CHAPTER II

MATERIALS AND METHODS

1. Mosquito strains

Two laboratory strains of *Ae. aegypti* used in this study, the PMD and PMD-R strains, were established from field caught mosquitoes from Ban Pang Mai Daeng, Mae Taeng District, Chiang Mai Province since 1997 (Somboon *et al.*, 2003) and were previously named RS and RR strains, respectively (Prapanthadara *et al.*, 2002). PMD is resistant to DDT whereas PMD-R is resistant to both DDT and permethrin. The colonies were maintained under insecticide selection pressure by exposure to standard WHO permethrin (0.25% and 0.75%) papers for at least 10 generations before the resistance ratios were determined. The eggs from these mosquitoes were harvested, dried and stored in ambient temperature. Under such conditions, the eggs can survive for several months.

2. Mosquito rearing

The method for mosquito rearing in the insectary followed the procedure mentioned in the manual rearing techniques for mosquitoes (Limsuwan *et al.*, 1987) with some modifications. The insectary room was maintained at 28°C and ~80% RH, with 12 hours day/night cycle. The filter paper containing the mosquito eggs were submerged in a plastic tray (25x35x6 cm) with 2 liters of tap water. Normally, the larvae will hatch in a few days. Growing larvae were fed every day with finely ground

dog-biscuit and the water was changed two or three times a week in order to avoid scum forming. After pupation, the pupae were separated and transferred to plastic cup containing tap water and then placed in the adult cages (30x30x30 cm). The emerging adults mosquitoes were fed with 10% w/v sucrose and 10% v/v multivitamin syrup soak in cotton wool that were changed every other day.

For this species, self mating will occur within the cage. The 3-5 days old adult female mosquitoes were fed on a blood-meal for egg production. Blood-meal was provided by a restrained white rat for 1-3 hours. Three days post feeding a filter paper wrapped in a conical shape was put in oviposition pot (plastic container, 9.5 cm in diameter and 9.0 cm high) containing distilled water, making sure that filter paper gets moist. The oviposition pot was kept inside the cage for 2-3 days. The container was removed and any excess water was drained out of the filter paper. The eggs were allowed to remain an additional 24 hours in the filter paper, and then removed and air dried for 4 days, at ambient temperature. Eggs can be stored for at least 4 months in a sealed plastic container before producing in the next generation.

3. Insecticide susceptibility test

3.1. Insecticide susceptibility test for larvae

The larval susceptibility test was conducted according to the WHO standard method (WHO, 1981a). Stock and serial dilutions of permethrin (Supelco, Belefonte, PA, USA) were prepared in ethanol. The bioassays were conducted in 400 ml beakers each containing 250 ml of distilled water with one of 5-7 different concentrations of insecticide (0.1 – 500 $\mu g\ liter^{-1}$), 4 replicates per concentration.

The ethanol content in each assay solution was limited to 0.4%. For each mosquito strain, batches of 25 early 4th instar larvae were tested per beaker. In the control experiments, 0.4% ethanol was included in 250 ml of water. Larval mortality was recorded after 24 hours exposure.

3.2 Insecticide susceptibility test for adult mosquitoes

The bioassay test was conducted according to the WHO standard method (WHO, 1981b). For each mosquito strain, bathes of 25 non-bloods fed, 1-day-old, adult females were exposed to 0.75% permethrin impregnated papers (Vector Control Research Unit, Penang, Malaysia) in standard WHO test tubes. Exposure tubes with permethrin impregnated papers were laid horizontally throughout the test. Control mosquitoes were exposed to paper without insecticide. All tests were undertaken at 25°C ± 2°C. The mosquitoes were exposed to the diagnostic dosages at 6-7 different time intervals. After the exposures periods, the mosquitoes were transferred into the holding tube lined with a clean paper and provided with 10% w/v sugar solution. The test mosquitoes and the controls were held for a 24-h recovery period and the mortality recorded.

4. Genetic inheritance of the F1534 and C1534 alleles in permethrin susceptible and resistant *Ae. aegypti*

The genetic inheritance characterization was determined according to Robertson *et al* (2007) and Robertson and Preisler *et al* (2007). About 250 virgin adults of the susceptible and the resistant strains, separated at the pupal stage, were reciprocally crossed (PMD_{female} X PMD-R_{male}; PMD-R_{female} X PMD_{male}) to produce F_1

hybrids. F_1 progeny from both reciprocal crosses were backcrossed to the resistant strain.

The larval susceptibility of the parents, crosses and backcrosses were determined by exposure to various concentrations of permethrin. To determine whether the resistance is conferred by oxidase, the synergist piperonyl butoxide; PBO (0.3 mg/ml) (Paul *et al.*, 2006) was added to each of the various concentration of permethrin in the bioassay of the F_1 progeney.

The adult mosquito susceptibility of the parents, crosses and backcrosses were also determined by exposure to 0.75% permethrin impregnated papers (Vector Control Research Unit, Penang, Malaysia) at various times.

5. Bioassay data analysis

The concentration-mortality and time-mortality responses of all bioassays were determined by probit analysis (Finney, 1971) using the software LdP Line (LdP Line, copyright 2000 by Ehab Mostofa Bakr, Cairo, Egypt). Mortality data were corrected by natural control mortality using Abbott's formular (Abbott, 1925). Bioassay data were calculated lethal concentration (LC_{50}) values for larvae and lethal time (LT_{50}) values for adult mosquitoes and their 95% fiducial limits, slopes and standard error. LC_{50} and LT_{50} values were considered significantly different if no overlap occurred between their 95% fiducial limits.

The degree of dominance (D) was determined according to Stone (1986) using the equation:

$$D = (2 \log LC_{RS} - \log LC_R - \log LC_S / (\log LC_R - \log LC_S),$$

where LC_R, LC_{RS} and LC_S are the lethal concentrations for the resistant, hybrid and susceptible individuals, respectively. $D = 1$ indicates complete dominance, $0 < D < 1$ incomplete dominance, $-1 < D < 0$ incomplete recessivity and $D = -1$ complete recessivity.

The backcross generation obtained from mating F_1 offspring with parental resistant strains were determined the larvae and adult susceptibility to test the single-gene hypothesis according to Roush and Daly (1990) and estimated the minimum number of genes affecting resistance by using the method of (Lande, 1981). In the single-gene hypothesis test, if dose response lines of the RR, RS and SS genotypes (R = resistant, S = susceptible) do not overlap, where a single-gene hypothesis is responsible for resistance then plateaus is occur in the regression line of the backcross progeny at about 50% mortality (Roush and Daly, 1990). The minimum number of independently segregating genes with equal effect contributing to resistance according to Lande's method as adapted by (Tabashnik *et al.*, 1992). In this method, if resistance is controlled by a single locus, a large increase in genetic variation in backcross progeny relative to parental strains and their F_1 progeny are observed. As the number of loci increase, decreases are expected in the extra genetic variation in backcross progeny relative to parental strains and their F_1 progeny. The slope of concentration-mortality lines is inversely related to variance (Tabashnik, 1991, Tabashnik *et al.*, 1992). Thus, relative to slopes for parental strains and their F_1 progeny indicates monogenic inheritance, whereas smaller decreases suggest that multiple loci are involved.

6. Isolation of total RNA and genomic DNA from mosquito

6.1. Total RNA extraction

Total RNA was isolated from the 4th instar larvae following the instruction manual from RNeasy Mini Kit (QIAGEN, Hilden, Germany). Thirty milligrams of the larvae were disrupted frozen by a mortar and pestle and ground to a fine powder under liquid nitrogen. The distrupted samples were added to the lysis buffer, containing guanidine isothiocyanate, and then swirled and mixed until homogeneity. Ethanol was added to the lysate, creating conditions that provide selective binding of RNA to the RNeasy silica-gel membrane. The samples were then applied to the RNeasy mini column to allow total RNA binding to the membrane, contaminants efficiently washed away. RNA was eluted in RNase-free water. Total RNA concentration was determined by absorption at 260 nm using a Spectra MR^{TM} Microplate spectrophotometer (DYNEX Technologies, Chantilly, VA, USA). This extracted RNA was used to synthesize first strand cDNA by RT-PCR.

6.2. Genomic DNA extraction from single mosquito

Genomic DNA was extracted using DNAzol Reagent (Invitrogen, Carlsbad, CA, USA) based on manufacturer's instruction. A single mosquito was homogenized with micropestle in 100 ul of DNAzol Reagent. The homogenate was centrifuged for 10 min at 10,000 g after a 5 min incubation period at room temperature. The supernatant was precipitated DNA by the addition of a half volume of 100% ethanol and centrifuged for 5 min at 8,000 g. The DNA pellets were washed twice with 70%

ethanol, air dried and resuspended in 20 ul of sterile distilled water. DNA concentration was determined by absorption at 260 nm using a Spectra MR™ Microplate spectrophotometer (DYNEX Technologies, Chantilly, VA, USA).

7. Amplification of the *Ae. aegypti* voltage-gated sodium channel gene

7.1. Amplification of the *Ae. aegypti* voltage-gated sodium channel gene using cDNA templates

First strand cDNA synthesis for RT-PCR

The total RNA was reversed transcribed to single stand cDNA using SuperScript™ III (Invitrogen, Carlsbad, CA, USA) for RT-PCR. The 20 ul of reaction volume was used for 5 ug of total RNA. The RNA mixture containing 25 ng of oligo $(dT)_{18}$ (Invitrogen, Carlsbad, CA, USA) and 0.5 mM dNTPs (New England Biolabs, Ipswich, MA, USA) was heated at 65 °C for 5 min and chilled in ice for at least 1 min. The cDNA Synthesis Mix contain 1X First-Strand Buffer, 5.0 mM DTT and 200 unit of SuperScript™ III RT was added to RNA mixture and then incubated at 50 °C for 50 min and 70 °C for 15 min to inactivate the enzyme. Finally, this cDNA was used as a template for PCR amplification.

Primer design

Optimal PCR primers were identified using the web-based Primer 3 program (Rozen and Skaletsky, 2000). Primers for amplifying the *Ae. aegypti* voltage-gated sodium channel gene fragments were designed using sequences from the *Ae. aegypti* Liverpool and China susceptible strains (Liverpool cDNAs are from GenBank accession numbers: XM_001657308 - 11 inclusive; China cDNAs are from

GenBank accession number: AY663385). Three pairs of specific primers were designed to encompass the region with putative amino acid mutations (Davies *et al.*, 2007) and used to amplify the DNA fragments (Table 2.1). Figure 2.1 shows a schematic diagram of voltage-gated sodium channel protein and the positions where putative amino acid mutations were reported.

Table 2.1 Sequences of primers for amplifying the *Ae. aegypti* voltage-gated sodium channel gene by using cDNA as template.

Primer name	Primer sequence (5'-3')	Product size (bp)	Region in sodium channel	Exon [a]	Voltage-gated sodium channel mutations [b]
IS4-6_F	ATCTCGCTGCATTGAGAACA	768	IS4 – IS6	1 - 6	I253N, V410M, E435K
IS4-6_R	CGTAGCTGTGGCAGGAAAAG				
IIS1-6_F	GGTCCAACGTTCAAGGACAA	812	IIS1 - IIS6	13 - 17	C785R, M827I, M918L, M918V, M918T, L925I,
IIS1-6_R	GAGGATGAACCGAAATTGGA				T929C, T929I, T929V, L932F, F979S, I1011M,
					L1014F, L1014S, L1014H, V1016G, S1020F
IIIS4-6_F	TTCAAGCATTCAAAACAATG	830	IIIS4 – IVS2	22 - 26	A1014V, A1494V, M1524I, F1528L, F1538I,
IIIS4-6_R	CATCAGACACTCGCTACTGA				D1549V, E1553G, L1595P

[a] Exon from the *Aedes aegypti* voltage-gated sodium channel gene. This transcript corresponds to VectorBase Transcript ID AAEL006019 and appears in supercont1.186 from nucleotide 18,685-163,945.

[b] Putative amino acid mutations were reported to correlate with insecticide resistance (Davies *et al.*, 2007) and position numbered according to the amino acid sequence of the house fly *Vssc1* voltage-gated sodium channel protein (Ingles *et al.*, 1996, Williamson *et al.*, 1996).

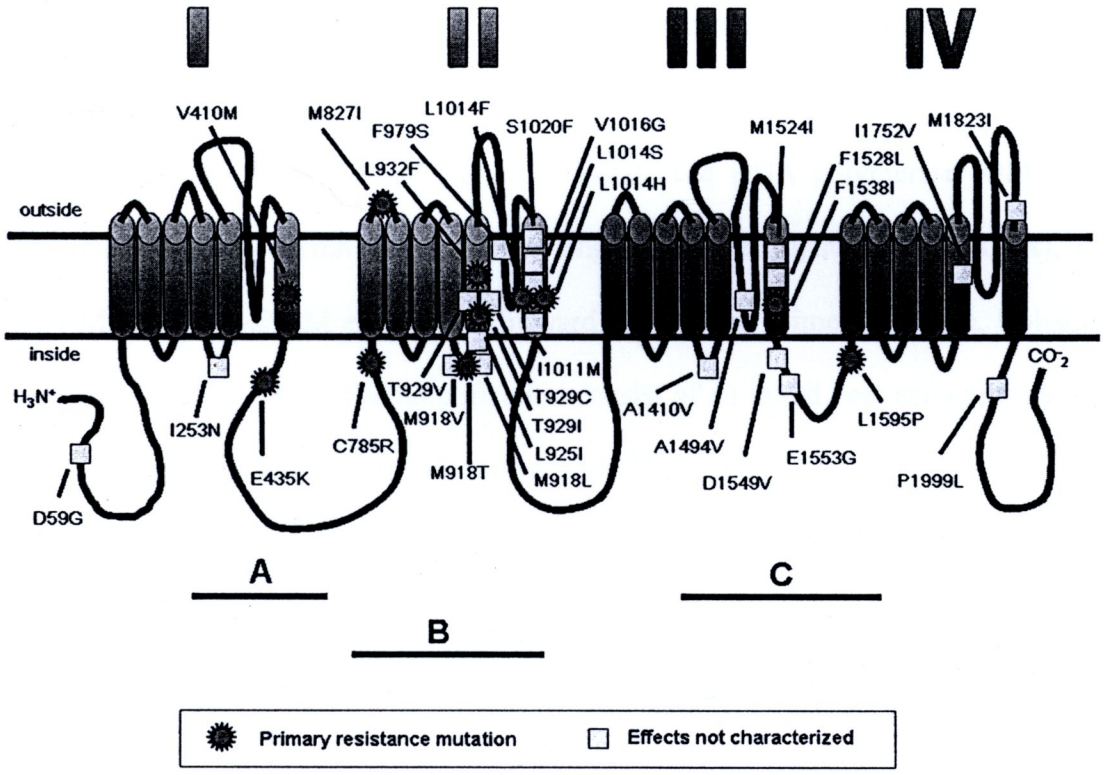

Figure 2.1 A schematic diagram of the extended transmembrane structure of voltage-gated sodium channel α subunits. Four internally homologous domains (labeled I-IV), each having six transmembrane helixes are shown. Positions where amino acid mutations reported to correlate with insecticide resistance (Davies *et al.*, 2007) are marked. The cDNA fragments A, B and C to be amplified are marked. Primers were used for each fragments are A: IS4-6 _F - IS4-6 _R, B: IIS1-6_F - IIS6_R and C: IIIS4-6_F - IIIS4-6_R, respectively.

7.1.3 Polymerase chain reaction

To determine the mutations in the voltage-gated sodium channel gene in *Ae. aegypti*, three fragments of cDNA were amplified. PCR were carried out in a 50 µl reaction volume containing 1.25 units of HotstarTaq DNA polymerase (QIAGEN, Hilden, Germany), 0.1mM dNTPs (New England Biolabs, Ipswich, MA, USA), 1.5 mM $MgCl_2$ and 0.5 µM each of the forward and reverse primers (Operon, Cologne, Germany) (Table 2.1). The amplification consist of an initial heat activation step at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 59 °C for 1 min and 72 °C for 30 s with a final extension step at 72 °C for 7 min. The amplified fragments were analyzed by electrophoresis on a 1.0% agarose gel (Invitrogen, Carlsbad, CA, USA) and visualized under UV light by ethidium bromide staining.

7.2. Amplification of the *Ae. aegypti* voltage-gated sodium channel gene using Genomic DNA templates

PCR primers were designed using the web-based Primer 3 program (Rozen and Skaletsky, 2000) to encompass the region with the V1016G and F1534C mutations (Table 2.2).

PCR was carried out in a 50 µl reaction volume containing 1.0 unit of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.1mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 µM each of the forward and reverse primers. The amplification consists of an initial heat activation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 30s with a final extension step at 72 °C for 2 min. The amplified fragments were analyzed by electrophoresis on a 1.0% agarose

gel (Invitrogen, Carlsbad, CA, USA) and visualized under UV light by ethidium bromide staining.

Table 2.2 Sequences of primers for amplifying the *Ae. aegypti* voltage-gated sodium channel gene by using genomic DNA as template.

Primer name	Primer sequence (5′-3′)	Product size (bp)	Region in sodium channel	Exon [a]
IIP_F	GGTGGAACTTCACCGACTTC	581	IIP – IIS6	16-17
IIS6_R	GGACGCAATCTGGCTTGTTA			
Ge-IIIS6_F	GCTGTCGCACGAGATCATT	635	IIIS4-IIIS6	24-26
IIIS6_R	GTTGAACCCGATGAACAACA			

[a] Exon from the *Aedes aegypti* voltage-gated sodium channel gene. This transcript corresponds to VectorBase Transcript ID AAEL006019 and appears in supercont1.186 from nucleotide 18,685-163,945.

8. Direct sequencing and sequence analysis

The voltage-gated sodium channel gene PCR fragments were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The purified PCR products were directly sequenced using the ABI Prism Big Dye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) on an ABI-PRISM 3100 automated DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequence data were analyzed using CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994) and Six Frame Translation program (Sequence Launcher, BCM) (Smith *et al.*, 1996).

9. Development of TaqMan SNP genotyping (TaqMan SNP) and AS-PCR assays for detection of the F1534C mutation in *Ae. aegypti*

9.1 TaqMan SNP Genotyping Assay

The TaqMan SNP Genotyping Assay is a single tube PCR assay that exploits the 5' exonuclease activity of AmpliTaq Gold DNA Polymerase (Livak, 1999) (Figure 2.2). The assay contains two specific primers that flank the SNP interest and two allele specific oligonucleotide TaqMan probes. These probes have a fluorescent reporter dye at 5'end and nonfluorescent quencher (NFQ) with the minor groove binding (MGB) at 3' end (Afonina *et al.*, 1997). A minor groove binder (MGB) stabilizes the double-stranded probe template structure thereby increases the probe melting temperature (T_m) without increasing probe length which allows the design of shorter probe (Kutyavin *et al.*, 2000). This results greater differences in T_m

values between matched and mismatched probes, which produce more accurate allelic discrimination.

The PCR primers amplify a specific locus on the template and each TaqMan probe anneals specifically to a complementary sequence. An intact probe emits minimal fluorescence signal when excited because the close physical proximity of the 5' fluorophore to the 3' quencher causes the fluorescent resonance (FRET) effect to quench the fluorescence emitted by the fluorophore (Livak *et al.*, 1995). A fluororescent signal is generated when the intact probe, which is hybridized to the target allele, is cleaved by the 5' exonuclease activity of AmpliTaq Gold DNA Polymerase during each cycle of the PCR reaction.

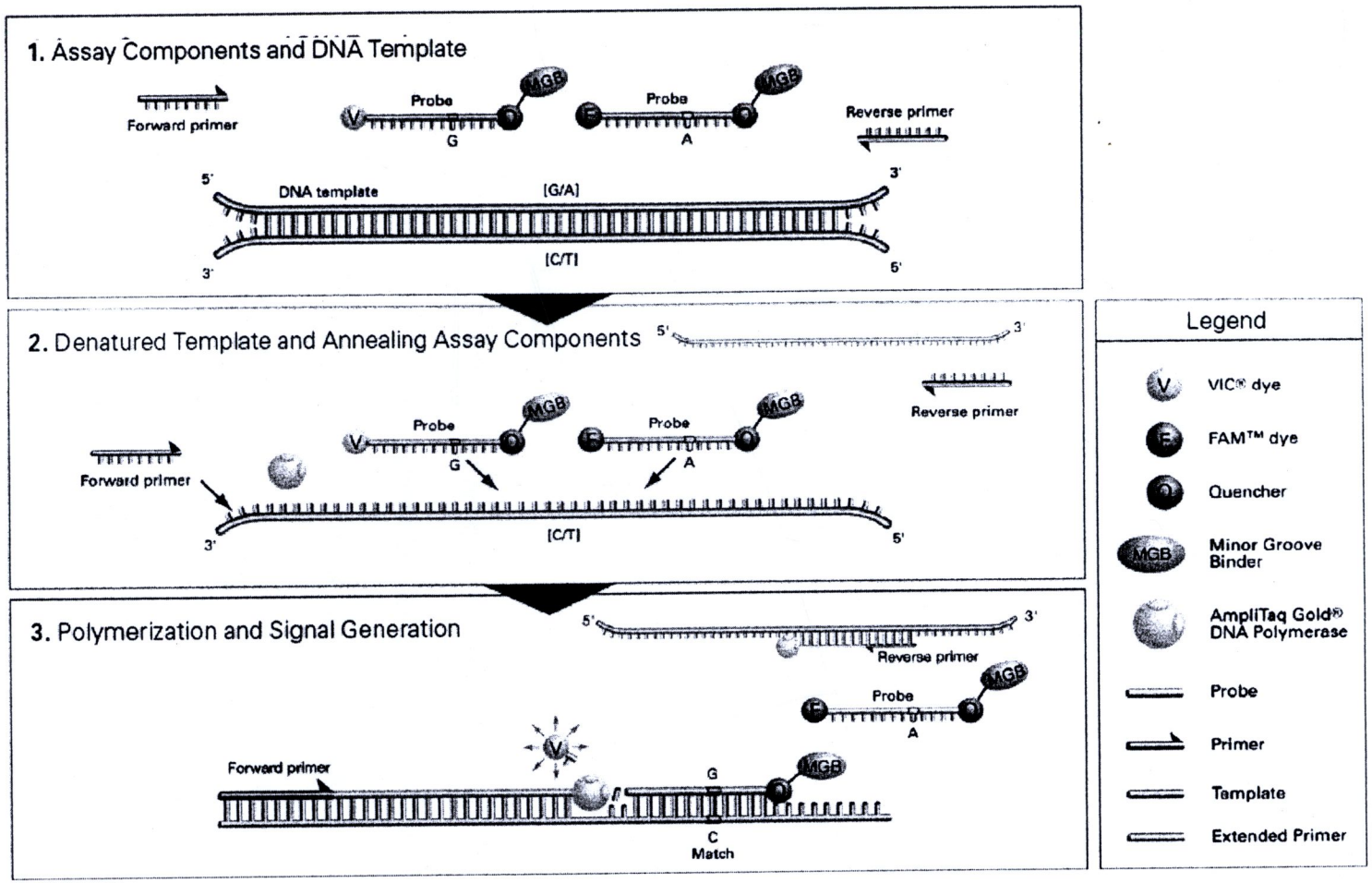

Figure 2.2 The principle of allelic discrimination using TaqMan SNP Genotyping Assay.

TaqMan Primer and Probe design

TaqMan primers and probes can be automatically designed with a propriety algorithm developed for Custom TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). These primers and probes design algorithm implements the thermodynamic and heuristic rules described in TaqMan assay design guidelines (Afonina *et al.*, 1997).

Two primers and two minor groove binding (MGB) probes (Applied Biosystems, Foster City, CA, USA) were designed using The Custom TaqMan SNP Genotyping Assay Service (Figure 2.3 and Table 2.3). Each probe consists of an oligonuclotide, a 5' reporter dye, a 3' nonfluorocence quencher (TAMRA) and a minor groove binder (MGB) at the 3' end. In both probe sets, VIC is linked to the 5' end of the probe for the detection of the wild type allele and 6-FAM is linked to the 5' end of the probe for the detection of the resistant allele.

Reaction Condition

The TaqMan SNP Genotyping assay was conducted in 25 ul volume in MicroAmp^{TM} Optical 96-well reaction plate (Applied Biosystems, Foster City, CA, USA) and sealed with MicroAmp Optical Adhesive film (Applied Biosystems, Foster City, CA, USA). Each reaction contains 12.5 ul of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1.25 ul of 20X Primer and Probe dye mix (Table 5) and 1 ul of genomic DNA making up to 25 ul with sterilised water. The assay was performed using an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) running the following thermal cycle condition of 10 min at 95 °C (AmpliTaq Gold Enzyme Activation) followed by 40 cycles of 92 °C

15 min and 60 °C for 1 min. Data were analyzed by the 7500 System SDS Software version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

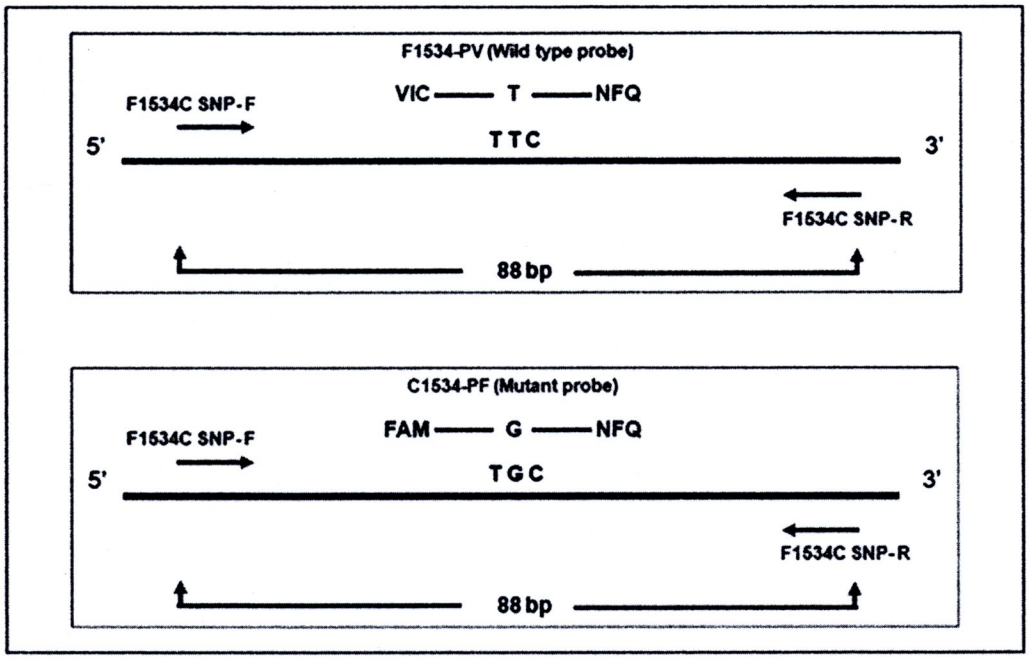

Figure 2.3 Schematic of the TaqMan SNP Genotyping for detection of the F1534C mutation. The primers and probes were designed based on the sequence of voltage-gated sodium channel from the PMD and PMD-R strains of *Ae. aegypti* (Genbank accession numbers: EU259810 and EU259811, respectively).

Table 2.3 Sequences of TaqMan primers and probes for TaqMan SNP Genotyping Assay.

Primers and Probes Name	Sequence (5'- 3')
F1534C SNP-F	CGAGACCAACATCTACATGTACCT
F1534C SNP-R	GATGATGACACCGATGAACAGATTC
F1534-PV	(VIC)-AACGACCCGAAGATGA-(MGBNFQ)
C1534- PF	(FAM)-ACGACCCGCAGATGA-(MGBNFQ)

9.2 Allele specific PCR

Primer design

Two forward allele-specific primers with the 3' base of each primer matching one of the single nucleotide polymorphism (SNP) allele bases and a reverse common primers were designed using the web-based Primer 3 program (Rozen and Skaletsky, 2000) (Table 2.4). Allele specific primers were designed first to have a T_m between 58.3 – 63.5 ^{0}C and a length of 14 – 27 bp. To improve the specificity of the assay, the two allele specific primers were introduced mismatch at the third nucleotide from the 3' terminus (Figure 2.4) as recommended by (Okimoto and Dodgson, 1996, Saavedra-Rodriguez *et al.*, 2007). If a purine naturally occurred at this site, it was replaced with a pyrimidine and vice versa. Next, a reverse common primer was identified to have a T_m between 62 – 75 ^{0}C with a length of 22 – 30 bp. To distinguish between the amplification products either base on size or melting temperature, 5' GC tails of different lengths were attached the allele specific primers (Germer and Higuchi, 1999, Wang *et al.*, 2005) (Figure 2.4).

Table 2.4 Sequences of oligonucleotides used for Allele Specific PCR. The long 26-bp GC tail has the sequence 5'- GCGGGCAGGGCGGCGGGGGCGGGGCC-3' and the short 6-bp GC tail has the sequence 5'-GCGGGC-3'.

Primer name	**Primer sequence (5'- 3')**	**Product size (bp)**
F1534-f	[Short GC-tail]TCTACTTTGTGTTCTTCATCATATT	93
C1534-f	[Long GC tail]TCTACTTTGTGTTCTTCATCATGTG	113
CP-r	TCTGCTCGTTGAAGTTGTCGAT	

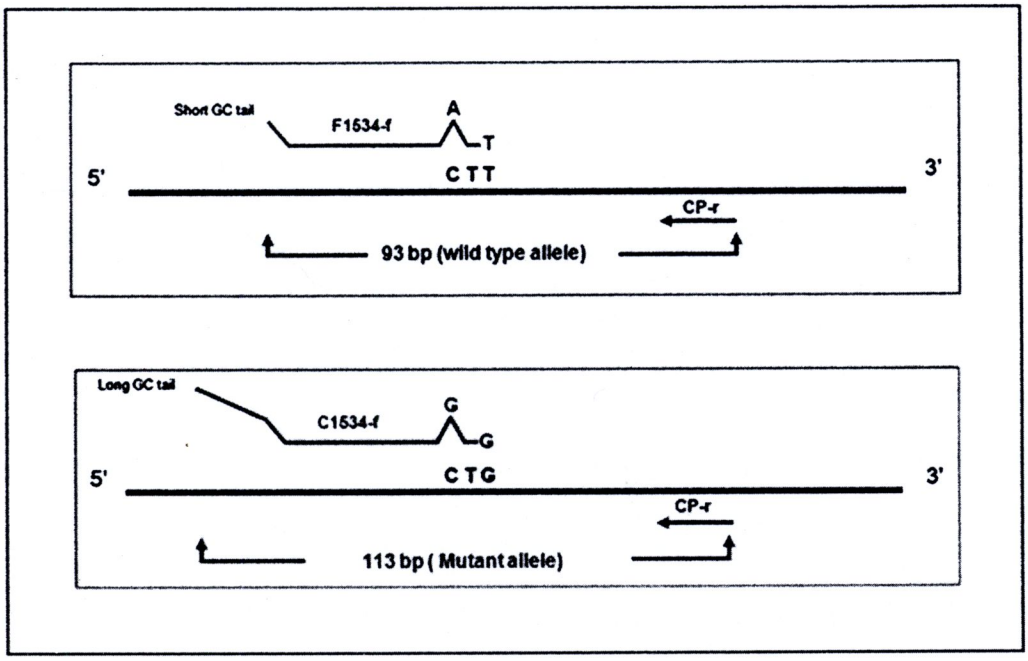

Figure 2.4 Schematic of the Allele Specific PCR assays for detection of the F1534C mutation. The primers and probes were designed based on the sequence of voltage-gated sodium channel from the PMD and PMD-R strains of *Ae. aegypti* (Genbank accession numbers: EU259810 and EU259811, respectively).

Allele Specific PCR Amplification

PCR was performed in a 50 ul reaction volume containing 1.0 unit of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM dNTPs (New England Biolabs, Ipswich, MA, USA), 1.5 mM $MgCl_2$, 0.5 uM F1534-f forward primer, 0.165 uM C1534-f forward primer and 0.5 uM common reverse primers (Invitrogen, Carlsbad, CA, USA). The amplification consists of an initial heat activation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s with a final extension step at 72 °C for 2 min. The amplified fragments were analyzed by electrophoresis on a 3.0% agarose gel (Agarose 3:1 HRB™, Amresco, Solon, OH, USA) for 45 min at 100 V alongside a low molecular weight DNA ladder (New England Biolabs, Ipswich, MA, USA).

10. Genotyping of the F1534C mutation in wild caught *Ae. aegypti* by AS-PCR

Wild *Ae. aegypti* mosquitoes were collected as larvae and pupae from 20 localities around Thailand (Table 2.5) and reared to adulthood in the insectary. Insecticide susceptibility tests were carried out for 14 localities using one-day-old non-blood fed females using WHO 0.75% permethrin paper for 60 min exposure time and mortality recorded after 24 hours. Survivors and dead individuals from this test were stored at -20 °C until tested. These mosquitoes were genotyped individually for the F1534C mutation with our developed AS-PCR method using the known genotyped laboratory strains as controls. A Pearson Chi-square test was used to compare the genotype frequency and also the allele frequency between the dead and survivor mosquito groups.

Table 2.5 Collection sites of *Ae. aegypti* in Thailand.

Region	Province	Locality	Social environment	Geographical coordinates	Sampling year
Northern	Chiang Mai	Chiang Mai City	Urban	18° 78' N, 98 ° 98' E	2007-2008
		Mae Taeng District	Rural	19° 12' N, 98° 82' E	2007-2008
	Lampang	Lampang city	Urban	18° 42' N, 99° 50' E	2007
		Donchai, Thoen District	Rural	17° 61' N, 99° 21' E	2009
	Mae Hong Son	Mae Sariang District	Urban	18° 31' N, 97° 74' E	2007
	Chiang Rai	Chiang Sane District	Rural	20° 27' N, 100° 00' E	2009
	Uttraradit	Uttraradit city	Urban	17° 65' N, 100° 12' E	2009
Central	Phitsanulok	Phitsanulok City	Urban	16° 76' N, 100° 20' E	2009
	Phetchabun	Phetchabun City	Urban	16° 40' N, 101° 15' E	2009
	Nakhonsawan	Nakhonsawon city	Urban	15° 65' N, 100° 06' E	2009
	Samutsongkhram	Amphawa District	Urban	13° 35' N, 99° 91' E	2009

Table 2.5 Collection sites of *Ae. aegypti* in Thailand (Continued).

Region	Province	Locality	Social environment	Geographic Coordinates	Sampling year
Eastern	Trat	Koh Chang Subdistrict	Rural	12° 05' N, 102° 29' E	2009
North-Eastern	Ubonratchathani	Ubon Ratchathani City	Urban	15° 29' N, 104° 85' E	2007
Western	Tak	Tak city	Urban	16° 90' N, 99° 10' E	2009
		Mae Kasa, Mae Sot District	Rural	16° 79' N, 98° 59' E	2009
		Mae Sot, Mae Sot District	Rural	16° 71' N, 98° 56' E	2009
	Kanchanaburi	Phutoey, Saiyok District	Rural	14° 48' N, 98° 84' E	2008
	Phetchaburi	Phetchaburi city	Urban	13° 11' N, 99° 94' E	2009
		Nongyapong District	Rural	13° 20' N, 99° 70' E	2009
Southern	Songkla	Ban Bo Tru, Ranot District	Rural	7° 76' N, 100 ° 32' E	2007