Chapter 3

Materials & Methods
3.1 Mosquito Strain: Establishment of *Culex quinquefasciatus* Colony

*Culex quinquefasciatus* larvae were collected from Tezpur City in Assam and were reared in the insectary of the Medical Entomology Division of Defence Research Laboratory, Tezpur, at 25 ± 3°C and 80 ± 10% relative humidity. Yeast powder was provided as larval food twice a day. Pupae was collected daily, transferred to small (250-ml) glass beakers containing clean aged water and placed in cages for the adult emergence. Adult mosquitoes were provided with cotton soaked with 10% sugar solution. Female mosquitoes were allowed to feed on a rabbit on the 3rd day after emergence. After 2-3 days, oviposition dishes were placed in the cages containing gravid females for egg laying. Eggs were collected and placed on enamel trays containing clean aged water for hatching. These adult mosquitoes were completely susceptible to deltamethrin, based on a standard WHO-recommended adult susceptibility assay (WHO 1998). The parent (F₀) colony was settled with approximately 6,000 adult mosquitoes.

3.2 Chemical Insecticides for This Study

3.2.1 Insecticide for Larval Selection

Technical grade deltamethrin (98.40% pure) was obtained free of cost from Heranba Industries Ltd., Gujarat, India. All deltamethrin-impregnated papers were made as per the WHO standard procedure. The papers were treated with 2.75ml per 180 cm² of diagnostic concentration insecticides solution.
3.2.2 Insecticides for Susceptibility Assays and Mechanisms Study

DDT (organochlorine) and deltamethrin (synthetic pyrethroid) were selected as insecticides for this study / project. The spraying of DDT within public health programme was introduced in India in 1950s and until today, DDT is the insecticide of choice in India. Synthetic pyrethroids were introduced in some parts of India in 1990s for selective control of multiple insecticide-resistant vectors (Singh et al. 2002). Deltamethrin (pyrethroid) have been popularly used for impregnation of bed nets and for indoor residual spraying for vector control in many parts of the country due to their effectiveness and their rapid excito-repellent action.

3.3 Study Sites and Its Eco-Environment

The Sonitpur district is situated on the North bank of the mighty river Brahmaputra of Assam. The district is on the longitude of 92° 20'E to 93° 45'E and latitude of 26° 20'N to 27° 05'N bounded by Arunachal Pradesh in north, river Brahmaputra is in the south, north Lakhimpur and Darrang district of Assam are in the east and west respectively (Figure 3.1). It covers an area of 5324 sq. km and is the second largest district of Assam after Karbianglong. Some parts of Sonitpur district are covered with grasses and scattered patches of rice field. On the north, it is elevated to the foothills, which is covered with dense green forest bordering Arunachal Pradesh.

In accordance with the census 2001, the total population of Sonitpur district was 1677874 comprising 864125 males and 813749 females with a density of 315 persons per sq. km. Rural and sub-urban population of the district was 1530043.
and 147831 respectively. Literacy rate was 50.79% of which male and female literacy was 57.20% and 43.98% respectively.

The Sonitpur district is having large number of tributaries originating from Himalayas flowing to the south to meet the river Brahmaputra. The drainage pattern is mainly dendritic in nature. There are lakes/swamps and ponds present every part of the district. The central and south portion of the main plain is dotted with hills and hillocks. The northern portion is made up of forest reserves and sparsely populated forest villages. The main plain has a slightly slope from east to west, and is at average latitude of 1800 feet from the mean sea level.

The climate of the region is warm, subtropical; the summer and the winter temperature ranged from 7°C to 36°C. The average annual rainfall is between 170 cm to 220 cm. This region is characterized by long rainy season starting from May to November. Winter extend from the month of October to February and is generally cold. Springs are pleasant, occurring in the month of late March to April. Hot and humid climatic conditions with high rainfall, presence of paddy fields, slow flowing streams, channels, irrigation drains, ditches, ponds provide conducive environment for breeding and proliferation of different mosquito species.

Study localities were selected based on different ecological conditions, strategic importance of the place and insecticide usage pattern. The field study was carried out in army cantonments and surrounding villages in Assam, India (Figure 3.1). The size of the entire study area was approximately 248km². The study sites were located approximately 1 to 10 km apart from each other. Following locations were selected for mosquito collection based on use of insecticides and/or the eco-environmental settings. 1) Benganajuli village, a malaria prone area where public
health workers apply DDT regularly. 2 & 3) two army animal transport Field Units (-I & -II) which are mainly surrounded by rice fields, with deep forest and tea gardens to the east of Field Unit-II. 5) Rikamari village, which is surrounded by forest. 6) The Missamari cantonment areas, where there is little or no use of DDT inside the cantonment, but pyrethroids are regularly used and some organophosphates are occasionally used. 7 & 8) The Solmara cantonment areas (-I and -II) in Tezpur, the town where the Defense Research Laboratory is situated, which is mainly surrounded by rice fields. A Geographical Information System (GIS)-based map of the study area is presented in Figure 3.1, which was created with ArcGIS 9.2 software (ESRI®ArcMap™9.2).

Figure 3.1 Study sites and its eco-environmental settings
3.4 Adult Mosquito Collection and Identification

Adult mosquitoes were collected by aspiration from human dwellings in villages and barracks in cantonments in the evening from 18.30 to 20.30 hours and early in the morning from 05.00 to 07.00 hours. Mosquitoes were identified as *Cx. quinquefasciatus* Say based on morphological characteristics after bioassays were performed.

3.5 Insecticide Susceptibility Adult Bioassay (WHO Test Kit)

Insecticide susceptibility assays were performed using World Health Organization (WHO) test kits (WHO, 1998). The tests were carried out using the diagnostic doses recommended by the WHO. The WHO test kit consisted of two plastic tubes (125mm in length, 44mm in diameter), with each tube fitted at one end with a 16-mesh screen. One tube (exposure tube) was marked with a red dot, the other (holding tube) with a green dot. The holding tube was screwed to a slide unit with a 20mm hole, into which mosquitoes were introduced into the holding tube by an
aspirator. The exposure tube was then screwed to the other side of the slide unit. Sliding the partition in this unit opens an aperture between the tubes so that the mosquitoes can be gently blown into the exposure tube to start the treatment and then blown back to the holding tube after the timed exposure. The filter papers were held in a position against the walls of the tubes by four spring wire clips: two steel clips for attaching the plain paper to the walls of the holding tube and two copper clips for attaching the insecticidal paper inside the exposure tube (Figure 3.3).

The control assays were carried out with papers treated with only carrier oils. For mosquitoes exposed to DDT, mortality rates were recorded after the recovery period, and for mosquitoes exposed to deltamethrin, the numbers knocked down were recorded every 10 minutes for up to 1 hour during exposure. All mosquitoes were provided with 10% sugar water during the 24-hour recovery period. Resistance status was evaluated according to the WHO criteria (WHO 1992), which classify mortality rates of less than 80% as indicative of resistance, while those greater than 98% indicate susceptibility. Mortality rates between 80–98% suggest the possibility of resistance that needs to be verified.
3.6 Biochemical Techniques

3.6.1 Equipments and Reagents

Equipments:

a) Ice Machine

b) Water purification system (Direct-QTM5, Millipore India Pvt. Ltd., Bangalore, India)

c) Microcentrifuge for 1.5ml Eppendorf tubes.

Figure 3.3 WHO test procedure for insecticide susceptibility in mosquitoes
d) Microplate Reader (Bio-Rad Laboratories, Philadelphia, PA)
e) Single channel micro pipette, P20, P200, P1000 variable volume
f) Multichannel pipette (8 channel & 12 channel), 10 to 100 μL.
g) pH Meter
h) Analytical balance
i) Refrigerator
j) Freezer -20 °C
k) Magnetic stirrer

Glassware & Plasticware:

a) Beakers (volume: 20, 50, 100, 250, 500 & 1000 mL)
b) Reagent Bottles (50, 100, 500 & 1000 mL)
c) Graduated cylinders
d) Falcon tubes (15 & 50 mL)
e) Pipetting reservoir
f) 96-well microplate, flat-bottom, well volume: 300 μL
g) Tips for single and multichannel pipette

Reagents (general use reagents):

Sodium acetate, anhydrous, monobasic potassium phosphate, anhydrous, Bibasic potassium phosphate, anhydrous, Bibasic potassium phosphate, tri-hidrated, monobasic sodium phosphate, anhydrous, Bibasic sodium phosphate, anhydrous

Assay specific use reagents:

Total protein assay

Commercial protein assay kit (Bangalore GENEI, Bangalore, India)
Mixed Function Oxidases assay

a) Cytochrome C (Sigma, storage: -20°C)
b) TMBZ (*) (Sigma, storage: 4°C)
c) Methanol (Merck, storage: Room temp)
d) Acetic acid glacial (Sigma storage: Room temperature)
e) 30% H₂O₂ (**) [Sigma, storage: 4°C]

Alpha (1-) & Beta (2-) Esterase assay

a) Beta-naphthyl acetate (Sigma, storage: -20°C)
b) Alpha-naphthyl acetate (Sigma storage: -20°C)
c) Beta-naphthol (*) [Sigma storage: Room temperature]
d) Alpha-naphthol (Sigma, storage: Room temperature)
e) SDS (Promega, storage: Room temperature)
f) Fast blue (Sigma, storage: 4°C)
g) PNPA (Sigma, storage: -20°C)
h) Acetonitrile (Sigma, storage: Room temperature)
i) Acetone (Merck, storage: Room temperature)

Glutathion-S-Transferase assay

a) GSH (Sigma, storage: 4°C)
b) CDNB [Sigma, storage: 4°C (*)]

[Note: (*) light sensitive reagent. Store protected from light. (**) Although storage of these reagents at room temperature is originally recommended, in the laboratory I keep them at 4°C, because of our tropical climate.]
3.6.2 Sample Preparation

Adult non blood fed female *Culex quinquefasciatus*, collected from the field and stored in liquid nitrogen, were homogenized individually in 1.5-mL microfuge tubes in 30 µL Milli-Q water, obtained from Direct-QTM5 (Millipore India Pvt. Ltd., Bangalore, India) and then diluted with additional 270 µL Milli-Q water. Tubes were kept in ice during the whole homogenization procedure. The homogenates were spun at 10000g for 2 min at 4 °C in an ultracentrifuge. The supernatant was used as a crude enzyme extract for the biochemical assays. Three microplates with duplicate mosquito homogenates were used for three enzymes and one microplate for total protein. Each biochemical assay was replicated twice with new individuals from the same mosquito population on two different days. Sample sizes for each biochemical assay ranged from 24 to 48 mosquitoes per location, depending on the availability in the field population. A minimum of three positive and three negative controls were used per plate. Absorbance was measured using a Bio-Rad Microplate Reader (Bio-Rad Laboratories, Philadelphia, PA).

3.6.3 Total protein assay

The total protein content of individual *Culex quinquefasciatus* mosquitoes was determined to correct for size variation among the specimens (Brogdon, 1984) using a commercial protein assay kit (Bangalore GENEI, Bangalore, India) according to the user's guide. The results were compared with a bovine serum albumin (BSA) standard curve. The plates were read at 550 nm wavelength.
3.6.4 **Alpha (1-) & Beta (2-) esterase assay**

The method of Peiris & Hemingway (1990) was used with alpha- and beta-naphthyl acetate as the substrate. 1-Naphthyl Acetate (NA) and 2-NA were used as substrate and Fast Blue B salt was used as stain in this assay. 30mM stock solution of 1- and 2-NA was prepared by dissolving 0.2793g NA in 50ml acetone. Working solution of 0.3mM naphthyl acetate (1 & 2) was prepared freshly by dissolving 30mM stock solution of NA in 0.02M sodium phosphate buffer pH 7.2. Stain was prepared freshly using 0.3% Fast Blue B in 3.5% SDS. 10 μl of mosquito homogenates were distributed, in duplicate, in one microplate; “blank” wells contained 10 μl of water. Then 200 μl of 1-NA/Na phosphate working solution was added in each well and incubate at room temperature for 15 minutes. After that 50 μl of Fast Blue stain was added to each well and incubate for another 5 minutes. The plates were then read at 550 nm wavelength.

3.6.5 **Glutathion-S-Transferase assay**

GST activity was assayed following the method of Brogdon and Barber (1990) with some minor modifications. Reduced glutathione (GSH) was used as the substrate. 10mM GSH was prepared by dissolving 0.0648 g GSH in 20 ml of 0.1 M potassium phosphate buffer (pH 6.5). 63mM chlorodinitrobenzene (CDNB) was prepared by dissolving 0.1278 g of CDNB in 10 ml methanol. Working solution (GSH/CDNB) was prepared by mixing 20 ml of GSH/phosphate buffer solution with 1 ml of CDNB/methanol solution. All chemicals were freshly prepared 1 hour before experiment. 10 μl of mosquito homogenates, in duplicate, were distributed in microplate plate; “blank” wells contained 10-μl water. 195 μl of working
solution (GSH/CDNB) were added to each well. The plate was read at 340 nm after 20 minutes incubation as the end point.

3.7 Molecular Techniques

3.7.1 Genomic DNA Extraction

Genomic DNA extractions from individual mosquito were performed by either manual method (modified Coen et al. 1982) or using High Pure PCR Template Preparation® kit (Roche Applied Science, Mannheim, Germany).

**DNA Preparation: Manual Method**

i. Individual mosquitoes were put into separate 1.5 ml microcentrifuge tube.

ii. The mosquitoes were dry at 95°C for two hours.

iii. 25μl of grinding buffer [0.1M NaCl, 0.2M Sucrose, 0.1M Tris-HCl (pH 9.1), 0.05M EDTA (pH 8.0), 0.05% SDS (pH 7.2)] was added to each of the tubes and then the mosquitoes were grinded with pestles until a homogenous mixture was formed with minimum cell debris. The pestles were then washed with additional 25μl of grinding buffer inside the respective micro centrifuge tubes so that each of the tubes contained 50μl of homogenate solution.

iv. Then the homogenates were briefly microfuged at 4000 rpm to precipitate the cell debris at the bottom of the tube.

v. Homogenates were incubated at 65°C for 30 minutes and 7μl of 8M potassium acetate solution was added into it while the tubes were warm.
vi. Then the tubes were incubated in ice for 30 minutes (to precipitate down SDS & proteins).

vii. The tubes were centrifuged at 14000 rpm for 15 minutes at 4°C.

viii. The supernatant was collected in a fresh 1.5 ml microfuge tube.

ix. 100µl of chilled 100% ethanol (stored at -20°C) was then added to the supernatant and gently mixed. The tubes were incubated at room temperature for 10 min to precipitate the nucleic acid.

x. After that, the tubes were centrifuged at 17000 rpm for 15 minutes at 4 °C. Ethanol was carefully removed and 70% alcohol was added.

xi. The tubes were centrifuged at 17000 rpm for 5 minutes at 4 °C and 70% ethanol was carefully removed.

xii. 100µl of 100% ethanol was added to each tube and centrifuged at 14000 rpm for 15 minutes.

xiii. Finally, ethanol was carefully removed and the pellet was air-dried.

xiv. The pellets were re-suspended in 100µl of TE buffer and stored at 4°C.

**DNA Preparation: High Pure PCR Template Preparation® kit**

The kit was standardized for preparation of genomic DNA from mosquito sample by modifying the supplier’s protocol.

i. Individual mosquitoes were taken in nuclease-free 1.5ml microcentrifuge tubes.

ii. 50 µl Tissue Lysis Buffer (kit content) and 10 µl Proteinase K were added to each tube. It was then immediately mixed and incubated for 1 hr at 55°C (or until the sample was digested completely).
iii. After incubation, 50 µl Binding Buffer (kit content) was added and mixed immediately. It was then incubated for 10 min at 70°C.

iv. 25 µl of isopropanol was added and mixed properly. The insoluble segments were withdrawn using a pipette tip.

v. Spin columns (kit content) were then inserted into collection tubes (kit content). Homogenates were pipette into the high filter of the Spin column-collection tube assembly and centrifuged for 1 min at 8000 x g (10,573 rpm).

vi. After centrifugation, collection tubes along with the flow through liquid were discarded.

vii. The spin columns were then combined with new collection tubes.

viii. Then 150 µl Inhibitor Removal Buffer (kit content) was added. It was then incubated for 2-3 min and centrifuged for 1 min at 8000 x g (10,573 rpm).

ix. Collection tubes were discarded along with the flow through liquid.

x. 150 µl Wash Buffer (kit content) was added and incubated for 2-3 min. Then it was centrifuged for 1 min at 8000 x g (10,573 rpm).

xi. After discarding the flow through, additional 150 µl of Wash Buffer was added and centrifuged for 1 min at 8000 x g (10,573 rpm). Flow through was discarded and the spin columns were centrifuged for additional 10 second at high speed (15,000 rpm).

xii. DNA was eluted into 1.5 ml microcentrifuge tubes by 75 µl pre-warmed Elution Buffer (kit content). It was incubated for 2-3 min and centrifuged for 1 min at 8000 x g (10,573 rpm).
3.7.2 Polymerase Chain Reaction (PCR)

The components used in a standard 50µL Polymerase Chain Reaction (PCR) are shown in Table 3.1. PCR amplification was performed in an Eppendorf Mastercycler using the conditions: 1 cycle at 94°C for 5 min followed by 29 cycle of PCR at 94°C for 1 min, 48°C for 2 min and 72°C for 2 min; followed by 1 cycle at 72°C for 10 min as the final extension.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Buffer (Sigma &amp;/Roche)</td>
<td>1 X</td>
<td>5µl</td>
</tr>
<tr>
<td>10mM dNTPs (Sigma &amp;/Roche)</td>
<td>200 µM</td>
<td>1µl</td>
</tr>
<tr>
<td>Forward primer (cq1)</td>
<td>10 picomole</td>
<td>1µl</td>
</tr>
<tr>
<td>Reverse primer (cq2)</td>
<td>10 picomole</td>
<td>1µl</td>
</tr>
<tr>
<td>Taq Polymerase (Sigma &amp;/Roche)</td>
<td>0.05 unit/µl</td>
<td>0.5µl (2.5 unit)</td>
</tr>
<tr>
<td>Template DNA</td>
<td>10 ng</td>
<td>5µl</td>
</tr>
<tr>
<td>PCR grade water (Fermentas &amp;/Roche)</td>
<td>50µl</td>
<td>36.5µl</td>
</tr>
</tbody>
</table>

3.7.3 Allele Specific Polymerase Chain Reaction (AS-PCR) Assay

For allele specific PCR assay, two PCR reactions were run in parallel and in each of them, the template was 10 ng of single mosquito DNA. In one PCR reaction, the primers Cq1, Cq2 and Cq3 were combined (10 pmol each) and in the other one Cq3 were replaced by Cq4. The components used in a standard 50µL AS-PCR are shown in Table 3.2. AS-PCR amplification was performed in an Eppendorf Mastercycler. The PCR conditions were 5 min at 94°C for the first cycle followed
by 1 min at 94°C, 2 min at 49°C and 2 min for 72°C for 29 cycles and 10 min at 72°C for the final extension.

**Table 3.2 Components used for AS-PCR assay standardization**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Buffer (Sigma &amp;/Roche)</td>
<td>1 X</td>
<td>5μl</td>
</tr>
<tr>
<td>10mM dNTP (Sigma &amp;/Roche)</td>
<td>200 μM</td>
<td>1μl</td>
</tr>
<tr>
<td>Forward Primer (1cq1)</td>
<td>10 picomole</td>
<td>1μl</td>
</tr>
<tr>
<td>Reverse Primer (2cq2)</td>
<td>10 picomole</td>
<td>1μl</td>
</tr>
<tr>
<td>Forward Primer (3cq3 / cq4)</td>
<td>10 picomole</td>
<td>1μl</td>
</tr>
<tr>
<td>Taq Polymerase (Sigma &amp;/Roche)</td>
<td>0.05 unit/μl</td>
<td>0.5μl (2.5 unit)</td>
</tr>
<tr>
<td>Template DNA</td>
<td>-</td>
<td>2μl</td>
</tr>
<tr>
<td>PCR grade water (Fermentas &amp;/Roche)</td>
<td>50μl</td>
<td>38.5μl</td>
</tr>
</tbody>
</table>

**3.7.4 Agarose Gel Electrophoresis**

Agarose gels were prepared by adding the required amount of powdered agarose to 1 X TAE buffer (50 X stock: 24.2g Tris base, 10 ml 0.5M EDTA, 5.71 ml glacial acetic acid – adjust to 100 ml dH₂O and dissolve). 2μL of 10μg/μL ethidium bromide was added to gels, they were poured and allowed to cool. Samples were separated on 0.7-4.0% agarose gel, depending on the expected size of the product, at 50-250V.
3.7.5 PCR Purification

PCR products were purified using the QIAquick PCR purification kit (Qiagen). Five volumes of the PBI buffer were added to one volume of the PCR reaction solution and mixed. The samples were applied to the spin column (kit content) and centrifuged for 30-60 seconds. The flow-through was discarded and 0.75 ml PE buffer was added to each spin column and centrifuged for 30-60 seconds. The flow-through was discarded and the spin columns were centrifuged for 1 min. Each of the spin columns were placed in a fresh clean 1.5ml microfuge tubes and 50μl elution buffer (EB buffer) was added to elute the DNA. For increased DNA concentration, additional 30μl of the elution buffer was added. The column is allowed to stand for one minute, and then it is centrifuged for 60 seconds.

3.7.6 DNA Sequencing

PCR products were directly sequenced on both strands. DNA sequencing was carried out using Applied Biosystems PRISM® BigDye™ with standard protocol. Sequencing was performed in University of Delhi, South campus, New Delhi and/or Bangalore Geneci, Bangalore, India.

3.7.7 Real-time Polymerase Chain Reaction

Real-time RT-PCR was performed on a Light Cycler 480® Realtime PCR machine using SimpleProbe® probe and Light Cycler 480 Genotyping Master© mix (Roche, Germany), which contained genotyping master; 5X concentration [Taq DNA Polymerase, reaction buffer, dNTPs (with dUTP instead of dTTP)], 15 mM MgCl₂, PCR grade water. LightCycler®480 Genotyping Master was diluted 1:10
to prepare the PCR mix for SimpleProbe probe. The final concentration of MgCl₂ used was 3 mM. The components and conditions used are shown in Table 3.3 and 3.4.

The 96-well Real-time PCR plate was taken and subset was selected according to the number of DNA sample along with one negative control. 15μl of PCR mixture (Table 3.3) was added to each well of the subset and mixed well. Then 5μl of sample DNA was added and gently tapped. PCR plate was sealed with sealing foil and loaded into the Light Cycler480 machine.

**Table 3.3 Component used for Real-time PCR genotyping**

<table>
<thead>
<tr>
<th>Components</th>
<th>Per sample (μl)</th>
<th>Total volume (μl) for each 25 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>7.8</td>
<td>195</td>
</tr>
<tr>
<td>Forward Primer (2.5 μM)</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Reverse Primer (0.5 μM)</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>SimpleProbe® (0.2 μM)</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>MgCl₂ (1.5mM)</td>
<td>1.2</td>
<td>30</td>
</tr>
<tr>
<td>5X PCR Master Mix</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>(include 1.5mM MgCl₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td>15</td>
<td>375</td>
</tr>
<tr>
<td>Sample (DNA)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Program Name</td>
<td>Accusation</td>
<td>Ramp rate</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>Pre-Incubation:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 95°C for 10 minutes</td>
<td>None</td>
<td>4.4°C/Sec</td>
</tr>
<tr>
<td><strong>Amplification:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 95°C for 10 seconds</td>
<td>None</td>
<td>4.4°C/Sec</td>
</tr>
<tr>
<td>(b) 53°C for 20 seconds</td>
<td>Single</td>
<td>2.2°C/Sec</td>
</tr>
<tr>
<td>(c) 72°C for 20 seconds</td>
<td>None</td>
<td>4.4°C/Sec</td>
</tr>
<tr>
<td><strong>Melting Curve:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 95°C for 10 seconds</td>
<td>None</td>
<td>4.4°C/Sec</td>
</tr>
<tr>
<td>(b) 48°C for 20 seconds</td>
<td>None</td>
<td>1.5°C/Sec</td>
</tr>
<tr>
<td>(c) 40°C for 4 minutes</td>
<td>None</td>
<td>1.5°C/Sec</td>
</tr>
<tr>
<td>(d) 75°C for continues</td>
<td>Continues</td>
<td>1.5°C/Sec</td>
</tr>
<tr>
<td><strong>Cooling:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40°C for 30 seconds</td>
<td>None</td>
<td>4.4°C/Sec</td>
</tr>
</tbody>
</table>

Table 3.4 Thermal cycling conditions used in Real-time PCR genotyping