approximately 2,000 bp [**Figure 4.3(A)**]**.** Then, this fragment was cloned into pGEM-T® easy vector and verification by *Eco* RI digestion [**Figure 4.3(B)**]. The sequence analysis showed some nucleotide variations (**Figure 4.4**). The sequences analysis result showed the connection between 3'-RACE part and 5'-RACE (**Figure 4.5**) part and translated amino acids were inframe (**Figure 4.6**). This sequence also was analyzed by BLASTn, the result showed the high similarity to LvToll. So, the gene specific primers (Toll_gen_cDNA, GSP3 and GSP4) were designed to use in next experiment.



(A) (B)

Figure 4.3 Agarose gel electrophoresis of 5'-RACE (second fragment) of PmToll gene. The PCR product were excised from gel for purification and run on 0.8% gel electrophoresis (A) and verification of recombinant clones by digested with *Eco* RI (B).

(A) Lane M : 1 kb ladder (Fermentas)
Lane 1 : 2 kb 5'-RACE fragment
Lane M1 : λ/*Hin* dIII marker

(B) Lane M : 1 kb ladder
Lane 1 : Clone 3
Lane 2 : Clone 4
Lane 3 : Clone 5

Figure 4.4 Alignment of second fragment sequence. The second fragment PmToll receptor cDNA from clone 3, clone 4, clone 5 and clone 17 were aligned using ClustalX2 software. Black highlight indicates identical nucleotides among the four sequences. The nucleotide primers are boxed.

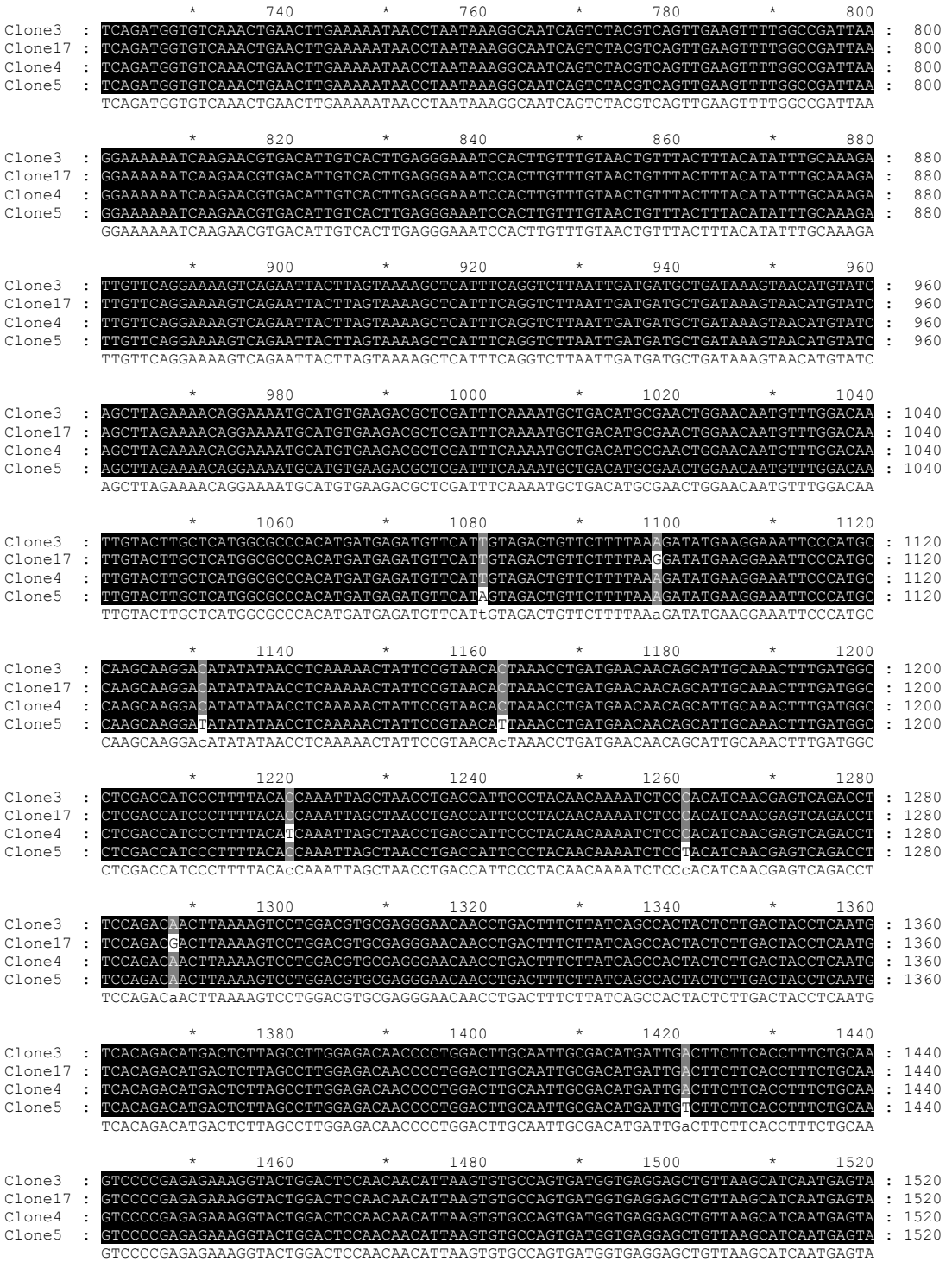


Figure 4.4 Alignment of second fragment sequence (Cont.).

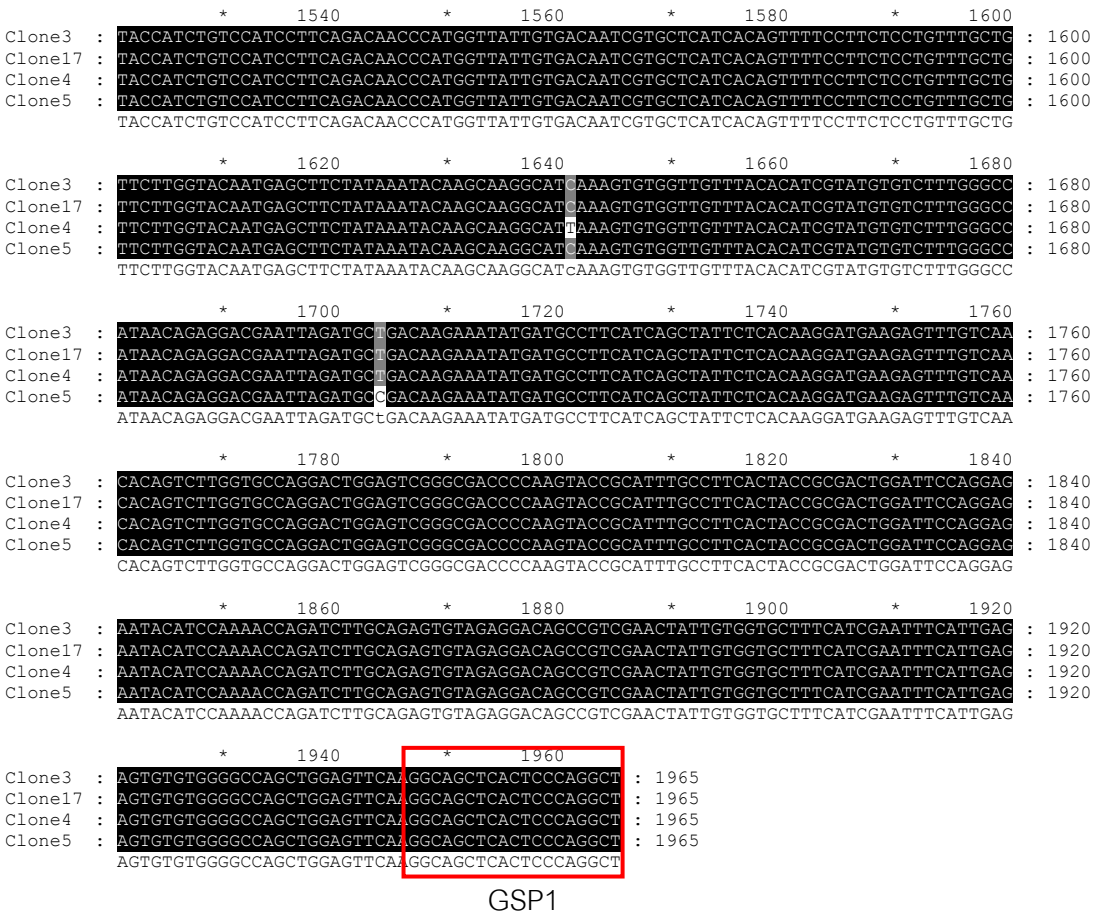


Figure 4.4 Alignment of second fragment sequence (Cont.).

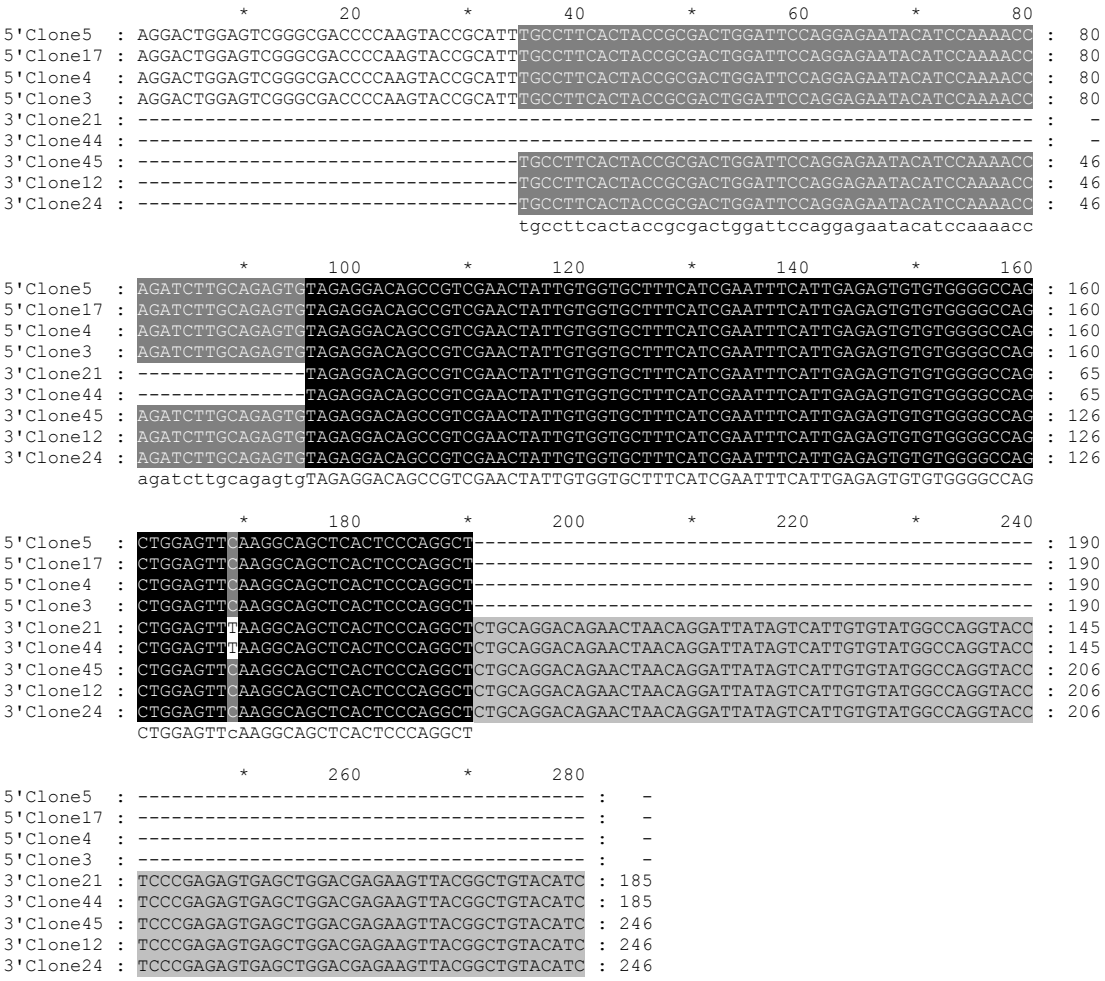


Figure 4.5 The overlapping sequence between 3'-RACE (first fragment) and 5'-RACE (second fragment) product. The chromatogram was used to assist for obtain the correct nucleotide which vary.

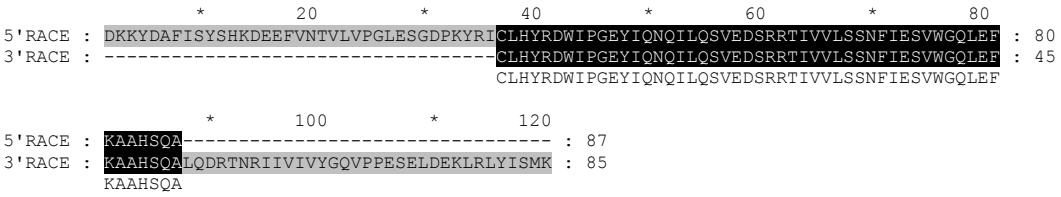
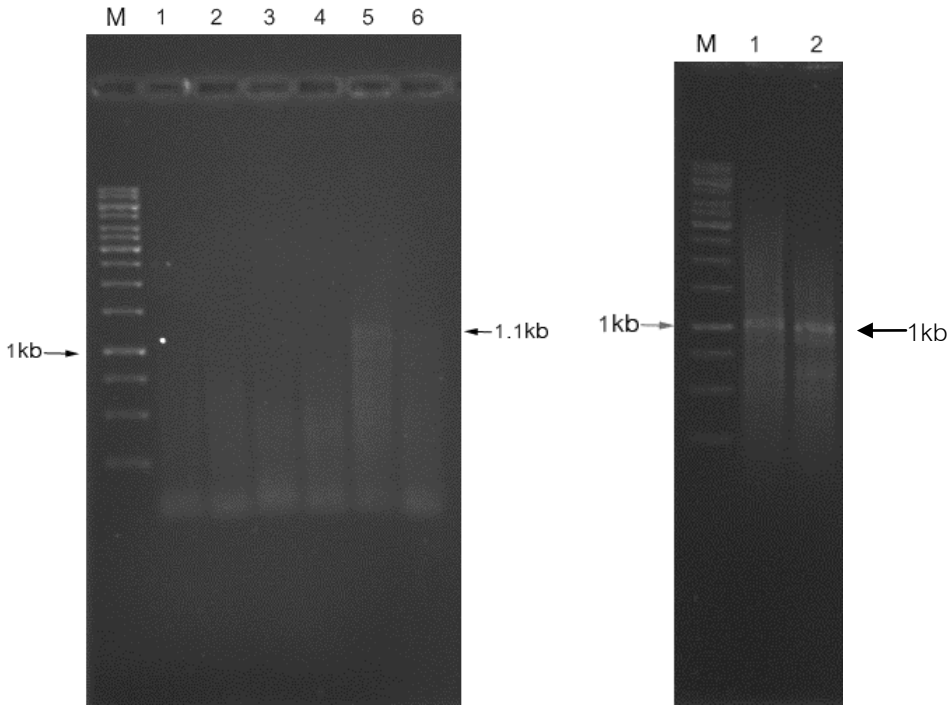


Figure 4.6 Translation of connection region. The inframe translation of 3'-RACE (first fragment) and 5'-RACE (second fragment).

4.2.3 Amplification and analysis of 5-end product using 5'-RACE

The position of start codon and 5'-UTR sequence were found in this experiment. The Toll_gen_cDNA [Table 3.5] primer was used to generate cDNA. The cDNA product was tailed with poly dC using TdTase. PCR reaction was performed by using PM1 and GSP3 primers. The result was shown in [**Figure 4.7 (A)**]. The band approximately 1.1 kb was found in heart from shrimps in lane 5 and 6. Then, the semi-nested PCR was performed to confirm this fragment. The band approximately 1 kb was appeared, which presented in [**Figure 4.7 (B)**]. This fragment was cloned into pGEM-T® easy vector and verified by *Eco* RI digestion (**Figure 4.8**). The result showed the insert approximately 1 kb was released from recombinant plasmid. The recombinant plasmids were sent for sequencing. The sequences alignment was show in **Figure 4.9**. BLASTn result showed high similarity to LvToll and the translated sequences were inframe with second fragment (**Figure 4.10**). Then, sequence was merged with second fragment (**Figure 4.11**).



(A) (B)

Figure 4.7 5'-RACE PCR product of PmToll. First and nested PCR (A and B respectively) product were analyzed on 1% agarose gel electrophoresis in 1X TAE buffer using 1kb ladder as the markers.

(A) Lane M : 1 kb ladder
Lane 1 : sample 1 from heart
Lane 2 : sample 2 from heart
Lane 3 : sample 3 from heart
Lane 4 : sample 4 from heart
Lane 5 : sample 5 from heart (use for semi-nested PCR)
Lane 6 : sample 6 from heart (use for semi-nested PCR)

(B) Lane M : 1 kb ladder
Lane 1 : semi-nested product from sample 5
Lane 2 : semi-nested product from sample 6

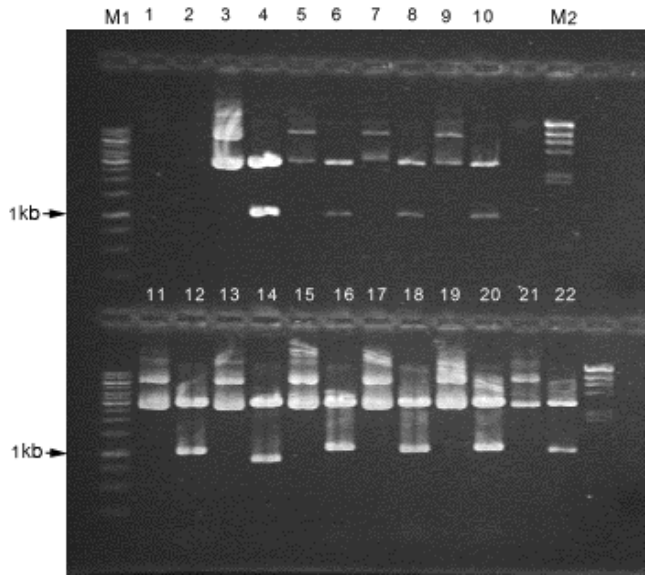


Figure 4.8 Verification of recombinant clones containing 5' cDNA end fragment of *P. monodon* Toll receptor using *Eco* RI. Recombinant plasmids (lane1, 3, 5…21) were extracted and digested with *Eco* RI (2, 4, 6…22), analyzed on 1% agarose gel electrophoresis. Lanes M1 and M2 are 1kb ladder and λ/*Hin*d III, respectively.

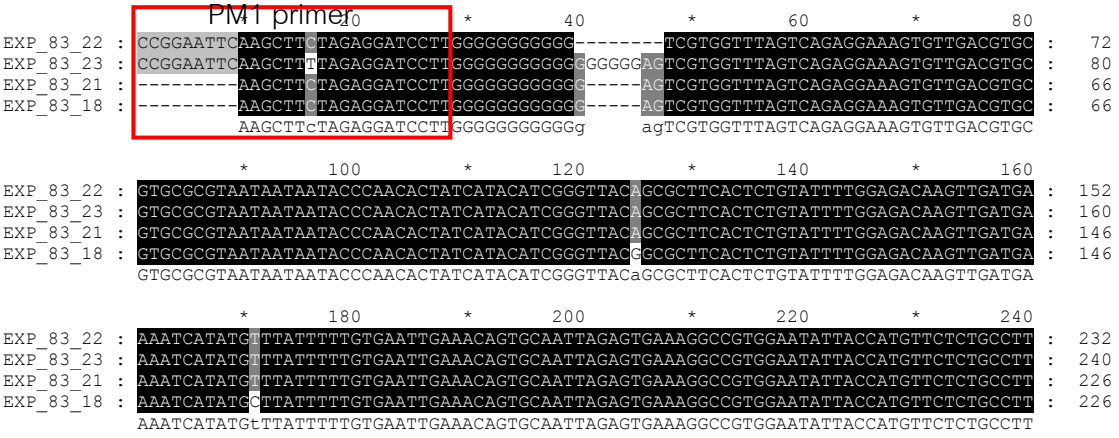


Figure 4.9 5'-RACE of 1 kb fragment sequence alignment. The 5'-RACE sequence of PmToll cDNA (third fragment) from clone 18, clone 21, clone 22 and clone 23 were aligned using ClustalX2 software. Black highlight indicates identical nucleotides among the four sequences.

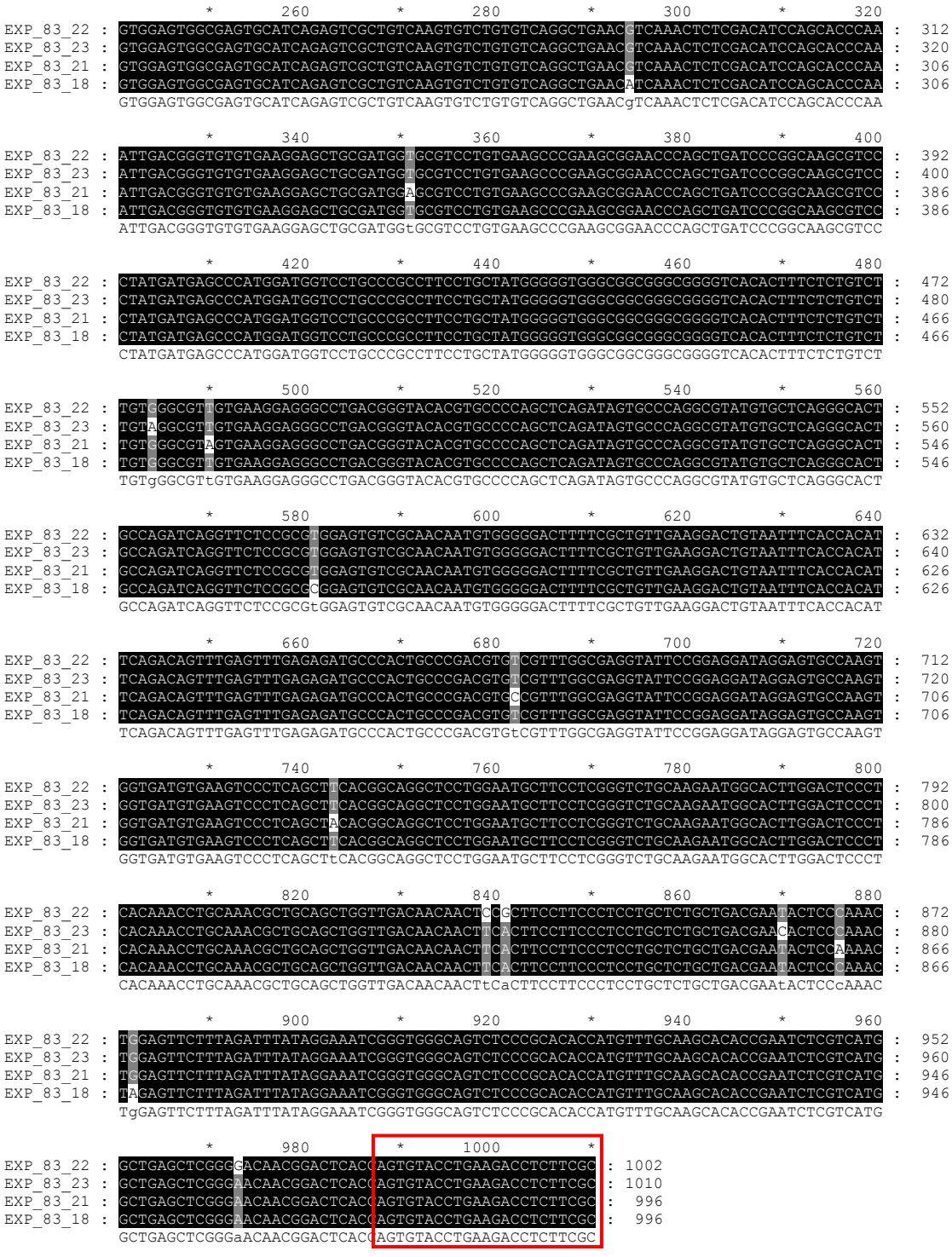


Figure 4.9 5'-RACE of 1 kb fragment sequence alignment (Cont).

```
                        *         20
5'END     : LGNNGLTSVPEDLF---------- : 14
5'Second  : -------SVPEDLFANLTKLLNVSL : 18
                    SVPEDLF
```

Figure 4.10 Translation of connection region. The alignment of translation of 5' end and 5' second fragment.

```
                    *        20         *        40         *        60         *
EXP_33_5  : ---------------------AGTGTACCTGAAGACCTCTTCGGCAACCTCACAAAGCTGCTCAATGTTAGTCTCTG : 56
EXP_33_17 : ---------------------AGTGTACCTGAAGACCTCTTCGGCAACCTCACAAAGCTGCTCAATGTTAGTCTCTG : 56
EXP_33_4  : ---------------------AGTGTACCTGAAGACCTCTTCGGCAACCTCACAAAGCTGCTCAATGTTAGTCTCTG : 56
EXP_33_3  : ---------------------AGTGTACCTGAAGACCTCTTCGGCAACCTCACAAAGCTGCTCAATGTTAGTCTCTG : 56
EXP_83_18 : CTCGGGAACAACGGACTCACCAGTGTACCTGAAGACCTCTTCGG---------------------------------- : 44
EXP_83_21 : CTCGGGAACAACGGACTCACCAGTGTACCTGAAGACCTCTTCGG---------------------------------- : 44
EXP_83_23 : CTCGGGAACAACGGACTCACCAGTGTACCTGAAGACCTCTTCGG---------------------------------- : 44
EXP_83_22 : CTCGGGGACAACGGACTCACCAGTGTACCTGAAGACCTCTTCGG---------------------------------- : 44
                                  AGTGTACCTGAAGACCTCTTCGC
```

Figure 4.11 Merging of 3'end from second fragment and 5'end sequence.

4.3 Full-length and amino acid sequence analysis

When the 3 DNA fragments were combined together, the result showed that PmToll cDNA was 4,129 bp long with open reading frame of 2,793 bp which encoding a protein of 931 amino acids, this result was showed in **Figure 4.12**. The deduce amino acids were predicted by using Vector NTI 9.0.0. Prosite was used to predict the signal peptides and protein domains. The extracellular domain was observed (residues 132-712), LRRs (residues 132-646), transmembrane domain (residues 713-735) and TIR domain (residues 766-904) and a signal peptide of 19 amino acids. Position of N-linked glycosylation sites were predicted using NetNGlyc 1.0 server, the result showed 12 N-linked glycosylation potential sites.

```
  1    AGTCGTGGTTTAGTCAGAGGAAAGTGTTGACGTGCGTGCGCGTAATAATAATACCCAACACTATCATACATCGGGTTACAGCGCTTCACT
 91    CTGTATTTTGGAGACAAGTTGATGAAAATCATATGTTTATTTTTGTGAATTGAAACAGTGCAATTAGAGTGAAAGGCCGTGGAATATTAC
```

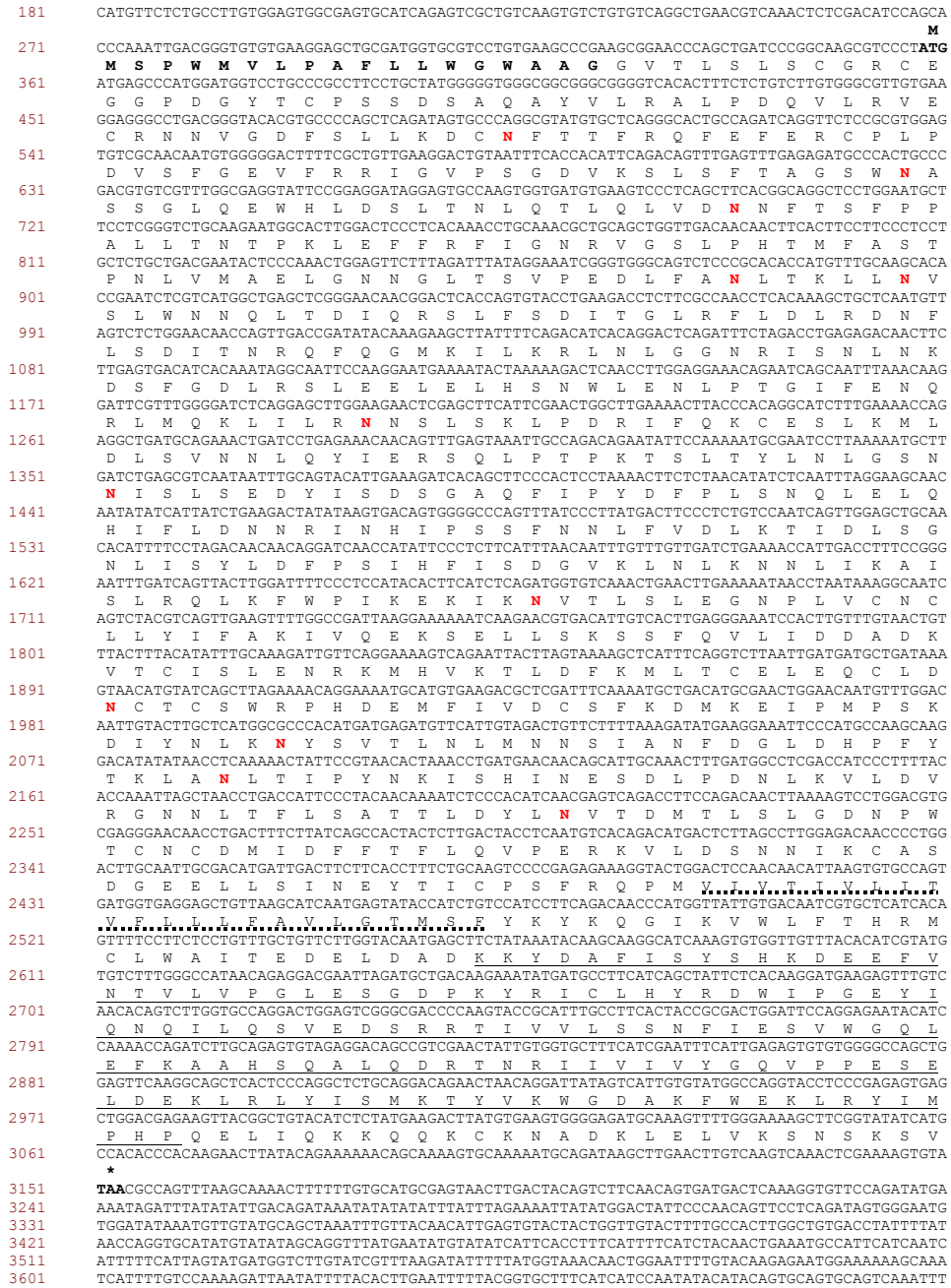


Figure 4.12 Nucleotide sequence and putative amino acid sequence of the PmToll. The amino acid sequence is shown with one-letter codes over the nucleotide sequence. The predicted signal peptide is shown in bolds. The potential N-linked glycosylation sites in the extracellular domain are reds. The transmembrane region and the TIR domain are shown using a dotted line and underline, respectively.

The PmToll was compared to 2 PmToll from GenBank database. The result showed in **Figure 4.13**. There are few differences between PmToll from this research and GenBank database.

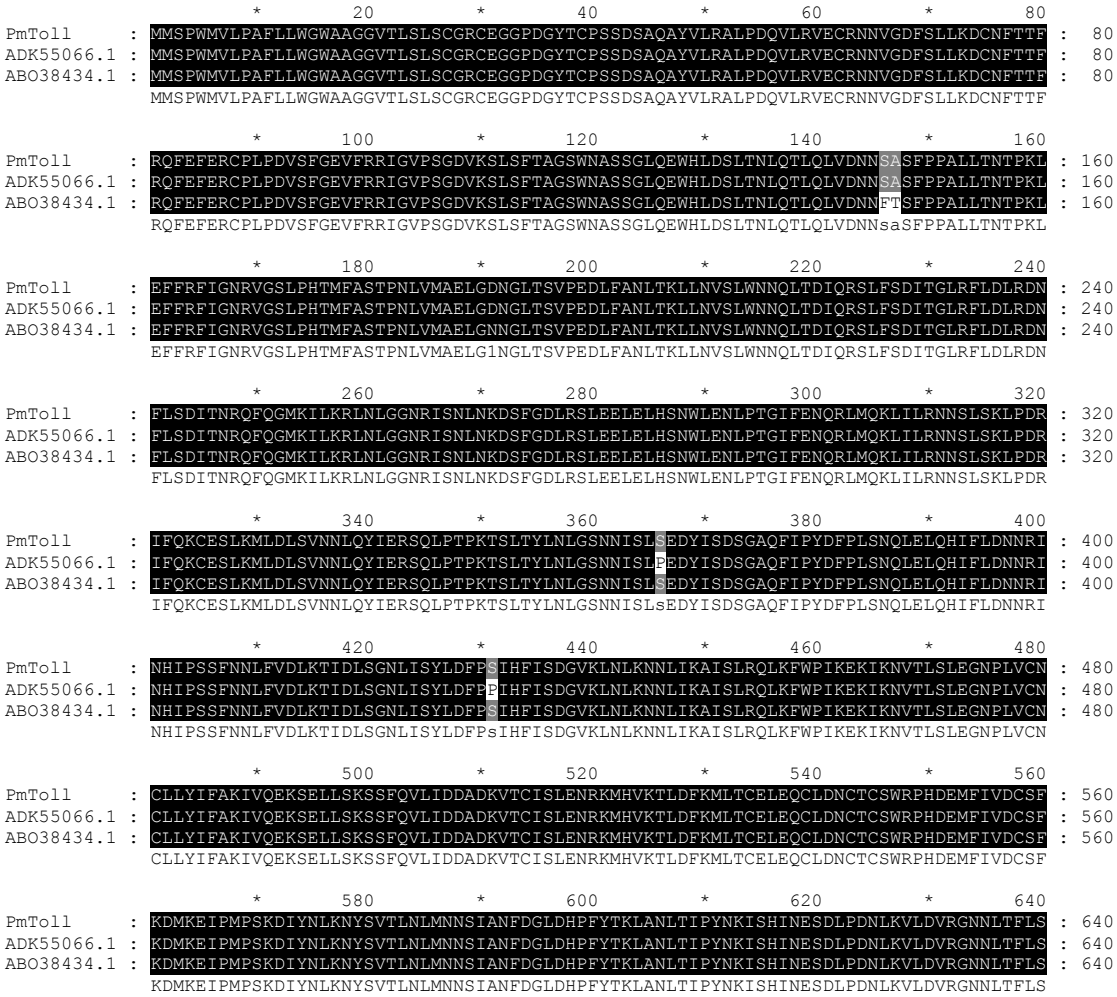


Figure 4.13 Alignment between PmToll and PmToll from GenBank database (ADK55066.1 and ABO38434.1).

```
                    *       660         *       680         *       700         *       720
PmToll      : ATTLDYLNVTDMTLSLGDNPWTCNCDMIDFFTFLQVPERKVLDSNNIKCASDGEELLSINEYTICPSFRQPMVIVTIVLI : 720
ADK55066.1  : ATTLDYLNVTDMTLSLGDNPWTCNCDMIDFFTFLQVPERKVLDSNNIKCASDGEELLSINEYTICPSFRQPMVIVTIVLI : 720
ABO38434.1  : ATTLDYLNVTDMTLSLGDNPWTCNCDMIDFFTFLQVPERKVLDSNNIKCASDGEELLSINEYTICPSFRQPMVIVTIVLI : 720
              ATTLDYLNVTDMTLSLGDNPWTCNCDMIDFFTFLQVPERKVLDSNNIKCASDGEELLSINEYTICPSFRQPMVIVTIVLI

                    *       740         *       760         *       780         *       800
PmToll      : TVFLLLFAVLGTMSFYKYKQGIKVWLFTHRMCLWAITEDELDADKKYDAFISYSHKDEEFVNTVLVPGLESGDPKYRICL : 800
ADK55066.1  : TVFLLLFAVLGTMSFYKYKQGIKVWLFTHRMCLWAITEDELDADKKYDAFISYSHKDEEFVNTVLVPGLESGDPKYRICL : 800
ABO38434.1  : TVFLLLFAVLGTMSFYKYKQGIKVWLFTHRMCLWAITEDELDADKKYDAFISYSHKDEEFVNTVLVPGLESGDPKYRICL : 800
              TVFLLLFAVLGTMSFYKYKQGIKVWLFTHRMCLWAITEDELDADKKYDAFISYSHKDEEFVNTVLVPGLESGDPKYRICL

                    *       820         *       840         *       860         *       880
PmToll      : HYRDWIPGEYIQNQILQSVEDSRRTIVVLSSNFIESVWGQLEFKAAHSQALQDRTNRIIVIVYGQVPPESELDEKLRLYI : 880
ADK55066.1  : HYRDWIPGEYIQNQILQSVEDSRRTIVVLSSNFIESVWGQLEFKAAHSQALQDRTNRIIVIVYGQVPPESELDEKLRLYI : 880
ABO38434.1  : HYRDWIPGEYIQNQILQSVEDSRRTIVVLSSNFIESVWGQLEFKAAHSQALQDRTNRIIVIVYGQVPPESELDEKLRLYI : 880
              HYRDWIPGEYIQNQILQSVEDSRRTIVVLSSNFIESVWGQLEFKAAHSQALQDRTNRIIVIVYGQVPPESELDEKLRLYI

                    *       900         *       920         *
PmToll      : SMKTYVKWGDAKFWEKLRYIMPHPQELIQKKQQKCKNADKLELVKSNSKSV : 931
ADK55066.1  : SMKTYVKWGDAKFWEKLRYIMPHPQELIQKKQQKCKNADKLELVKSNSKSV : 931
ABO38434.1  : SMKTYVKWGDAKFWEKLRYIMPHPQELIQKKQQKCKNADKLELVKSNSKSV : 931
              SMKTYVKWGDAKFWEKLRYIMPHPQELIQKKQQKCKNADKLELVKSNSKSV
```

Figure 4.13 Alignment between PmToll and PmToll from GenBank database (ADK55066.1 and ABO38434.1) (Cont.).

The 15 LRRs consensus sequences were found and alignment of LRRs consensus was showed in **Figure 4.14**. The LRRs consensus sequence is ectodomain (ECD) of TLRs, the general LRR pattern composed of 24 amino acids, XLXXLXLXXNXϕXXϕXXXXFXXLX, X refers to any amino acid, ϕ is any hydrophobic residue, and L and F are frequently replaced by other hydrophobic residues (Bell et al., 2003).

```
            XLXXLXLXXNXϕXXϕXXXXFXXLX
LRR1  : -NLQTLQLVDNNSASFPPALLTN-- : 22  (135-156)
LRR2  : -NLVMAELGDNGLTSVPEDLFAN-- : 22  (183-204)
LRR3  : -KLLNVSLWNNQLTDIQRSLFSD-- : 22  (207-228)
LRR4  : -GLRFLDLRDNFLSDITNRQFQG-- : 22  (231-252)
LRR5  : -ILKRLNLGGNRISNLNKDSFGD-- : 22  (255-276)
LRR6  : -SLEELELHSNWLENLPTGIFEN-- : 22  (279-300)
LRR7  : -LMQKLILRNNSLSKLPDRIFQK-- : 22  (303-324)
LRR8  : -SLKMLDLSVNNLQYIERSQLPT-- : 22  (327-348)
LRR9  : -SLTYLNLGSNNIS-LSEDYISDS- : 22  (352-373)
LRR10 : -ELQHIFLDNNRINHIPSS-FNNL- : 22  (389-410)
LRR11 : VDLKTIDLSGNLISYLDFPSIHF-- : 23  (413-434)
LRR12 : DGVK-LNLKNNLIKAIS---LRQLK : 21  (437-457)
LRR13 : YSVT-LNLMNNSIANFDG--LDHPF : 22  (579-600)
LRR14 : -KLANLTIPYNKISHINESDLP--- : 21  (603-623)
LRR15 : -NLKVLDVRGNNLTFLSATTLDY-- : 22  (625-646)
```

Figure 4.14 The alignment of leucine-rich repeats (LRRs) of *P. monodon* Toll receptor with 24 prevailing consensus sequence of TLRs.

The phylogenetic, which used for study in genetic evolution, was constructed using amino acids sequences were shown in **Figure 4.15**. The PmToll showed high similarity to FcToll (93.65% identity), followed by LvToll (89.48%), MjToll2 (86.79%), LvToll2 (42.39%), MjToll (47.89%) and DmToll5 (29.87%). Moreover, the alignment of PmToll with other type I of penaeid toll receptors was showed in **Figure 4.16**.

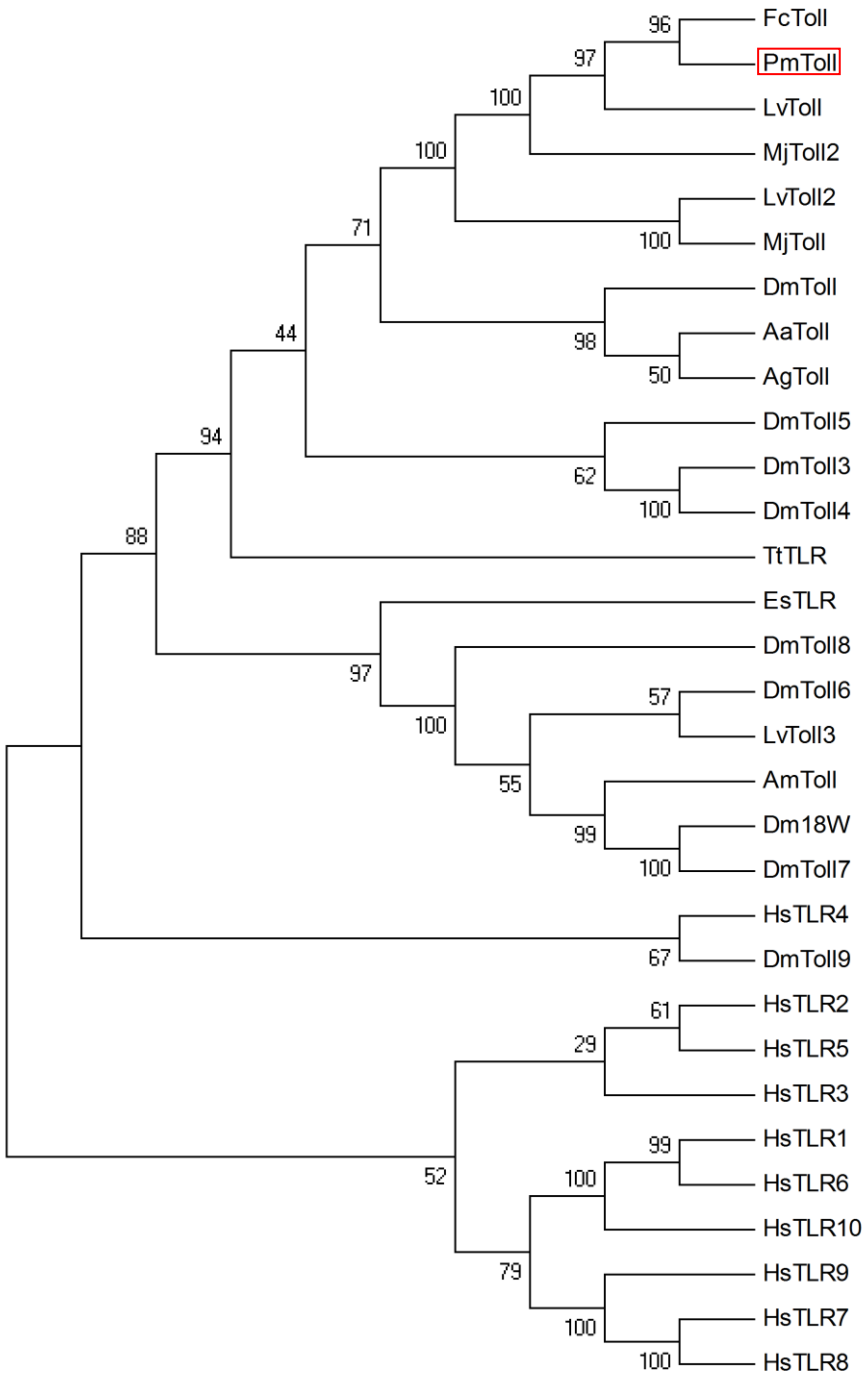


Figure 4.15 Phylogenetic of PmToll compared to different organism. Amino acids sequences were aligned using ClustalX2 and the phylogenetic was constructed using Mega4 software. The Toll receptor and Toll-like receptor sequences were obtained from Genbank: AaToll (EAT48962.1), AgToll (AAL37901.1), AmToll (AAX33677.1), Dm18W (AAF57509.1), DmToll (AAQ64938.1), DmToll3 (AAF86229.1), DmToll4 (AAF52747.3), DmToll5 (AAF86227.1), DmToll6 (AAF49645.1), DmToll7 (AAF49645.1), DmToll8 (AAF86224.1), DmToll9 (AAF51581.1), EsTLR

(AAY27971.1), FcToll (ABQ59330.1), HsTLR1 (AAC34137.1), HsTLR2 (AAM23001.1), HsTLR3 (AAC34134.1), HsTLR4 (AAY82270.1), HsTLR5 (ACM69034.1), HsTLR6 (ABY67133.1), HsTLR7 (AAF78035.1), HsTLR8 (AAF78036.1), HsTLR9 (BAB19259.1), HsTLR10 (AAK26744.1), LvToll (ABK58729.1), MjToll (BAF99007.1), MjToll2 (BAG68890.1), PmToll and TtTLR (BAD12073.1). Abbreviations : Aa, *Aedes aegypti*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Fc, *Fenneropenaeus chinensis*; Lv, *Litopenaeus vannamei*; Mj, *Marsupenaeus japonicus*; Pm, *Penaeus monodon*; and Tt, *Tachypleus tridentatus.*

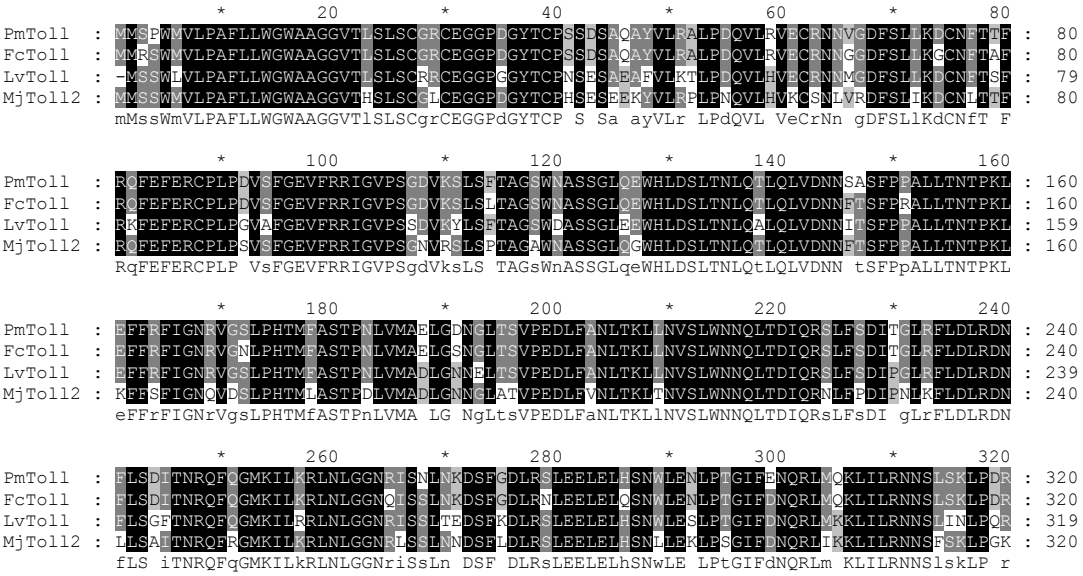


Figure 4.16 Amino acids alignment of type 1 penaeid toll receptor. This alignment was showed high similarity of the type 1 penaeid toll receptor including PmToll, FcToll, LvToll and MjToll2 especially TIR domain.

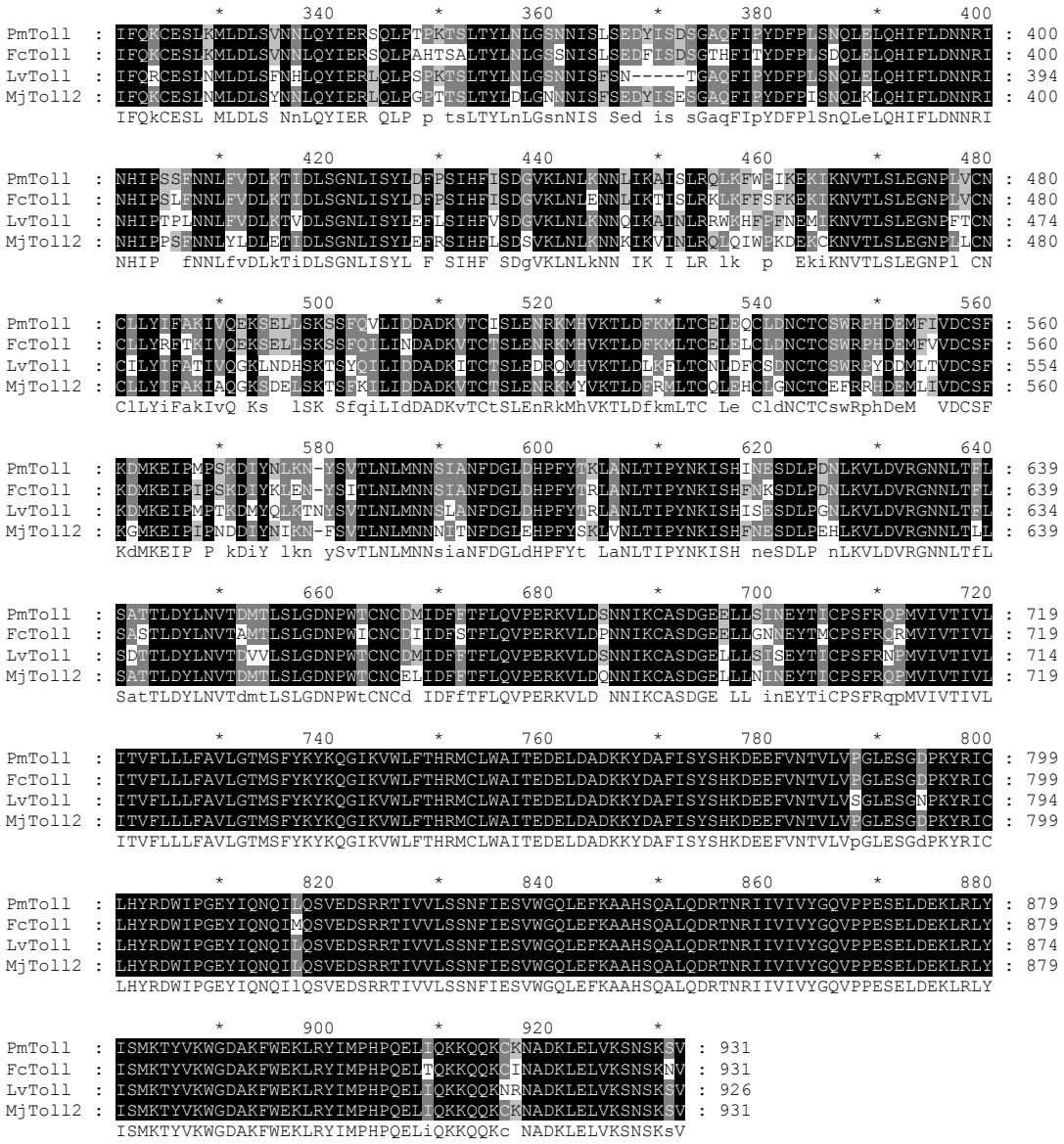


Figure 4.16 Amino acids alignment of type 1 penaeid toll receptor (Cont.).

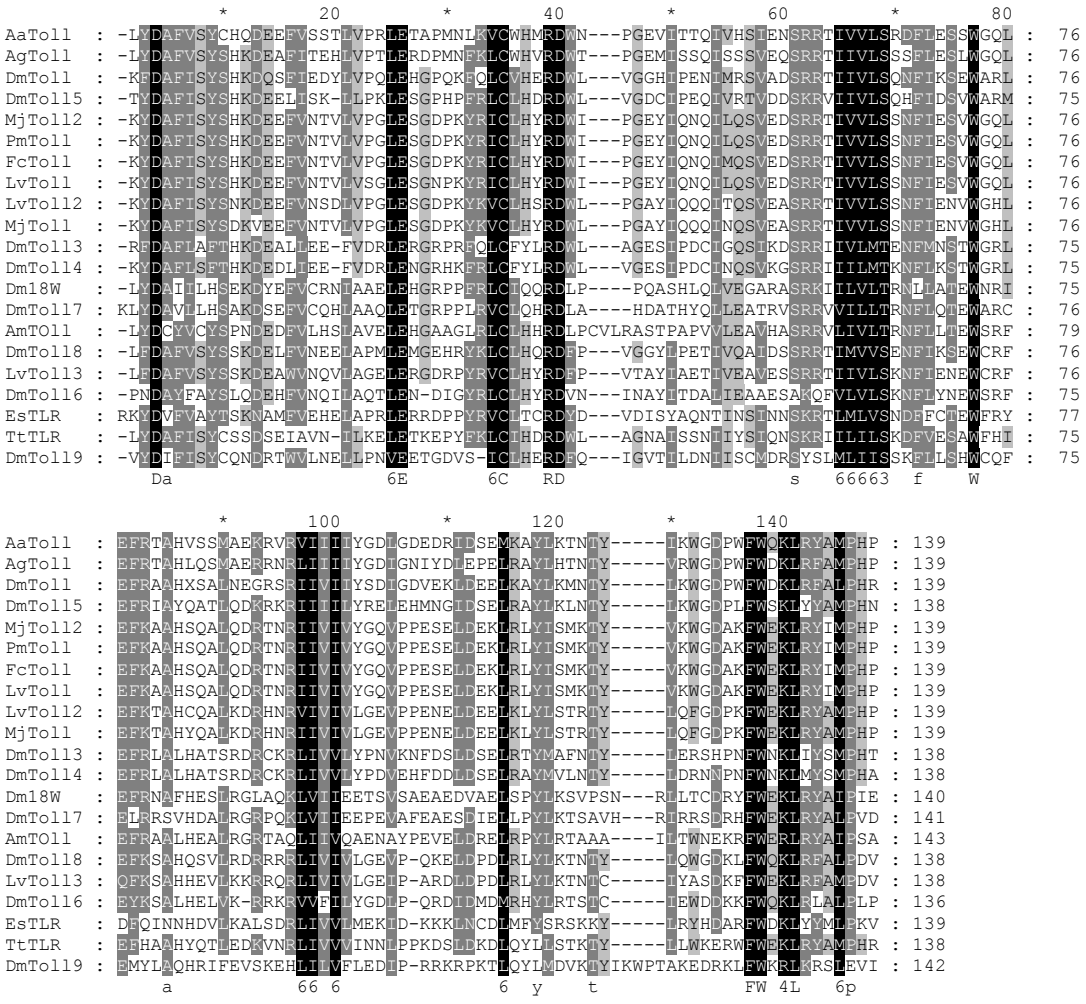


Figure 4.17 Alignment of TIR domain of PmToll to related Toll and TLR. Identical or highly conserved residues are shaded in black while similar residues are shaded in grey. Sequences for the alignment were obtained from the GenBank**.**

The alignment of PmToll TIR domain with other organisms showed high similarity to type I and II penaeid toll receptor and Toll/TLR in other organisms included MjToll (100% identitiy), MjToll2 (100%), FcToll (99.28%), LvToll (98.56%), LvToll2 (77.7%), DmToll5 (52.6%), DmToll (55.1%), AaToll (53.6%) and AgToll (56.5%).

To study the distribution of PmToll gene, the total RNA was extracted from various tissues. Then, RT-PCR was used to analyze expression of various tissues by using Toll-F, Toll-R, Actin-F and Actin-R as primers. The PCR products were analyzed on 1.8% agarose gel electrophoresis. The result showed the PmToll was expressed in gill, heart, lymphoid, muscle, nerve, pleopod and stomach but less expression in hepatopancreas.

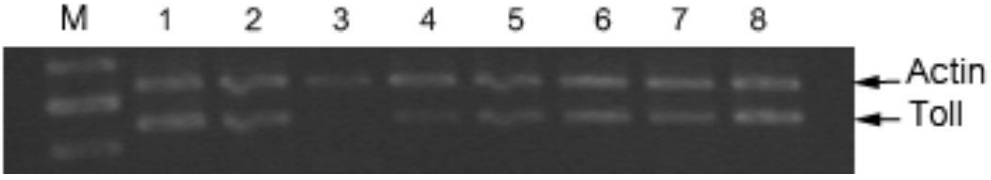


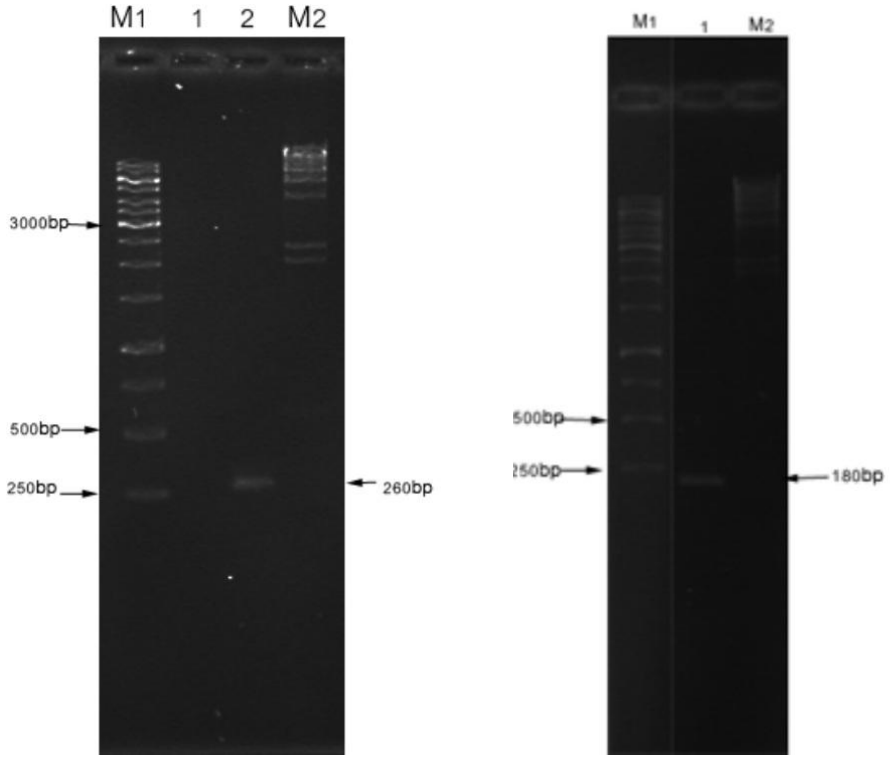
Figure 4.18 Tissue distribution of PmToll in various tissues.

Lane M : Marker
Lane 1 : Gill
Lane 2 : Heart
Lane 3 : Hepatopancreas
Lane 4 : Lymphoid
Lane 5 : Muscle
Lane 6 : Nerve
Lane 7 : Pleopod
Lane 8 : Stomach

4.4 Contruction of PmToll dsRNA

The non-structural region of PmToll was chosen to construct dsRNA. To construct the recombinant dsRNA-PmToll pET17b, sense fragment of PmToll was amplified using dsToll-F1 and dsToll-R1 primers by using 5'-RACE plasmid (from 4.2.2 clone4) as the template. This DNA fragment was cut with *Xba* I and *Xho* I **Figure 4.19 (A)** and ligated into pET17b which was cut with the same restriction enzymes. Then, ligation reaction was used to transform into *E. coli* DH5α and the recombinant clones were analyzed on 1.8% agarose gel electrophoresis by using simplified rapid size screening (data not showed). The recombinant plasmids (pET17: Toll sense) were sequenced. The dedicated plasmid was used as template to integrate the anti-sense fragment. The pET17b: Toll sense was extracted from *E. coli* DH5α. Then, anti-sense fragment of PmToll was amplified using dsToll-F2 and dsToll-R2 as primers. The anti-sense fragment was cut with *Eco* RI and *Xho* I **Figure 4.19 (B)**. The ligation reaction was used to transform into *E. coli* DH5α. The recombinant clones were analyszed by simplified rapid size screening (data not showed). Then, the dedicated dsRNA-PmToll

pET17b plasmids were extracted and linearized using *Eco* RI for DNA sequencing. The sequence analysis was showed in **Figure 4.20**.



(A) **(B)**

Figure 4.19 Lane 1(A) and Lane 1(B) are sense and anti-sense product. M1 and M2 are represented 1kb ladder and λ/*Hin* dIII, respectively. (A) Lane 1 is purified pET17b which was digested *Xho* I and *Xba* I.

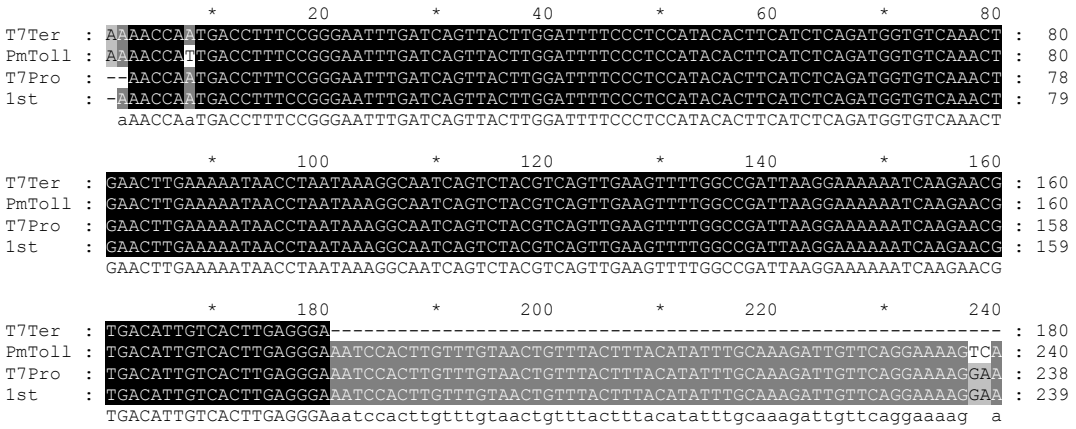


Figure 4.20 Toll dsRNA alignment. Alignment sequence from clone 1, 1st is represented sequence from pET17b: Toll sense. T7 Promoter and T7 Terminator are represented sequencing of sense and anti-sense strand. PmToll is represented *P. monodon* Toll receptor.

4.5 Expression of PmToll and GFP dsRNA

The dsRNA of Toll and GFP (dsToll and dsGFP) were 5-fold serial diluted and analyzed on 1% agarose gel electrophoresis. The intensity of diluted dsToll and dsGFP were compared to λ/*Hin* dIII. Then, the intensity was back calculated to obtain dsRNA concentration. For example dsToll (1/125) was estimate same density to 564 bp of λ/*Hin* dIII, which was amount 1.2% (total = 200 ng). So, the concentration of dsToll was 125 x 1.2% x 200 = 300 ng/μl.

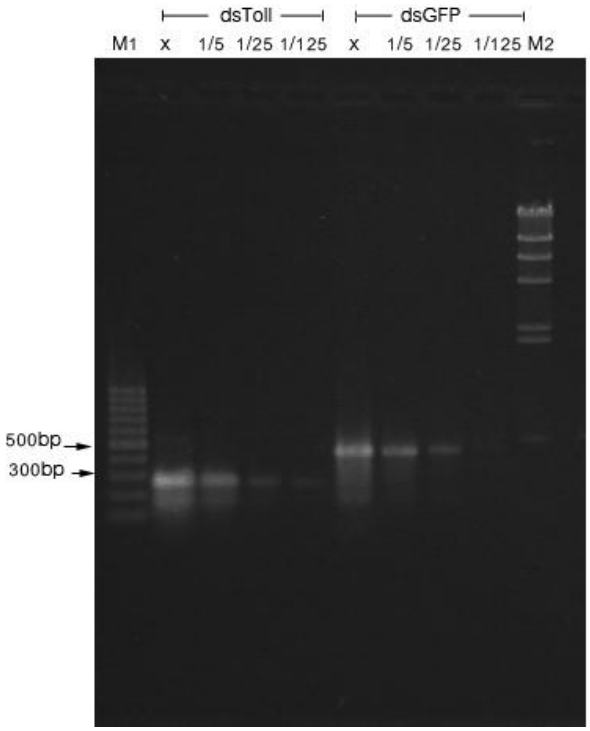


Figure 4.21 Extraction of dsRNA using Tri-reagent® (Molecular Research Center). To estimate the concentration dsRNA were serial diluted with 100mM NaCl, x mean undiluted sample and 1/5, 1/25 and 1/125 are diluted sample. Lane 2-5 are Toll dsRNA and Lane 6-9 are GFP dsRNA. M1 and M2 are 100 bp ladder and λ/*Hin* dIII, respectively.

4.6 Knocked-down expression of Toll receptor by injected dsRNA to shrimp

4.6.1 Injection 2.5 μg/gram shrimp, collected 24 hours interval for 5 days

The healthy shrimps (5-8 grams) were chosen to inject dsRNA (dsToll, dsGFP) and 150 mM NaCl. After that, Shrimps were collected 24 h intervals for 5 days. RNAs were extracted from gills. Then, RT-PCR and PCR were performed using Toll-F, Toll-R, Actin-F and Actin-R and analyzed on 1.8% agarose gel electrophoresis for detection of Toll expression compared to expression of Actin. The result showed in **Figure 4.22**. The density analysis showed that expression of PmToll was decreased in day 3 post injection when compared to samples which injected with dsGFP and 150 mM NaCl (**Figure 4.23**).

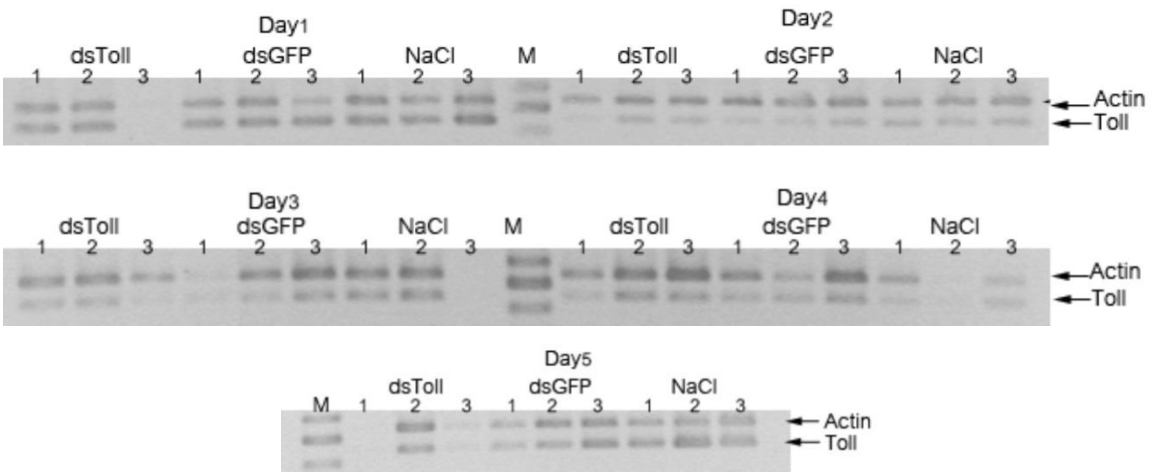


Figure 4.22 Expression of Toll compared to β-Actin.

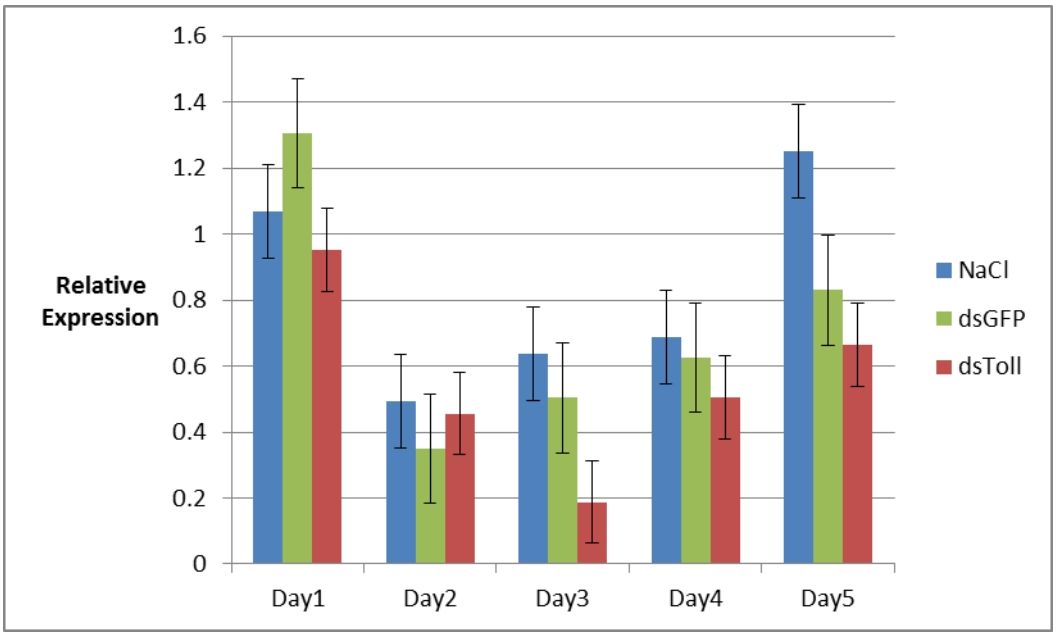


Figure 4.23 Relative expression of Toll compared to β-Actin expression.

4.6.2 Injection 2.5 μg/gram shrimp, collected 24 hours interval for 5 days (2)

The healthy shrimps (3 grams) were chosen to inject dsRNA (dsToll, dsGFP and 150 mM NaCl). Then, samples were collected 24 hours interval, for 5 days. RNAs were extracted from gills. Then, RT-PCR and PCR were performed using Toll-F, Toll-R, Actin-F and Actin-R and analyzed on 1.8% agarose gel electrophoresis for detection of PmToll expression compared to expression of Actin. The result showed in **Figure 4.24**. The expression of PmToll was significantly decreased in day 1 when compared to samples which injected with dsGFP and 150 mM NaCl (**Figure 4.25**).

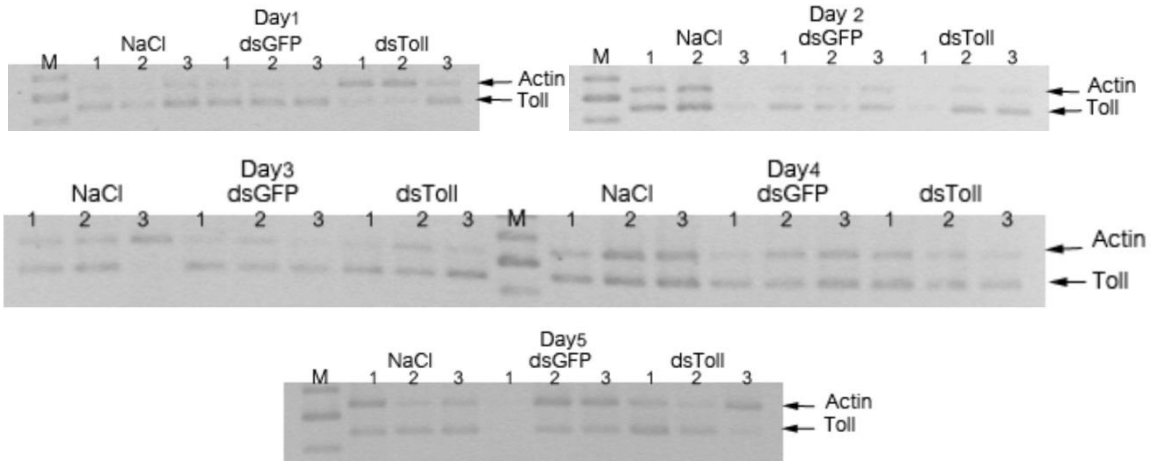


Figure 4.24 Expression of Toll compared to β-Actin.

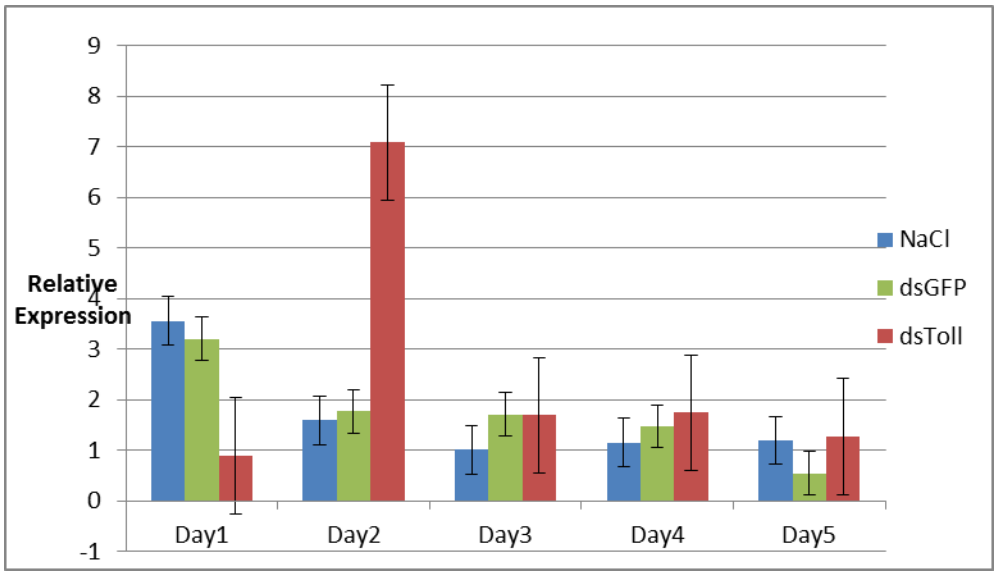


Figure 4.25 Relative expression of Toll compared to β-Actin.

4.6.3 Injection 2.5 μg/gram shrimp, collected 12 hours interval for 3 days

The healthy shrimps (1-2 grams) were chosen to inject dsRNA (dsToll and dsGFP) and 150 mM NaCl and shrimps were injected again 24 hours after first injection. Then, shrimps were collected 12 hours intervals for 3 days. RNAs were extracted from gills. Then, RT-PCR and PCR were performed using Toll-F, Toll-R, Actin-F and Actin-R and analyzed on 1.8% agarose gel electrophoresis for detection of Toll expression compared to expression of Actin. The result was show in **Figure 4.26**. The expression of PmToll was not significantly decreased at any time point when compared to samples which injected with dsGFP and 150 mM NaCl (**Figure 4.27**).

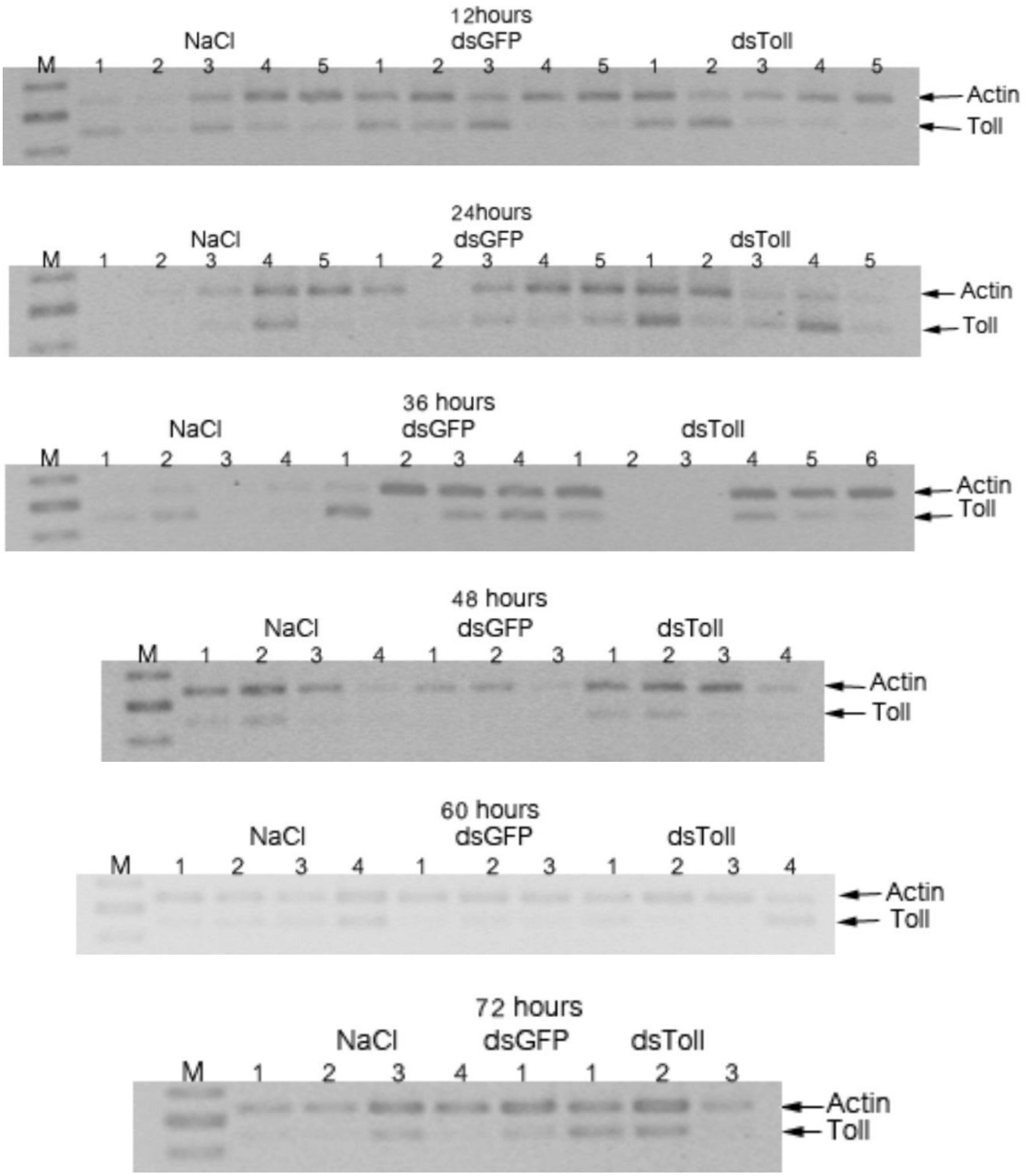


Figure 4.26 Expression of Toll compared to β-Actin.

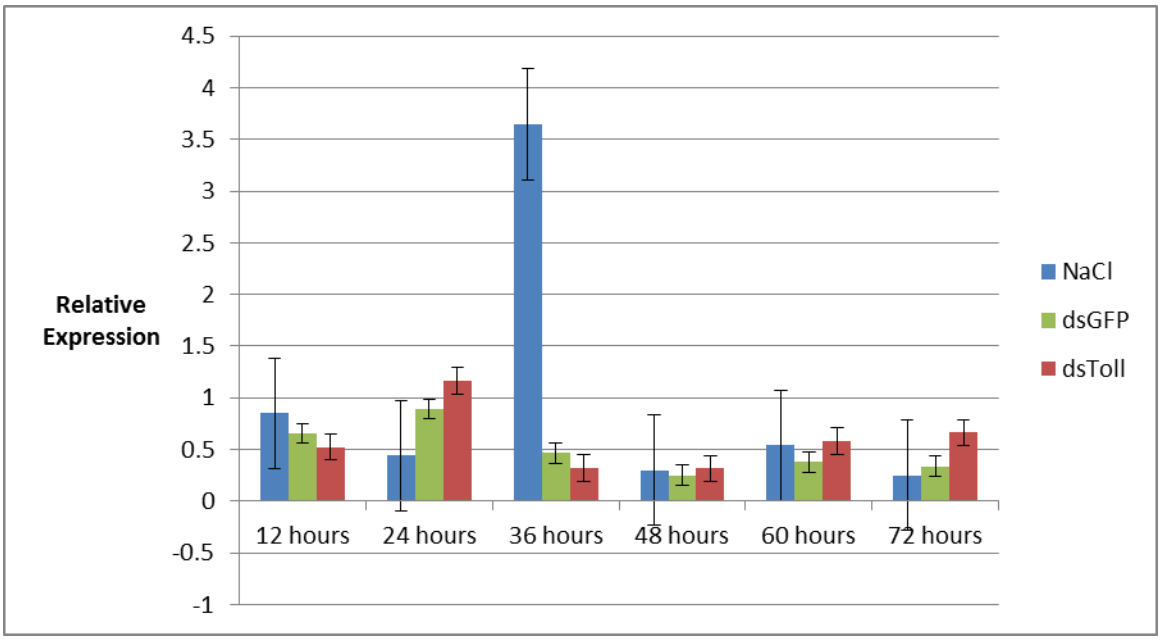


Figure 4.27 Relative expression of Toll compared to β-Actin.

4.6.4 Injection 5 μg/gram shrimp, collected 6 hours interval for 1.5 days

The healthy shrimps (1-2 grams) were chosen to inject dsRNA (dsToll and dsGFP) and 150 mM NaCl and shrimps were injected again 24 hours after first injection. Then, shrimps were collected 12 hours intervals for 3 days. RNAs were extracted from gills.Then, RT-PCR and PCR were performed using Toll-F, Toll-R, Actin-F and Actin-R and analyzed on 1.8% agarose gel electrophoresis for detection of Toll expression compared to expression of Actin. The result was show in **Figure 4.28**. The expression of PmToll was decreased in 24 h post injected when compared to samples which injected with dsGFP and 150 mM NaCl (**Figure 4.29**).

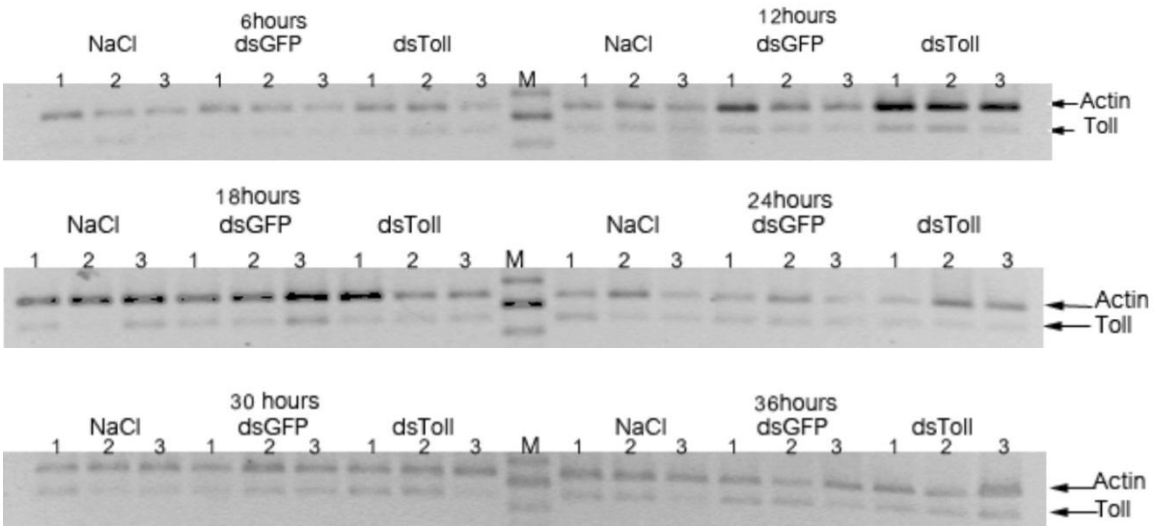


Figure 4.28 Expression of Toll compared to β-Actin.

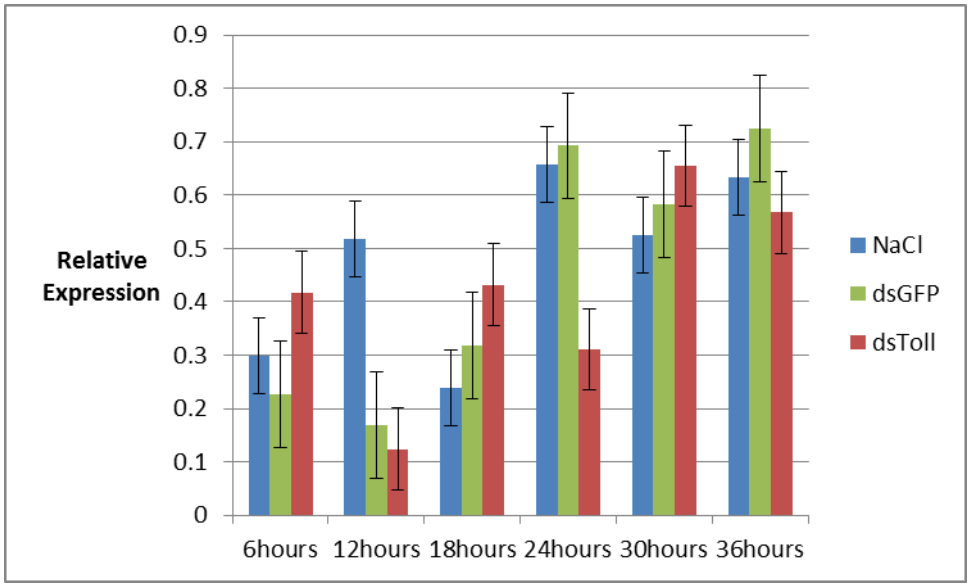


Figure 4.29 Relative expression of Toll compared to β-Actin expression.

CHAPTER V

DISCUSSION AND CONCLUSION

Recently, toll receptors have been identified in many penaeid shrimps. The first penaeid toll receptor gene has been found and identified in *Penaeus monodon* (Arts et al., 2006), then follow identified in *Litopenaeus vannamei* (Yang C. et al., 2007; Wang et al., 2012), *Feneropenaeus chinensis* (Yang L-S et al., 2008), *Marsupenaeus japonicus* (Mekata et al., 2008). In this study, the full-length of PmToll cDNA was cloned and identified from heart. It has 4,129 nucleotides which contain 2,793 nucleotides ORF that encode 931 amino acids of putative PmToll. The PmToll was blast against to the GenBank database. The result found that it has closely similar to two Tolls of *P. monodon* (GenBank Access No.ADK55066.1 and ABO38434.1). The phylogenetic relationship study showed that PmToll shared high similarity to penaeid toll receptor such as FcToll (Yang C et al., 2008), LvToll1 (Yang L-S et al., 2007) and MjToll2 (GenBank Access No. AB385869.1). Moreover, the penaeid toll receptors have been classified into 3 types which are type I, II and III. The all types of penaeid toll receptor showed mostly similarity in TIR domain but different in amino acids sequences and organization in LRRs of extracellular domains, suggested in different of ligand binding sites (Wang et al., 2012). Analysis of deduced amino acid of PmToll suggested that it might be classified into to type I penaeid toll receptor.

In *F. chinensis*, the expression level of FcToll during infected with *V. aguillarum* and WSSV was studied by qRT-PCR. The result showed that FcToll gene highly expressed during both bacterial and viral infection when comparing with the control group (Yang C., et al., 2008). Moreover, the injection of non-specific of siRNA duplex and long dsRNA did not affect to LvToll1 mRNA expression level in *L. vannamei* when analyzed by qRT-PCR. In addition, the silenced of LvToll1 in *L. vannamei* and follow by co-injected with non-specific dsRNA and WSSV was also studied. The result found that the silenced LvToll1 group did not have higher cumulative mortality than control group (not knocked down LvToll1 and followed by co-injected with non-specific dsRNA and WSSV). Taken these results together, it suggested that there are less or no relationship between LvToll1 and dsRNA (Labreuche et al., 2009).

The previous researches of *Drosophila* Toll pathway showed that Spätzle proteins act as a ligand to *Drosophila* Toll receptor, and then it will activate the intracellular signaling for the expression of either antimicrobial genes, other immune genes or development (Hoffmann, 2003). Additionally, many penaeid spätzle-like proteins have been discovered and characterized in penaeid shrimp such as *F. chinensis* (FcSpz) (Shi et al., 2009) and *L. vannamei* (LvSpz1, LvSpz2 and LvSpz3) (Wang et al., 2012). These spätzle-like protein shared homology to *Drosophila* spätzle protein. However, the interaction between penaeid toll receptor and spätzle-protein need to be further study for conclusion in penaeid toll receptor ligand.

In study of PmToll tissue expression was studied, the results showed that PmToll was expressed in gill, heart, lymphoid, muscle, nerve, pleopod, stomach and low expressed in hepatopancreas. This result was similar to LvToll1 in *L. vannamei* (Yang L-S et al., 2007) and FcToll in *F. chinensis* (Yang C. et al., 2008). However, only one black tiger shrimp was used in tissue distribution expression in this study. This might be a factor which affect in plausibility in this experiment. Therefore, the many shrimps should be used to investigate tissue distribution. Moreover, the quality of RNA is one important factor in this experiment especially the good quality of total RNA from hepatopancreas is quite difficult to extract and less intact when compared to other organs. This result might occur from a lot of nucleases in the hepatopancreas which is a digestive organ in shrimp.

RNA interference, known as a post transcriptional silencing, was found in various organisms. RNAi has been found in many biological functions such as a role in pathogen resistance and regulation of endogeneous protein-coding genes (Hannon, 2002). Moreover, RNAi has been purposed in antiviral response (Silva et al., 2002). In shrimp, RNAi technique was used in endogenous gene silencing experiment (Labreuche et al., 2009, Ongvarrasopone et al., 2008 and Wang et al., 2010) and induction in antiviral immunity (Phetrungnapha et al., 2011, Robalino et al., 2004, Tirasophon et al., 2007, Xu et al.,2007 and Yodmuang et al., 2006). In this study, RNAi technique was used in knock-down expression of PmToll for further characterization experiment by injected with pathogen and investigated the expression of PmToll and other immune genes. The PmToll dsRNA was injected into the hemolymph of shrimp. The first (5-8 g shrimp) and second (3 g shrimp) experiment the amount of dsRNA which used to inject in shrimps are 2.5 μg/g shrimp and collected 24 h intervals for 5 days. Then, gills were collected and

extracted total RNA and analyzed by RT-PCR. The result showed that the relative expression of PmToll was decreased in day 3 and 5 in first experiment, whereas, the relative expression of PmToll was decreased in day 1 in the second experiment. In the third experiment, the PmToll dsRNA was injected at amount 2.5 μg/g shrimp and followed by second injected 2.5 μg/g shrimp after 24 h post- injection. The shrimps were collected at 12 h intervals for 3 days. Then, gills were collected and extracted total RNA and analyzed by RT-PCR. The result showed that relative expression of PmToll was not significantly decreased in any time point when compared to controls. In the last experiment, the 5 μg PmToll dsRNA per gram shrimp was injected twice 24 h interval. Then, the shrimps were collected every 6 h for 2 days. After that, gills were collected and extracted total RNA and analyzed by RT-PCR. The result showed that the expression of PmToll was significantly decreased at 24 h post second injection. However, the other studies showed that some of endogenous genes such as Tudor (Phetrungnapha et al., 2011), Rab7 (Ongvarransopone et al., 2008) and LvToll1 (Wang et al., 2010) were significantly reduction after injected with specific dsRNA. Even though, some knocked down experiment in this study can decrease the expression of PmToll but each experiment was performed only one time. Therefore, the further knocked down study should be performed.

The comparison between knocked down of PmToll (this study) and LvToll1 (Wang et al., 2010) showed some differences materials and methodologies such as region of Toll specific dsRNA, size of dsRNA and shrimp sizes. First, the region of each Toll receptor which was chosen for constructed dsRNA is totally different. The non-structural of LRR region was chosen in PmToll but TIR domain was chosen to construct the dsRNA in LvToll1. Therefore, the sequences of generating siRNAs are different. Then, the targeted mRNA which siRNAs will be complement and induced in nucleolytic cleavages are different. Moreover, the secondary structure of mRNA affects to complement of siRNA (Yoshinari et al., 2004). In case of the length of dsRNA, the PmToll dsRNA is180 bp while LvToll1 is 460 bp. The transfection of *D. melanogaster S2* cell by using various sizes of dsRNA, the result showed that the 400 bp and 540 bp dsRNAs were more effective than 200 bp and 300 bp dsRNAs, while 50–100 bp dsRNAs were ineffective in induction of RNAi pathway (Hammond et al., 2000). The size and age of the shrimp might affect to response of RNAi pathway because the metabolism of shrimp in different lifespan could be different which affect in RNAi process.

CONCLUSION

A full-length of PmToll cDNAs is 4,129 nucleotides long, which containing a 2,793 nucleotides ORF that translated into a putative PmToll of 931 amino acids. The predicted PmToll protein composed of 15 LRRs, transmembrane domain and TIR domain. The PmToll gene expresses in gill, heart, lymphoid, muscle, nerve, pleopod and stomach but less expresses in hepatopancreas.

The gene silencing step, the expression of PmToll was decreased within 24 h post injection but not completely knocked down with this dsRNA-PmToll.

The further researches might aim to construct long dsRNA (400-500 bp) and find the optimal condition for PmToll silencing. After silencing and/or challenge with pathogens, the relative expression of PmToll and other immune genes could be studied.

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APPENDICES

Appendix A

Medium and preparation

1. Luria-Bertani medium (LB broth)

Tryptone	10	grams
Yeast extract	5	grams
NaCl	5	grams

Dissolve 3 chemical agents in 800 ml distilled water, adjust pH to 7 with 1 N NaOH and then, added distilled water to 1,000 ml. Sterile the medium in autoclave at 15 ppi, 121 °C for 15 min.

2. Luria-Bertani agar (LB agar)

Prepare medium same as LB broth and added 15 grams per 1 litre and Sterile the medium in autoclave at 15 ppi 121 °C for 15 min.

3. Luria-Bertani medium (LB) plus appropriated anti-biotic

Prepare medium same as LB broth or LB agar. After sterile, leave the medium until temperature reach to 55 °C and then, add the appropriated anti-biotic to final concentration as below.

Ampicillin	100	μg/ml
Tetracycline	12.5	μg/ml

Appendix B

Chemical reagents and instrument

1. 100 % glycerol

100% Glycerol is steriled by autoclave at 15 ppi, 121 °C for 15 min. Then, dry in hot air oven at 80 °C for 24 hours. Repeat sterile and dry for 2 times.

2. 10 mM each mixed dNTP solution

Mix 10 μl each of 100 mM dATP, dGTP, dCTP and dTTP and add deionized water to volume 100 μl. Store at -20 °C until use.

3. 50X Tris-acetate EDTA (TAE) buffer

Trisma base	121	grams
Glacial acetic acid	28.55	ml
0.5 M EDTA	50	ml

Dissolve 3 chemical agents in 300 ml deionized water, added deionized water to 1,000 ml. Sterile the solution in autoclave at 15 ppi, 121 °C for 15 min.

4. 6X loading dye

Bromphenolblue	0.25	%
Glycerol	40	%

Dissolve 2 chemical agents in deionized water and store at 4 °C until use.

5. Geneaid® Gel/PCR extraction kit

DF Buffer

W1 Buffer

DF column

Wash Buffer

Add 24 ml of absolute ethanol in wash buffer before use and follow the manufacture protocol.

6. Geneaid® High-speed Plasmid Mini Kit

   PD1 Buffer

   PD2 Buffer

   PD3 Buffer

   W1 Buffer

   Wash Buffer

   RNase A

   PD column

Add 20 μl RNase A to PD1 Buffer and store at 4 °C until use. Add 24 ml of absolute ethanol in wash buffer before use and follow the manufacture protocol.

Appendix C

Scion density data

The density of each band and background were measured for 3 times.

Mean=(Measure1+Measure2+Measure3)/3

Adjust Volume=Mean(Actin or Toll)-Mean(Background)

Relative expression= Adjust Vol. (Toll)/Adjust Vol. (Actin)

Result: 4.6.1 Injection 2.5 μg/gram shrimp, collected 24 hours interval for 5 days

Index		Measure1	Measure2	Measure3	Mean	Adjust	Relative
	Day1					Volume	Expresson
T1.1		71.46	70.29	71.29	71.01333	16.25	-
T1.2		69.27	68.05	69.27	68.86333	14.1	0.867692
T2.1		72.45	71.31	72.39	72.05	17.28667	-
T2.2		73.15	71.73	73.15	72.67667	17.91333	1.036251
G1.1		70.19	69.27	70.19	69.88333	15.12	-
G1.2		71.29	70.11	71.29	70.89667	16.13333	1.067019
G2.1		76.59	75.36	76.59	76.18	21.41667	-
G2.2		75.28	73.82	74.92	74.67333	19.91	0.92965
G3.1		65.53	64.88	65.51	65.30667	10.54333	-
G3.2		75.47	73.95	75.5	74.97333	20.21	1.916851
N1.1		78.78	77.29	78.79	78.28667	23.52333	-
N1.2		77.1	75.58	77.12	76.6	21.83667	0.928298
N2.1		71.23	70.53	71.16	70.97333	16.21	-
N2.2		73.78	72.56	73.78	73.37333	18.61	1.148057
N3.1		82.1	81.08	82.1	81.76	26.99667	-
N3.2		85.98	83.78	85.98	85.24667	30.48333	1.129152
	Day2						
T1.1		68.34	67.65	68.45	68.14667	13.38333	-
T1.2		60.27	60.09	60.22	60.19333	5.43	0.405729
T2.1		74.69	73.67	75.04	74.46667	19.70333	-
T2.2		65.14	64.63	65.14	64.97	10.20667	0.518017
T3.1		73.22	71.93	72.88	72.67667	17.91333	-
T3.2		62.9	62.43	62.9	62.74333	7.98	0.445478
G1.1		75.7	73.91	75.37	74.99333	20.23	-
G1.2		60.29	60	60.29	60.19333	5.43	0.268413
G2.1		72.99	71.78	72.99	72.58667	17.82333	-
G2.2		61.48	61.15	61.4	61.34333	6.58	0.369179
G3.1		75.7	74.5	75.7	75.3	20.53667	-
G3.2		63.56	62.85	63.26	63.22333	8.46	0.411946
N1.1		68.2	67.36	68.07	67.87667	13.11333	-
N1.2		63.12	62.29	63.16	62.85667	8.093333	0.617184
N2.1		69.07	68.31	69.03	68.80333	14.04	-

N2.2		61.28	60.72	61.28	61.09333	6.33	0.450855
N3.1		70.1	68.89	70.1	69.69667	14.93333	-
N3.2		61.04	60.46	61.11	60.87	6.106667	0.408929
Background		56.16	53.99	54.14	54.76333	-	
	Day3						
T1.1		74.93	72.01	74.77	73.90333	14.93	-
T1.2		60.71	59.81	60.71	60.41	1.436667	0.096227
T2.1		80.21	77.4	80.29	79.3	20.32667	-
T2.2		65.13	63.71	65.15	64.66333	5.69	0.279928
T3.1		68.57	67.02	68.63	68.07333	9.1	-
T3.2		57.85	57.28	58.08	57.73667	-1.23667	-0.1359
G1.1		61.29	60.94	61.17	61.13333	2.16	-
G1.2		60.63	60.22	60.59	60.48	1.506667	0.697531
G2.1		83.58	80.41	83.4	82.46333	23.49	-
G2.2		67.4	66.65	67.27	67.10667	8.133333	0.346247
G3.1		97.09	93.47	97.09	95.88333	36.91	-
G3.2		76.68	75.1	76.71	76.16333	17.19	0.465727
N1.1		90.38	86.86	90.41	89.21667	30.24333	-
N1.2		78.26	76.16	77.85	77.42333	18.45	0.610052
N2.1		93.72	90.87	94.41	93	34.02667	-
N2.2		82.34	80.09	82.4	81.61	22.63667	0.665263
	Day4						
T1.1		83.71	81.29	83.29	82.76333	23.79	-
T1.2		71.12	70.51	71.27	70.96667	11.99333	0.504133
T2.1		100.3	95.91	99.62	98.61	39.63667	-
T2.2		82.6	80.79	82.61	82	23.02667	0.580944
T3.1		107.59	103	107.59	106.06	47.08667	-
T3.2		79.88	78.34	79.86	79.36	20.38667	0.43296
G1.1		87.35	84.72	87.36	86.47667	27.50333	-
G1.2		73.41	72.43	73.41	73.08333	14.11	0.513029
G2.1		74.29	73.43	74.38	74.03333	15.06	-
G2.2		72.89	71.67	72.89	72.48333	13.51	0.897078
G3.1		100.66	95.81	100.66	99.04333	40.07	-
G3.2		78.6	76.4	78.6	77.86667	18.89333	0.471508
N1.1		73.69	71.76	73.41	72.95333	13.98	-
N1.2		65.11	64.58	65.11	64.93333	5.96	0.426323
N2.1		61.09	59.92	61.09	60.7	1.726667	-
N2.2		61.01	59.91	60.93	60.61667	1.643333	0.951737
Background		57.08	63.08	56.76	58.97333	-	
	Day5						
T1.1		71.41	75.62	74.47	73.83333	30.47	-
T1.2		60.01	62.76	62.2	61.65667	18.29333	0.600372
T2.1		46.93	47.44	47.36	47.24333	3.88	-
T2.2		45.91	46.33	46.33	46.19	2.826667	0.728522
G1.1		54.78	56.45	56.01	55.74667	12.38333	-
G1.2		52.78	53.98	53.61	53.45667	10.09333	0.815074
G2.1		67.23	70.59	69.77	69.19667	25.83333	-

G2.2		59.05	61.65	61.1	60.6	17.23667	0.667226
G3.1		69.98	73.08	72.35	71.80333	28.44	-
G3.2		69.77	73.58	72.58	71.97667	28.61333	1.006095
N1.1		61.26	63.52	62.9	62.56	19.19667	-
N1.2		64.91	68.31	66.97	66.73	23.36667	1.217225
N2.1		64.25	65.79	64.84	64.96	21.59667	-
N2.2		74.27	78.37	77.36	76.66667	33.30333	1.542059
N3.1		63.93	66.24	65.37	65.18	21.81667	-
N3.2		63.35	65.88	65.83	65.02	21.65667	0.992666
Background		43.32	43.14	43.63	43.36333	-	

Result: 4.6.2 Injection 2.5 μg/gram shrimp, collected 24 hours interval for 5 days (2)

Index		Measure1	Measure2	Measure3	Mean	Adjust	
	Day1					Volume	
N1.1		59.91	60.29	59.93	60.04333	2.54	-
N1.2		64.47	65.38	64.45	64.76667	7.263333	2.85958
N2.1		58.44	58.41	58.31	58.38667	0.883333	-
N2.2		62.2	62.69	62.27	62.38667	4.883333	5.528302
N3.1		63.36	63.57	63.16	63.36333	5.86	-
N3.2		70.65	71.55	70.56	70.92	13.41667	2.289534
G1.1		61.73	61.79	61.68	61.73333	4.23	-
G1.2		67.12	68.04	67.06	67.40667	9.903333	2.341214
G2.1		60.83	61.13	60.95	60.97	3.466667	-
G2.2		66.59	67.76	66.97	67.10667	9.603333	2.770192
G3.1		59.64	59.93	59.85	59.80667	2.303333	-
G3.2		67.5	68.36	67.66	67.84	10.33667	4.487699
T1.1		68.97	70.02	69.21	69.4	11.89667	-
T1.2		61.48	61.88	61.56	61.64	4.136667	0.347716
T2.1		70.11	71.14	70.12	70.45667	12.95333	-
T2.2		60.42	60.52	60.68	60.54	3.036667	0.234431
T3.1		61.79	62.38	61.98	62.05	4.546667	-
T3.2		66.56	67.64	66.92	67.04	9.536667	2.097507
Background		57.4	57.08	58.03	57.50333	-	
	Day2						
N1.1		55.97	55.97	54.83	55.59	15.73	-
N1.2		62.7	62.7	60.97	62.12333	22.26333	1.415342
N2.1		61.83	61.83	60.54	61.4	21.54	-
N2.2		67.78	67.78	66.21	67.25667	27.39667	1.271897
N3.1		42.71	42.71	42.76	42.72667	2.866667	-
N3.2		45.88	45.88	45.62	45.79333	5.933333	2.069767
G1.1		45.3	45.3	45.08	45.22667	5.366667	-
G1.2		51.92	51.92	51.13	51.65667	11.79667	2.198137
G2.1		45.75	45.75	45.34	45.61333	5.753333	-
G2.2		48.73	48.73	48.03	48.49667	8.636667	1.501159
G3.1		46.16	46.16	45.83	46.05	6.19	-

G3.2		50.06	50.06	49.28	49.8	9.94	1.605816
T1.1		39.43	39.43	39.5	39.45333	-0.40667	-
T1.2		41.61	41.61	41.35	41.52333	1.663333	-4.09016
T2.1		41.38	41.38	41.15	41.30333	1.443333	-
T2.2		50.5	50.5	49.26	50.08667	10.22667	7.08545
T3.1		39.54	39.54	39.24	39.44	-0.42	-
T3.2		48.47	48.47	47.54	48.16	8.3	-19.7619
Background		39.76	39.76	40.06	39.86	-	
	Day3						
N1.1		61.69	61.88	62.58	62.05	8.73	-
N1.2		63.02	63.67	64.92	63.87	10.55	1.208477
N2.1		63.67	63.92	64.63	64.07333	10.75333	-
N2.2		67.68	68.39	69.54	68.53667	15.21667	1.415065
N3.1		68.45	68.98	70.56	69.33	16.01	-
N3.2		59.34	59.43	59.59	59.45333	6.133333	0.383094
G1.1		61.74	62.09	62.26	62.03	8.71	-
G1.2		66.93	67.42	68.34	67.56333	14.24333	1.635285
G2.1		61.58	61.64	62.23	61.81667	8.496667	-
G2.2		64.21	64.67	65.77	64.88333	11.56333	1.360926
G3.1		58.79	58.77	58.89	58.81667	5.496667	-
G3.2		64.47	64.82	65.59	64.96	11.64	2.117647
T1.1		62.46	62.48	62.71	62.55	9.23	-
T1.2		68.75	69.32	70.5	69.52333	16.20333	1.755507
T2.1		66.19	66.44	67.26	66.63	13.31	-
T2.2		69.01	69.35	70.56	69.64	16.32	1.226146
T3.1		64.58	65.11	65.42	65.03667	11.71667	-
T3.2		76.4	77.3	79.5	77.73333	24.41333	2.083642
	Day4						
N1.1		68.63	69.39	70.42	69.48	16.16	-
N1.2		73.59	74.22	76.33	74.71333	21.39333	1.323845
N2.1		78.58	79.06	81.78	79.80667	26.48667	-
N2.2		77.94	78.54	81.35	79.27667	25.95667	0.97999
N3.1		74.87	75.55	77.48	75.96667	22.64667	-
N3.2		78.06	78.57	81.21	79.28	25.96	1.146306
G1.1		59.46	59.81	60.18	59.81667	6.496667	-
G1.2		65.91	66.36	67.77	66.68	13.36	2.056439
G2.1		64.37	64.93	66.03	65.11	11.79	-
G2.2		65.49	65.62	67.01	66.04	12.72	1.07888
G3.1		66.25	66.67	68.04	66.98667	13.66667	-
G3.2		69.66	70.54	72.31	70.83667	17.51667	1.281707
T1.1		62.32	62.73	63.86	62.97	9.65	-
T1.2		67.14	67.52	69.64	68.1	14.78	1.531606
T2.1		56.93	57.14	57.88	57.31667	3.996667	-
T2.2		60.4	60.71	62.18	61.09667	7.776667	1.945788
T3.1		51.26	51.32	52	51.52667	-1.79333	-
T3.2		55.98	56.24	57.7	56.64	3.32	-1.8513
Background		54.15	54.97	50.84	53.32	-	

	Day5						
N1.1		84.27	87.24	87.83	86.44667	14.57667	-
N1.2		78.85	80.56	80.9	80.10333	8.233333	0.56483
N2.1		75.61	76.4	76.52	76.17667	4.306667	-
N2.2		78	80.04	80.04	79.36	7.49	1.739164
N3.1		77.3	78.5	78.96	78.25333	6.383333	-
N3.2		78.43	80.22	81.08	79.91	8.04	1.25953
G1.1		85.09	88.59	89.18	87.62	15.75	-
G1.2		78.11	79.85	80.17	79.37667	7.506667	0.476614
G2.1		84.77	87.64	87.67	86.69333	14.82333	-
G2.2		79.89	81.29	81.12	80.76667	8.896667	0.60018
T1.1		80.06	81.62	82.13	81.27	9.4	-
T1.2		83.74	87.35	87.33	86.14	14.27	1.518085
T2.1		75.48	76.1	76.27	75.95	4.08	-
T2.2		79.05	80.75	80.8	80.2	8.33	2.041667
T3.1		82.3	84.41	85.28	83.99667	12.12667	-
T3.2		74.57	75.2	75.33	75.03333	3.163333	0.260858
Background		71.68	72.15	71.78	71.87	-	

Result: 4.6.3 Injection 2.5 μg/gram shrimp, collected 12 hours interval for 3 days

Index		Measure1	Measure2	Measure3	Mean	Adjust	Relative
	12 hours					Volume	Expression
N1.1		84.6	84.62	84.62	84.61333	9.216667	-
N1.2		89.27	89.66	89.5	89.47667	14.08	1.527667
N2.1		82.33	82.5	82.5	82.44333	7.046667	-
N2.2		82.9	82.81	82.87	82.86	7.463333	1.05913
N3.1		91.27	91.33	91.28	91.29333	15.89667	-
N3.2		90.6	90.96	90.97	90.84333	15.44667	0.971692
N4.1		101.67	102.39	102.28	102.1133	26.71667	-
N4.2		86.42	86.23	86.21	86.28667	10.89	0.407611
N5.1		100.35	100.71	100.63	100.5633	25.16667	-
N5.2		82.45	82.73	82.38	82.52	7.123333	0.283046
G1.1		93.92	93.76	93.76	93.81333	18.41667	-
G1.2		91.45	91.5	91.5	91.48333	16.08667	0.873484
G2.1		102.37	103.35	102.38	102.7	27.30333	-
G2.2		89.36	89.7	89.72	89.59333	14.19667	0.519961
G3.1		93.48	93.74	93.45	93.55667	18.16	-
G3.2		98.26	98.79	97.94	98.33	22.93333	1.262849
G4.1		97.75	97.78	98.21	97.91333	22.51667	-
G4.2		82.45	82.45	82.44	82.44667	7.05	0.313101
G5.1		102.34	102.54	102.44	102.44	27.04333	-
G5.2		83.66	83.73	83.77	83.72	8.323333	0.307778
T1.1		100.67	100.6	100.6	100.6233	25.22667	-
T1.2		90.75	91.45	91.39	91.19667	15.8	0.626321
T2.1		86.69	86.91	86.71	86.77	11.37333	-
T2.2		95.05	95.74	95.12	95.30333	19.90667	1.750293

T3.1		85.85	86.21	85.88	85.98	10.58333	-
T3.2		79.97	80.01	80.23	80.07	4.673333	0.441575
T4.1		90.24	90.14	90.14	90.17333	14.77667	-
T4.2		78.9	78.83	79	78.91	3.513333	0.237762
T5.1		91.15	91.29	91.6	91.34667	15.95	-
T5.2		76.74	76.82	76.8	76.78667	1.39	0.087147
Background		75.48	75.31	75.4	75.39667	-	
	24 hours						
N1.1		81.74	81.8	81.66	81.73333	8.43	-
N1.2		76.38	76.53	76.47	76.46	3.156667	0.374456
N2.1		96.56	96.83	97.2	96.86333	23.56	-
N2.2		91.36	91.19	91.07	91.20667	17.90333	0.759904
N3.1		95.93	95.37	96.24	95.84667	22.54333	-
N3.2		77.74	77.55	77.65	77.64667	4.343333	0.192666
G1.1		87.42	87.8	88.14	87.78667	14.48333	-
G1.2		76.81	76.65	76.64	76.7	3.396667	0.234522
G2.1		75.45	75.55	75.49	75.49667	2.193333	-
G2.2		79.25	79.19	79.36	79.26667	5.963333	2.718845
G3.1		88.66	88.91	88.99	88.85333	15.55	-
G3.2		83.93	84.07	84.4	84.13333	10.83	0.696463
G4.1		101.17	101.69	101.88	101.58	28.27667	-
G4.2		82.05	82.43	82.86	82.44667	9.143333	0.323353
G5.1		102.9	103.94	104.32	103.72	30.41667	-
G5.2		87.63	88.02	88.7	88.11667	14.81333	0.487014
T1.1		103.68	103.46	104.4	103.8467	30.54333	-
T1.2		105.58	105.4	106.53	105.8367	32.53333	1.065153
T2.1		100.08	100.19	100.36	100.21	26.90667	-
T2.2		84.97	84.8	85.13	84.96667	11.66333	0.433474
T3.1		83.62	83.45	83.86	83.64333	10.34	-
T3.2		85.14	84.74	85.38	85.08667	11.78333	1.139587
T4.1		84.48	84.48	84.57	84.51	11.20667	-
T4.2		97.75	97.41	98.37	97.84333	24.54	2.189768
T5.1		74.75	74.59	74.79	74.71	1.406667	-
T5.2		76.27	76.23	76.45	76.31667	3.013333	2.14218
Background		72.55	73.68	73.68	73.30333	-	
Index		Measure1	Measure2	Measure3	Mean	Adjust	Relative
	36 hours					Volume	Expression
N1.1		74.14	74.47	74.32	74.31	-3.31	-
N1.2		76.47	77.2	77.26	76.97667	-0.64333	-
N2.1		77.9	78.34	78.36	78.2	0.58	-
N2.2		81.09	82.12	82.38	81.86333	4.243333	7.316092
N3.1		79.63	80.11	80.2	79.98	2.36	-
N3.2		77.87	78.26	78.38	78.17	0.55	0.233051
N4.1		82.28	82.69	83.03	82.66667	5.046667	-
N4.2		92.87	95.31	95.95	94.71	17.09	3.386394
G1.1		100.34	103.87	104.7	102.97	25.35	-
G1.2		80.05	80.18	80.27	80.16667	2.546667	0.10046

G2.1		98.28	101.27	101.98	100.51	22.89	-
G2.2		88.26	89.58	89.91	89.25	11.63	0.508082
G3.1		95.92	98.4	98.9	97.74	20.12	-
G3.2		92.6	94.58	95.11	94.09667	16.47667	0.81892
G4.1		98.28	101.3	102	100.5267	22.90667	-
G4.2		86.69	87.86	88.14	87.56333	9.943333	0.43408
T1.1		96.74	99.63	100.18	98.85	21.23	-
T1.2		87.04	88.44	88.78	88.08667	10.46667	0.493013
T2.1		92.3	94.78	95.2	94.09333	16.47333	-
T2.2		81.46	82.23	82.33	82.00667	4.386667	0.266289
T3.1		95.62	98.85	99.56	98.01	20.39	-
T3.2		79.05	79.52	79.61	79.39333	1.773333	0.086971
Background		77.65	77.29	77.92	77.62	-	
	48 hours						
N1.1		93.87	95.73	95.63	95.07667	20.51	-
N1.2		81	81.57	81.48	81.35	6.783333	0.330733
N2.1		101.14	103.89	104.11	103.0467	28.48	-
N2.2		84.03	84.95	85.02	84.66667	10.1	0.354635
N3.1		88.1	89.47	89.65	89.07333	14.50667	-
N3.2		77.32	77.62	77.64	77.52667	2.96	0.204044
N4.1		78.25	78.46	78.49	78.4	3.833333	-
N4.2		75.66	75.76	75.82	75.74667	1.18	0.307826
G1.1		82.45	83.34	83.34	83.04333	8.476667	-
G1.2		75.46	75.64	75.57	75.55667	0.99	0.116791
G2.1		85.66	86.66	86.66	86.32667	11.76	-
G2.2		76.11	76.09	76.19	76.13	1.563333	0.132937
G3.1		79.03	79.36	79.33	79.24	4.673333	-
G3.2		76.81	77	76.87	76.89333	2.326667	0.49786
T1.1		93.87	95.65	95.68	95.06667	20.5	-
T1.2		83.17	83.75	83.73	83.55	8.983333	0.438211
T2.1		97.94	100.98	100.98	99.96667	25.4	-
T2.2		83.63	84.18	84.3	84.03667	9.47	0.372835
T3.1		97.38	99.88	99.71	98.99	24.42333	-
T3.2		77.68	77.89	78.07	77.88	3.313333	0.135663
T4.1		78.37	79.03	79.1	78.83333	4.266667	-
T4.2		74.5	74.56	74.62	74.56	-0.00667	-0.00156
Background		73.47	76.62	73.61	74.56667	-	
Index		Measure1	Measure2	Measure3	Mean	Adjust	
	60 hours					Volume	
N1.1		40.27	41.26	40.68	40.73667	7.03	-
N1.2		35.52	35.79	35.62	35.64333	1.936667	0.275486
N2.1		39.97	40.85	40.37	40.39667	6.69	-
N2.2		36.78	37.08	36.91	36.92333	3.216667	0.480817
N3.1		39.26	39.89	39.51	39.55333	5.846667	-
N3.2		37.59	37.96	37.73	37.76	4.053333	0.693273
N4.1		43.46	44.58	43.97	44.00333	10.29667	-
N4.2		40.62	41.4	40.89	40.97	7.263333	0.705406

G1.1		41.22	42.23	41.57	41.67333	7.966667	-
G1.2		35.96	36.09	35.94	35.99667	2.29	0.287448
G2.1		42.99	43.85	43.41	43.41667	9.71	-
G2.2		37.98	38.34	38.12	38.14667	4.44	0.457261
G3.1		42.55	43.57	43.05	43.05667	9.35	-
G3.2		37.34	37.53	37.34	37.40333	3.696667	0.395365
T1.1		41.48	42.26	41.86	41.86667	8.16	-
T1.2		38.19	38.65	38.58	38.47333	4.766667	0.58415
T2.1		43.62	45.05	44.14	44.27	10.56333	-
T2.2		36.31	36.51	36.44	36.42	2.713333	0.256863
T3.1		40.92	42.03	41.44	41.46333	7.756667	-
T3.2		35.17	35.24	35.17	35.19333	1.486667	0.191663
T4.1		37.92	38.42	38.35	38.23	4.523333	-
T4.2		39.23	40.04	39.44	39.57	5.863333	1.296242
Background		33.78	33.65	33.69	33.70667	-	
	72 hours						
N1.1		73.13	74.57	75.18	74.29333	12.11333	-
N1.2		65.04	65.42	65.54	65.33333	3.153333	0.260319
N2.1		72.75	74.11	74.42	73.76	11.58	-
N2.2		63.93	64.3	64.31	64.18	2	0.172712
N3.1		85.28	88.93	89.79	88	25.82	-
N3.2		71.44	72.96	73.23	72.54333	10.36333	0.401368
N4.1		80.4	83.3	83.23	82.31	20.13	-
N4.2		65.25	65.6	65.43	65.42667	3.246667	0.161285
G1.1		88.19	91.84	92.75	90.92667	28.74667	-
G1.2		71.14	72.3	72.18	71.87333	9.693333	0.337199
T1.1		84.9	87.71	88.4	87.00333	24.82333	-
T1.2		81.08	83.41	83.9	82.79667	20.61667	0.830536
T2.1		93.55	97.71	98.5	96.58667	34.40667	-
T2.2		82.33	84.42	85.01	83.92	21.74	0.631854
T3.1		77.29	78.93	79.26	78.49333	16.31333	-
T3.2		70.49	70.9	71.03	70.80667	8.626667	0.528811
Background		62.14	62.4	62	62.18	-	

Result: 4.6.4 Injection 5 μg/gram shrimp, collected 6 hours interval for 1.5 days

Index		Measure1	Measure2	Measure3	Mean	Adjust	Relative
	6 hours					Volume	Expression
N1.1		74.82	77.16	76.71	76.23	18.13	-
N1.2		57.61	57.67	57.63	57.63667	-0.46333	-
N2.1		67.47	68.54	68.32	68.11	10.01	-
N2.2		61.54	62.03	61.93	61.83333	3.733333	0.37296
N3.1		66.78	67.92	67.39	67.36333	9.263333	-
N3.2		60.08	60.25	60.17	60.16667	2.066667	0.223102
G1.1		74.19	76.47	75.64	75.43333	17.33333	-
G1.2		59.61	59.69	59.64	59.64667	1.546667	0.089231
G2.1		70.23	71.89	71.32	71.14667	13.04667	-

G2.2		61.44	61.75	61.48	61.55667	3.456667	0.264946
G3.1		66.82	67.74	67.26	67.27333	9.173333	-
G3.2		61	61.15	61.07	61.07333	2.973333	0.324128
T1.1		77.34	78.92	78.94	78.4	20.3	-
T1.2		63.66	63.89	63.83	63.79333	5.693333	0.28046
T2.1		79.19	81.16	80.51	80.28667	22.18667	-
T2.2		66.53	67.03	67.08	66.88	8.78	0.395733
T3.1		69.7	70.62	70.31	70.21	12.11	-
T3.2		64.88	65.22	65.11	65.07	6.97	0.575557
	12 hours						
N1.1		77.96	79.83	79.28	79.02333	20.92333	-
N1.2		66.98	67.67	67.53	67.39333	9.293333	0.444161
N2.1		83.71	85.93	85.09	84.91	26.81	-
N2.2		70.23	70.98	70.94	70.71667	12.61667	0.470596
N3.1		78.88	80.47	79.87	79.74	21.64	-
N3.2		71.59	72.19	72.01	71.93	13.83	0.639094
G1.1		106.23	110.87	109.16	108.7533	50.65333	-
G1.2		68.25	69.45	68.67	68.79	10.69	0.211042
G2.1		87.04	89.75	88.75	88.51333	30.41333	-
G2.2		65.45	66.09	66.07	65.87	7.77	0.25548
G3.1		79.67	80.77	80.96	80.46667	22.36667	-
G3.2		62.45	63	62.61	62.68667	4.586667	0.205067
T1.1		131.77	143.36	138.27	137.8	79.7	-
T1.2		70.02	71.16	71.14	70.77333	12.67333	0.159013
T2.1		121.99	128.28	125.99	125.42	67.32	-
T2.2		68.82	70.34	69.83	69.66333	11.56333	0.171767
T3.1		113.83	121.23	118.78	117.9467	59.84667	-
T3.2		60.13	60.77	60.82	60.57333	2.473333	0.041328
Background		58.99	60.1	55.21	58.1	-	
Index		Measure1	Measure2	Measure3	Mean	Adjust	Relative
	18 hours					Volume	Expression
N1.1		90.67	84.73	87.13	87.51	39.10333	-
N1.2		59.65	58.62	58.97	59.08	10.67333	0.272952
N2.1		103.08	94.73	98.62	98.81	50.40333	-
N2.2		56.19	56.16	56.15	56.16667	7.76	0.153958
N3.1		111.57	103	107.33	107.3	58.89333	-
N3.2		66	64.53	65.31	65.28	16.87333	0.286507
G1.1		92.89	87.54	90.47	90.3	41.89333	-
G1.2		63.06	61.82	62.71	62.53	14.12333	0.337126
G2.1		102.21	95.35	98.1	98.55333	50.14667	-
G2.2		63.98	62.8	63.35	63.37667	14.97	0.298524
G3.1		135.81	123.23	129.06	129.3667	80.96	-
G3.2		75.17	72.32	74.29	73.92667	25.52	0.315217
T1.1		129.77	117.65	123.18	123.5333	75.12667	-
T1.2		65.12	64.56	64.67	64.78333	16.37667	0.217987
T2.1		81.54	78.45	79.65	79.88	31.47333	-
T2.2		65.63	64.65	64.94	65.07333	16.66667	0.529549

T3.1		82.19	78.37	79.49	80.01667	31.61	-
T3.2		66.31	65.24	65.52	65.69	17.28333	0.546768
	24 hours						
N1.1		74.19	72.12	73.11	73.14	24.73333	-
N1.2		70.02	68.58	69.03	69.21	20.80333	0.841105
N2.1		81.62	78.95	80.18	80.25	31.84333	-
N2.2		61.86	61.15	61.57	61.52667	13.12	0.412017
N3.1		65.12	64.07	64.6	64.59667	16.19	-
N3.2		60.49	59.67	60	60.05333	11.64667	0.719374
G1.1		68.66	66.79	67.79	67.74667	19.34	-
G1.2		62.33	61.31	61.91	61.85	13.44333	0.695105
G2.1		73.8	71.02	72.56	72.46	24.05333	-
G2.2		61.58	60.3	61	60.96	12.55333	0.521896
G3.1		59.09	58.12	58.58	58.59667	10.19	-
G3.2		57.56	56.73	57.21	57.16667	8.76	0.859666
T1.1		60.12	58.53	59.2	59.28333	10.87667	-
T1.2		55.86	55.06	55.62	55.51333	7.106667	0.653386
T2.1		78.4	74.69	75.81	76.3	27.89333	-
T2.2		53.37	53.09	52.85	53.10333	4.696667	0.16838
T3.1		69.25	65.54	67.51	67.43333	19.02667	-
T3.2		50.86	50.26	50.41	50.51	2.103333	0.110547
Background		48.42	48.43	48.37	48.40667	-	
Index		Measure1	Measure2	Measure3	Mean	Adjust	Relative
	30 hours					Volume	Expression
N1.1		76.12	77.57	77.83	77.17333	21.54	-
N1.2		68.5	69.34	69.79	69.21	13.57667	0.6303
N2.1		81	83.17	83.59	82.58667	26.95333	-
N2.2		67.94	68.29	67.81	68.01333	12.38	0.459312
N3.1		84.61	85.34	86.47	85.47333	29.84	-
N3.2		69.77	70.17	70.47	70.13667	14.50333	0.486037
G1.1		81.08	82.3	83.01	82.13	26.49667	-
G1.2		71.82	72.57	72.38	72.25667	16.62333	0.627375
G2.1		93.64	96.61	97.07	95.77333	40.14	-
G2.2		75.61	76.09	75.97	75.89	20.25667	0.50465
G3.1		87.5	88.29	90.13	88.64	33.00667	-
G3.2		75.41	75.76	76.83	76	20.36667	0.617047
T1.1		87.76	89.5	89.97	89.07667	33.44333	-
T1.2		79.14	80.57	81.14	80.28333	24.65	0.737068
T2.1		93.02	94.83	95.54	94.46333	38.83	-
T2.2		82.05	84.05	84.38	83.49333	27.86	0.717486
T3.1		96.89	98.77	99.81	98.49	42.85667	-
T3.2		77.11	77.73	77.69	77.51	21.87667	0.510461
	36 hours						
N1.1		98.32	101.2	101.75	100.4233	44.79	-
N1.2		84.32	85.57	85.77	85.22	29.58667	0.660564
N2.1		94.32	96.89	97.4	96.20333	40.57	-
N2.2		80.98	82.25	82.44	81.89	26.25667	0.647194

N3.1		89.5	91.46	91.78	90.91333	35.28	-
N3.2		76.2	76.65	76.55	76.46667	20.83333	0.590514
G1.1		86.47	88.14	88.56	87.72333	32.09	-
G1.2		78.54	79.27	80.15	79.32	23.68667	0.738132
G2.1		80.17	81.05	81.37	80.86333	25.23	-
G2.2		76.86	78.05	78.72	77.87667	22.24333	0.881622
G3.1		84.85	85.9	87.03	85.92667	30.29333	-
G3.2		71.78	72.35	72.86	72.33	16.69667	0.551166
T1.1		84.69	86.47	87.46	86.20667	30.57333	-
T1.2		72.51	72.23	73.55	72.76333	17.13	0.560292
T2.1		75.66	77.09	77.31	76.68667	21.05333	-
T2.2		69.38	69.94	70.26	69.86	14.22667	0.675744
T3.1		85.06	86.97	88.86	86.96333	31.33	-
T3.2		69.24	70.4	71.24	70.29333	14.66	0.467922
Background		55.86	55.6	55.44	55.63333	-	

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