CHAPTER III
MATERIALS AND METHODS

1. Bed bugs and Mosquitoes

1.1 Bed bugs (*C. hemipterus*)

Bed bugs were collected from a hotel in Bangkok, Thailand, and were reared at the experimental animal Unit, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

1.2 Mosquitoes (*Ae. aegypti*)

Mosquitoes were reared and maintained at the experimental animal Unit, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

2. Materials

- Water bath (Memmert; LIO LAB Ltd. Part.)
- Incubator (Memmert; LIO LAB Ltd. Part.)
- Incubator shaker (Bio-Shaker BR 300 L; TAITEC)
- Digital balancing (Sartorious; Scientific Promotion Co. Ltd)
- pH meter (Orion 420A+, USA)
- Microwave (Intellowave; LG)
- Refrigerator
- Autoclave (HIRAYAMA HA-3D, Japan)
- Vortex mixer (FineVortex)
- Centrifuge (Centrifuge 5804 R: Eppendorf)
- Microcentrifuge (MICRO-12; Hanil Science Industrial. Co. Ltd)
- Spectrophotometry (BIO-RAD)
- Thermal cycler (2720 Thermal cycler, Applied Biosystem)
- Electrophoresis (SUB-Cell® GT; BIO-RAD)
- Gel Photodocumentation system (BIO-RAD)
- Microscope (Olympus: SZ60)
- Microinjector (Tram cell oil; Eppendorf)

- Plastic straw
- Dropper for collection of larvae and pupae
- Manual suction
- Urine plastic collection container
- Cages size 9 x 9 x 9 inch
- Clear plastic container cup
- White paper 80 gram
- Petri dish
- Tray
- LightCycler® Instrument (LightCycler® 1.5 Instrument, Roche)
- Sealed plastic container
- Ice cold box
- Safety Cabinet (Augustin™)
- Auto-pipette (Eppendorf)

3. Chemicals (Appendix)

3.1 Chemicals for prepare the media; Lueria-Bertani broth (LB), LB agar, SOB solution and SOC medium

3.2 Chemicals for prepare the competent cell

3.3 Chemicals for cloning

3.4 DNA plasmid extraction kit (QIA Spin Miniprep Kit); QIAGEN®

3.5 Solutions for DNA analysis use for separate DNA size on agarose gel electrophoresis

3.6 Chemicals for Polymerase Chain Reaction (PCR); Invitrogen®

3.7 Chemicals for *Wolbachia* extraction

3.8 Chemicals for DNA extraction

3.9 Chemicals for DNA ligation

4. Methods

4.1 Maintenance of Bed bugs

Bed bug colonies were maintained in plastic containers cup size ϕ3 x 6 inch at room temperature and a relative humidity between 70% and 80% for laying eggs. Bed bug colonies were kept at Medical Entomology Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Thailand.

4.2 Bed bug *Wolbachia* Extraction

Wolbachia were crudely extracted from 5 newly laid bed bug (*C. hemipterus*) eggs which infected with F-supergroup *Wolbachia*. The bed bug eggs were homogenized in 50 µl of homogenize buffer. The homogenate was gently centrifuge to remove cellular debris. The supernatant were used immediately for mosquito microinjection.

4.3 Microinjection of Bed bug *Wolbachia* into *Ae. aegypti* mosquitoes

Approximately 0.2 µl of bed bug *Wolbachia* extraction solution was microinjected into a newly emerged twenty-four-hour-old adult virgin female of naturally uninfected *Ae. aegypti* recipient. The mosquitoes were anesthetized on an ice cold box, the extraction solution was directly microinjected into the region between the posterior pronotum and the sternopleuron of the mosquito through a glass needle using a microinjector (Figure 3.2). 41 female mosquitoes were injected, the injected mosquitoes were allowed to recovery in a c age size 9 x 9 x 9 inch under controlled laboratory conditions at a temperature of 28^{o}C and a relative humidity between 70% and 80% supplement with 8% sucrose and 2% vitamin C. The survived mosquitoes were allowed to mate with naturally uninfected males to establish isofemale lines.

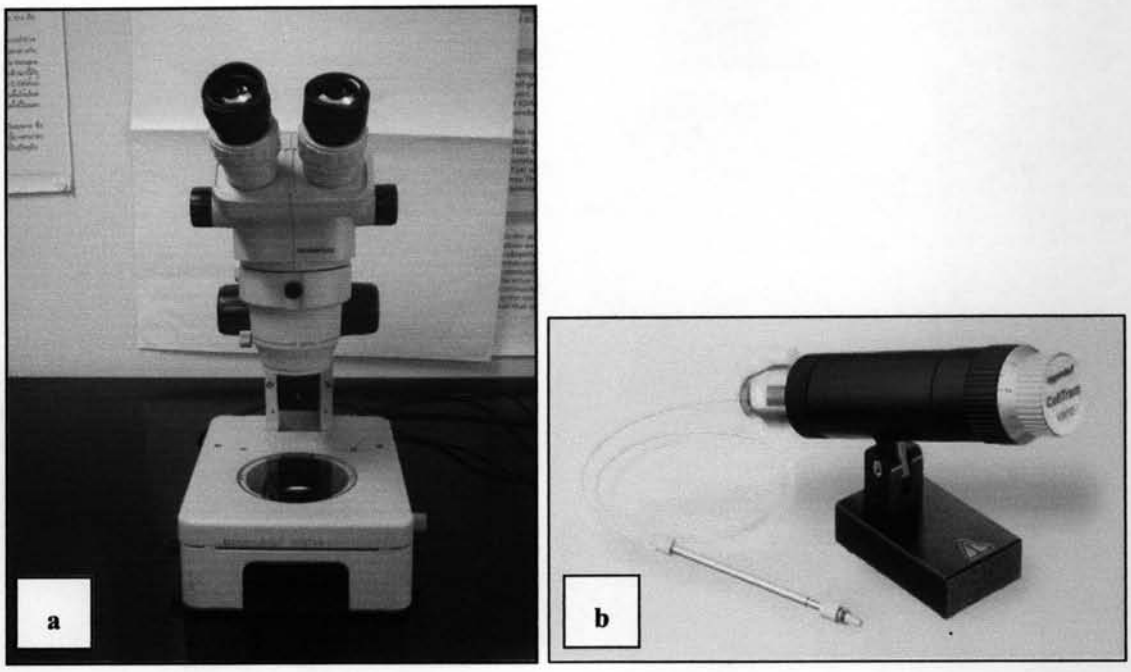

Figure 3.1 Instrument for Microinjection.

a. Microscope

b. The Tram cell oil microinjection

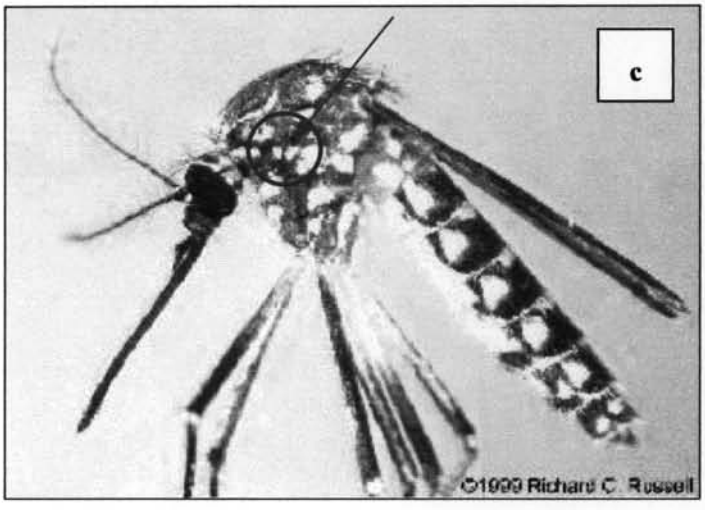

Figure 3.2 Region between the posterior pronotum and the sternopleuron of the mosquito were microinjected directly through a glass needle using a microinjector (Arrow)

4.4 Establishment of isofemale lines

Mosquitoes survived after the injection were allowed to mate with naturally uninfected males and were maintained in a cage size 9 x 9 x 9 inch. The female mosquitoes were set to feed on mice because the female mosquitoes usually need blood meals for ovarian development and produce yolk protein. Individual transinfected survived female mosquitoes were reared in a plastic container provided with a piece of the moisture cotton wool and white paper for laying eggs. Cotton wool soaked with 8% sucrose solution and 2% vitamin C was placed on top of the gauze cover and changed every other day (Figure 3.3). After female mosquitoes laid eggs on the surface of the moisture paper, the paper was removed from the plastic container and allowed to dry at room temperature (Figure 3.4). The egg paper was kept in a sealed plastic container for hatching. The female mosquitoes were killed by placing at -20°C for 10 minutes and DNA extraction was performed, present of *Wolbachia* transinfection in mosquito was demonstrated by PCR using the extracted transinfected mosquito DNA.

Figure 3.3 Transinfected female mosquito was reared individually in a clear plastic drinking cup with a piece of the moisture cotton wool and white paper.

4.4.1 Eggs

After remove the egg paper from a plastic container cup and allow air dried at room temperature. The number of egg laid was collected. Store the eggs paper in a sealed plastic container until hatching.

Figure 3.4 Eggs on the white paper were removed from the clear plastic drinking cup and allow air dried at room temperature and then were stored in a sealed plastic container.

4.4.2 Larvae

4.4.2.1 Put chlorine-free water in a rearing tray 6 x 12 inch.

4.4.2.2 Immerse the egg paper in chlorine-free water to hatch the eggs. Normally, hatching occurs within two-six hours. In this experiment, the egg paper was left in water for 1 day.

4.4.2.3 Add the larval food on the day of hatching.

4.4.2.4 Approximately 2-3 days after hatching, transfer larvae to the new rearing tray and count by a pipette. The hatching rate was calculated.

4.4.2.5 Feed the larvae with larval food from day 1 to 5. The larval development is completed within 5-6 days.

4.4.3 Pupae

4.4.3.1 An individual pupa was removed from the rearing tray with a pipette and transferred into plastic cup contained with1/4 chlorine-free water.

4.4.3.2 The adult will emerge about two days after pupation.

4.4.4 Adults

4.4.4.1 Remove the adult from the plastic container and separate of sex into the cage.

4.4.4.2 The adult mosquitoes are fed by means of the soak cotton wool providing the 8% sucrose solution and 2% Multi-vitamin syrup in the Petri dish and place in the cage.

4.4.4.3 Change the cotton wool with sucrose mixture every other day.

4.4.4.4 The next day females were mated with naturally uninfected males. And then, remove the Petri dish of cotton wool from the cage before feed mice blood meal about 6-8 hours.

4.4.4.5 Offer the mice blood meal to adult females by place the mice into the mice lock and place in the cage for 1 hr.

4.4.4.6 Remove individual female mosquito into the plastic cup with moisture white paper for laying. Place the soak cotton wool providing the sucrose mixture on the top of the gauze cover and change the soak cotton wool providing the sucrose mixture every other day.

4.4.4.7 Approximately, 3-5 days the eggs of mosquito are deposited on the surface of the moisture paper after blood feeding.

4.5 DNA extraction

Remove transinfected female mosquitoes from plastic container cup by using knock down at -20^{o}C refrigerator before the eggs were colleted. And then, DNA was extracted by using the modified salt procedure (46).

4.5.1 Individual mosquito or bed bug was homogenized in 100 µl of extraction buffer in 1.5 ml eppendorf and incubated at 65°C for one hour to cellular debris.

4.5.2 Add 15 µl of cold 8 M potassium acetate (8 M KAC) to each homogenate for precipitate the SDS.

4.5.3 Sample was incubated on ice for 45 min and centrifuged at 4°C and 12000 rpm for 10 minutes to remove cellular debris.

4.5.4 Transferred supernatant into a new 1.5 ml eppendorf. Add 250 µl of 100% ethanol to precipitate the DNA, followed by 5 min incubation at room temperature.

4.5.5 DNA was pelleted by a 15 min centrifugation at 4°C and 12000 rpm.

4.5.6 Finally, the supernatant was discarded and dried pellets. Pellets were resuspended in 10 µl of 0.1x SSC plus 20 µl of deionized water. The DNA was stored at -20°C until used.

4.6 *Wolbachia* Detection and DNA Analysis by Polymerase Chain Reaction (PCR) Technique

The PCR is a technique which is used to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested.

PCR reaction composed of

1. DNA template: a piece of DNA that want to amplify the number of copies.

2. Primers: a complementary strand of DNA in the 5' to 3' direction using a single stranded template.

3. *Taq* DNA Polymerase: enzymes which catalyze the synthesis of long polynucleotide chains from monomer.

4. Nucleotides: deoxynucleoside triphosphates (dNTPs) using one of the original parental strands as a template for the synthesis of a new complementary strands. The synthesis always proceeds in the 5' to 3' direction.

4.6.1 Normal PCR

PCR was performed by using known infected *C. hemipterus* specimens as a positive control and uninfected *Ae. aegypti* specimens as a negative control. We used a primer set of INTR2 (5'-AGTCATCATGGCCTTTATGGA-3') and INTF2 (5'-TCATGTACTCGAGTTGCAGAGT-3') (19, 47) that specifically amplifies a 136-bp fragment from *Wolbachia* 16s rDNA and a primer set of Def-F (5'-ATCACTGGTGCTTACCCACAGG-3') and Def-R2 (5'-GACGCACACCTTCTTGGAGTTG-3') (15) that specifically to defensin A of *Ae. aegypti*. This primer set were used for analyze the *Ae. aegypti* host cell and amplified a 302 bp fragment. Each 25 µl PCR reaction component were described in Table 3.1. Fragments were amplified on the ABI thermal cycler (2720 Thermal Cycler, Applied Biosystem) by using condition program as described below.

The PCR condition for *Wolbachia* and *Aedes aegypti*.

1. Initial denaturation at 95°C for 5 min 1 cycle
2. PCR consists
   - denaturation at 95°C for 1 min
   - annealing at 55°C for 1 min
   - extension at 72°C for 1 min

   } 40 cycle
3. Final extension at 72°C for 5 min 1 cycle
4. PCR products were store at 4°C

DNA fragments were resolved by 2% (wt / vol) agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light by using Gel Photodocumentation System (Bio-rad) compare with 50 bp of standard marker.

Table 3.1 Normal PCR reaction mixture consists.

Component	Volume (µl)
10x PCR buffer	2.5
25 mM $MgCl_2$	2.5
2 mM dNTP	2.5
0.5 µM Primer Forward	1.25
0.5 µM Primer Reverse	1.25
5U/ul *Taq* DNA polymerase	0.19
DNA template	5
Deionized water	9.81
Total reaction mixture	**25**

4.6.2 Re-amplify PCR product

PCR product was re-amplified by using as previous described on above except the DNA template was replaced by using 1 µl of previous PCR solution and deionized water were changed from 9.81 µl to 13.81 µl. Specific PCR re-amplification was confirmed by sequencing.

4.7 DNA Cloning and Sequencing

4.7.1 Preparation of ultra-competent E. coli cells for transformation

The purpose is to introduce a foreign plasmid into *E. coli* DH5α-bacteria and use bacteria to amplify the plasmid to make large quantities. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA. This is done by creating small holes in the bacteria cells by suspending them in a high concentration of calcium. DNA can be forced into the cells by incubating the cells and the DNA together on ice, placing fast at 42°C "heat shock" for 45 sec and then putting back on ice. This causes the bacteria to take in the DNA (48).

4.7.1.1 Culture DH5α cells from glycerol stock on LB-agar plate and incubate at 37°C overnight.

4.7.1.2 Pick up 1 colony into 2 ml of SOB solution and incubate shaker at 37°C overnight for starter.

4.7.1.3 Add 5 µl of starter as previously described on above into 250 ml of SOB solution in 1 L flask and vigorous shaking at 16°C to early log phase OD at 600 nm = 0.4-0.5 (normally it takes 24-36 hrs)

4.7.1.4 Place the flask in ice cold for 10 min

4.7.1.5 Pelleting the cells by spinning at 4000 rpm at 4°C for 10 min for select the cells.

4.7.1.6 Decant the supernatant and gently resuspend the pellet cells in 80 ml ice cold of TB solution and store on ice for 10 min.

4.7.1.7 Spin at 4000 rpm at 4°C for 10 min.

4.7.1.8 Decant the supernatant and gently resuspend the pellet cells in 20 ml ice cold of TB solution and 1.4 ml DMSO (the DMSO needs to be stored at -20°C overnight before use)

4.7.1.9 Aliquot 100 µl of the cell into 1.5 ml microcentrifuge tube for transformation or store at -70°C until use.

4.7.2 pGEM®-T Easy vector (Figure 3.4)

The pGEM®-T Easy vector of Promega® was percharged from Madison.

4.7.3 DNA ligation (Figure 3.5)

For cloning, PCR product was directly ligated into the pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5α competent cells.

4.7.3.1 Re-amplify PCR product from 4.6.2 was ligated with 50 ng/µl pGEM®-T Easy vector (Promega®) in 0.5 µl reaction tube as described in table 3.2.

Table 3.2 Ligation reaction of insert DNA into pGEM®-T Easy vector

Components	Volume (µl)
PCR product	5
pGEM®-T Easy vector	1
5X Ligation buffer	2
T4 DNA ligase	1
Deionized water	1
Total reaction mixture	10

4.7.3.2 Mix gently and stored at 4°C overnight for transformation into the ultra *E. coli* DH5α competent cells.

4.7.4 Plasmid transformation into competent cell

4.7.4.1 Pipette 100 µl of ultra *E. coli* DH5α competent cell into 1.5 ml eppendorf

4.7.4.2 Add 5 µl of ligation reaction from previous described on above (4.7.3) and place in ice cold box for 30 min.

4.7.4.3 Heat shock at 42°C water bath for 45 sec.

4.7.4.4 Place in ice cold box for 2 min.

4.7.4.5 Add 900 µl of LB broth.

4.7.4.6 Incubate shaker at 37°C for 1.30 hr.

4.7.4.7 Spread 100 µl on LB agar plate with 50 mg/ml ampicillin and 30 µl of 20 mg/ml X-Gal and 30 µl of 24 mg/ml IPTG.

4.7.4.8 Incubate at 37°C overnight.

4.7.4.9 Check colonies transformation (blue-white colonies).

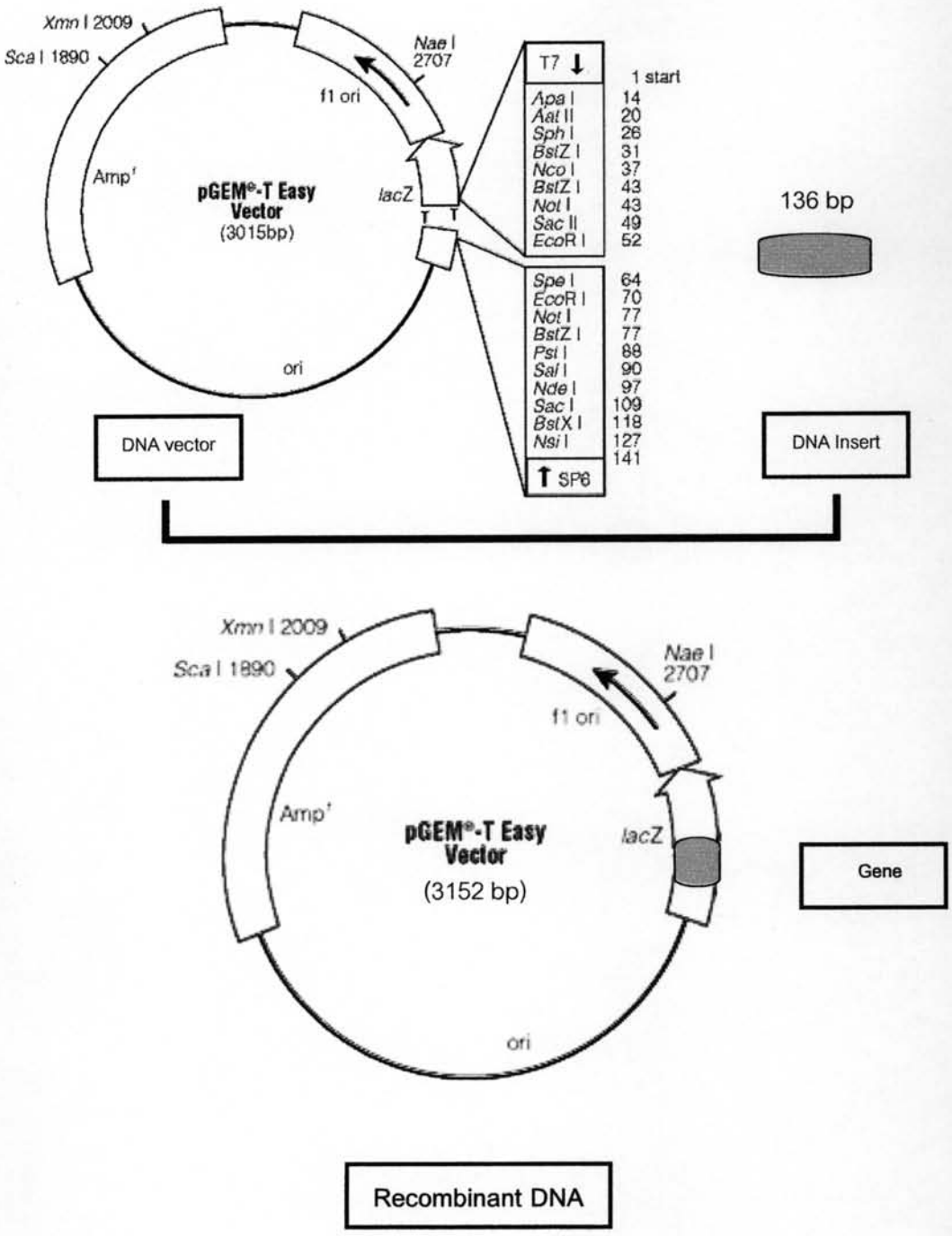

Figure 3.5 The DNA ligation (Modified from Promega. 2007). The vector is 3015 bp and carries the f1 origins of replication, the *amp* and *lacZ* markers and the SP6 and T7 promoter sequences.

4.7.5 Detection of the transformation colonies (Colony PCR)

Bacterial clones (*E. coli*) can be screened for the correct ligation products. Selection of the right (white) colonies "recombinants" is done on Luria-Bertani (LB) medium containing ampicilline, because the competent *E. coli* carry the ampicilline resistance gene. Furthermore, only those *E. coli* cells with the insert DNA will not express the *lacZ* gene, showing white colonies on LB medium containing IPTG and X-gal, whereas cells without insert will turn light to blue.

4.7.5.1 Pick up one of white colony with a sterile toothpick from LB plate into 2 ml LB broth and 2 µl of 50 mg/ml ampicillin in 15 ml centrifuge tube.

4.7.5.2 Dry shaker at 37°C overnight.

4.7.5.3 Check inserted DNA by normal PCR as a previous described on above by using 5 ul of culture transformed cell instead of the 5 ul of DNA template and resolved by 2% (wt / vol) agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light by using Gel Photodocumentation System (Bio-Rad) compare with 50 or 100 bp of standard marker.

4.7.6 Plasmid mini prep

After check inserted DNA of culture transformed cell that has been inserted DNA to plasmid. The culture cells were extracted to purify plasmid by using the Plasmid mini prep of QIA Spin Miniprep Kit; QIAGEN®.

4.7.6.1 Pipette 1.5 ml of cultured transformed cell from transformation on above into the 2 ml culture tube (provided with kit)

4.7.6.2 Centrifuge at 13000 rpm for 1 min to pellet the cells.

4.7.6.3 Remove medium by decanting.

4.7.6.4 Add 400 µl of ice-cold complete lysis solution.

4.7.6.5 Mix thoroughly by constant vortexing at the highest setting for a full 30 sec.

4.7.6.6 Incubate the lysate at room temperature for 3 min.

4.7.6.7 Transfer the lysate to a spin column assembly by decanting or pipetting.

4.7.6.8 Centrifuge the spin column assembly at 13000 rpm for 30 sec.

4.7.6.9 Add 400 µl of diluted wash buffer to the spin column assembly.

4.7.6.10 Centrifuge the spin column assembly at 13000 rpm for 30 sec.

4.7.6.11 Remove the spin column assembly from the centrifuge and decant the filtrate from the spin column assembly waste tube. Place the spin column assembly back into the waste tube and return into the centrifuge.

4.7.6.12 Centrifuged at 13000 rpm for 1 min to dry the spin column assembly.

4.7.6.13 Transfer the spin column assembly into a collection tube.

4.7.6.14 Add 50 µl of elution buffer directly to the center of the spin column membrane and cap the collection tube over the spin column.

4.7.6.15 Centrifuged at 13000 rpm for 30 sec.

4.7.6.16 Remove and *discard* the spin column.

4.7.6.17 The eluted DNA can be used immediately for downstream applications or stored at -20^{o}C.

4.7.7 Sequence analysis

To confirm the establishment of *Wolbachia* within the novel hosts *Ae. aegypti* populations. At least tree clones of all generations of transinfected lines were purified and sequenced. Plasmid DNA from each generation was measured concentration by OD absorbance at 260 nm. Analysis of the predicted nucleotides sequence, the plasmid was measured concentration and sent to First BASE Laboratories Sdn Bhd, Malaysia pass through Ward Medic Ltd., Part, Thailand. The plasmid DNA was directly sequenced on an ABI 3730 Sequencer (Applied Biosystems). Then, the nucleotide bases were performed using the NCBI BLAST programs like Nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/blast). The nucleotide bases

were aligned using a Cluster algorithm (CLUSTALX). Moreover, the nucleotide bases were translated to protein by GeneDoc MFC application (GENEDOC) program.

4.8 CI Expression

To determined the capability of CI expression. Transinfected lines from G_2 and G_4 were selected. All mating were set up with one virgin male and one virgin female. Individual crosses were done with 1 day-old virgin males and 1 day-old virgin females. Each cross was initiated by placing one male and one female that were fed blood meal before in a plastic container provided with a piece of the moisture cotton wool and white paper for laying eggs. The CI tests crosses were performed using individuals from infected male with naturally uninfected female. Levels of CI expression were compared with the tests crosses between uninfected male with uninfected female, uninfected male with infected female and infected male with infected female.

Levels of the CI expression were calculated by using SPSS version 11.5 program.

4.9 Quantitative Real-Time PCR

Real-Time PCR is quantitative PCR method for the determination of copy number of PCR templates. Real-Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the newly synthesized double-stranded DNA. Real-Time PCR assays are now easy to perform, have high sensitivity, more specificity and provide scope for automation (49).

4.9.1 LightCycler® 1.5 instrument Protocol

The following table (Table 3.3) shows the PCR parameters that must be programmed for normal LightCycler® PCR run with the LightCycler® FastStart DNA Master^{plus}SYBR Green I. In the detection channel within the photometer unit permit analyzes with the single color wavelength specific measurement at LD 530 nm only. The PCR was performed in glass capillaries.

Table 3.3 The LightCycler® Instrument program for analyzed *Wolbachia* copy number.

Analysis Mode	Cycle	Segment	Target Temperature	Hold Time	Acquistion Mode
			Denaturation		
None	1		95°C	10 min	None
			Amplification		
Quantification	45	Denaturation	95°C	10 sec	None
		Annealing	65°C	10 sec	None
		Extension	72°C	6 sec	None
			83°C	1 sec	Single
			Meating Curves		
Melting Curves	1	Denaturation	95°C	0 sec	None
		Annealing	65°C	15 sec	None
		Melting	95°C	0 sec	Continuous
			Slope =0.05°C/sec		
			Cooling		
None	1		40°C	30 sec	None

4.9.2 Preparation of the PCR Mix

A real-time PCR assay based on a single-copy gene (*wsp*) encoding a surface protein of *Wolbachia* was used to determine *Wolbachia* copy number in the hosts.

4.9.2.1 In a 1.5 ml reaction tube on ice box, prepare the PCR mix for one (20 ul) reaction by adding the following components in the order mentioned below (Table 3.4)

Table 3.4 The Real-time PCR reaction.

Component	Volume (µl)
Master Mix	4
0.5 µM PCR primer Reverse	1
0.5 µM PCR primer Forward	1
DNA template	1
H_2O (PCR grade)	13
Final Volume	20

4.9.2.2 Mix gently by pipetting up and down.

4.9.2.3 Pipette 19 µl of PCR mix into each LightCycler® Capillary (20 ul).

4.9.2.4 Add 1 µl DNA template or Standard.

4.9.2.5 Seal each capillary.

4.9.2.6 Place the capillary into the sample carousel. Centrifuge by using the LightCycler® Carousel Centrifuge for spinning the capillaries.

4.9.2.7 Place the sample carousel into the LightCycler® Instrument.

4.9.2.8 Cycle the samples as described on above (4.8.1)

4.9.3 Standard Curve of *Wolbachia*

4.9.3.1 Measures concentration of *Wolbachia* plasmid at OD absorbance 260 nm for dilution of standard curve.

4.9.3.2 Dilute the *Wolbachia* plasmid to 10^0, 10^1,10^2, 10^3, 10^4, 10^5 and 10^6ng/µl.

4.9.3.3 Each concentration were prepared as a previous described on Table 3.4 and were measured as program of the LightCycler® Instrument as a previous described on Table 3.3 based on the primer set of INTF2 and INTR2

4.9.4 Analysis of *Wolbachia* copy number

DNA was extracted as previously described. Replicate DNA from each generation of *Wolbachia* transinfected that positive with PCR methods were analysis for the copy number by using Real-Time PCR method based on the SYBR Green I.

4.9.4.1 Each generation DNA was prepared to reaction mix as a previous described on Table 3.4 by using the single-copy gene encoding a surface protein of *Wolbachia*. Specific primer set of INTF2 and INTR2 was used to measure the amount of *Wolbachia* copy number in the hosts (density).

4.9.4.2 The PCR reaction were analyzed as a program as previous described on Table 3.3 by compared with the one concentration of the standard curve.

4.9.5 Standard Curve of *Ae. aegypti*

4.9.5.1 Measures concentration of *Ae. aegypti* plasmid at OD absorbance 260 nm for dilution of standard curve.

4.9.5.2 Dilute the *Ae. aegypti* plasmid to 10^0, 10^1,10^2, 10^3, 10^4, 10^5 and 10^6ng/µl.

4.9.5.3 Each concentration were prepared as a previous described on Table 3.4 and were measured as program of the LightCycler® Instrument as show in Table 3.5 based on the primer set of Def-F and Def-R2.

Table 3.5 The LightCycler® Instrument program for *Ae. aegypti* copy number of hosts cell.

Analysis Mode	Cycle	Segment	Target Temperature	Hold Time	Acquistion Mode
			Denaturation		
None	1		95°C	10 min	None
			Amplification		
Quantification	45	Denaturation	95°C	10 sec	None
		Annealing	55°C	10 sec	None
		Extension	72°C	6 sec	None
			83°C	1 sec	Single
			Meating Curves		
Meltnig Curves	1	Denaturation	95°C	0 sec	None
		Annealing	65°C	15 sec	None
		Melting	95°C	0 sec	Continuous
			Slope =0.05°C/sec		
			Cooling		
None	1		40°C	30 sec	None

4.9.6 Analysis of *Ae. aegypti* copy number

Replicate DNA from each generation was used to measure the amount of host cell number of *Ae. aegypti* by using Real-Time PCR method based on the SYBR Green I.

4.9.6.1 DNA was prepared to reaction mix as a previous described on Table 3.4 by using the single-copy gene encoding an insect immunity. The primer set of Def-F and Def-R2 was used to measure the amount of host cell number.

4.9.6.2 The PCR reaction were analyzed as a program as previous described on above (Table3.5) by compared with the one concentration of the standard curve.

4.9.7 Performing Quantification Analyses

4.9.7.1 Standard Curves Analyses

In an absolute quantification analysis, a standard curve is used to determine the concentration of unknown samples. In a standard curve, the concentrations of standard samples are plotted against the crossing points of the samples. The X axis of the standard curve represents the log of the initial DNA concentration, and the Y axis represents crossing point in cycles. In general, the higher the crossing point, the lower the initial concentration of DNA.

4.9.7.2 Melting Curve Analyses

The melting temperature (Tm) was used to identify characteristic and specific PCR products samples. A Tm analysis uses the fluorescence measurements of the melt program to determine the melting temperature of each sample. The Tm of a sample is defined at the point at with half the dye have melts of the DNA. The specific PCR product was confirmed by 2% agarose gel electrophoresis.

Wolbachia copy number in the host cell was calculated from each generation.