# CHAPTER V

# RESULTS

## 1. Primes and probes design

Dengue virus consensus primer DEN, DENR, and DENG were designed from available published sequences with the aid of a ClustalX program and Primer3 (URL: http://frodo.wi.mit.edu/primer3-code.html). The following criteria were used in designing the primers: (i) maximum homology to the four genotypes and all strain of each genotype that available from NCBI (www.ncgi.nlm.nih.gov/), (ii) high melting temperature, and (iii) nonhomology to other regions of dengue virus genomes. To fulfill this criteria primer DEN and DENR, are longer than usual (29, 28 bp respectively) in order to compensate for the mismatch with some dengue genotype, as shown in table 3 along with their genome positions and product size. When used in enzymatic amplifications the type specific primers shown in table 3 (DEN1, DEN2, DEN3, and DEN4) were designed to anneal specifically to each of their respective genomes. The position of the type-specific primer was design in such a way that, when it was amplified the PCR products was generated a fragment of 204 bp for dengue genotype 2, 275 bp for dengue genotype 3, and 377 bp for dengue genotype 4, and dengue genotype 1 is 467 bp (Figure 3). This result will aid user for genotype identification by gel electrophoresis.

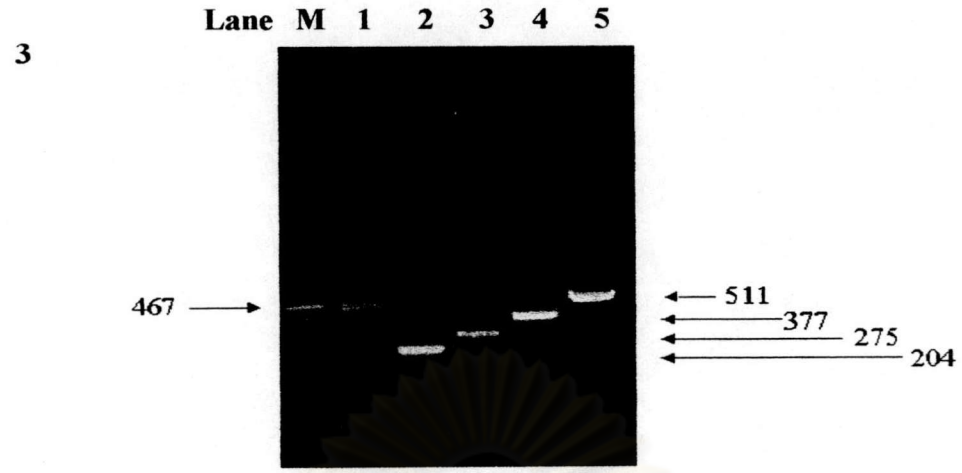



Figure 3. Gel electrophoresis from conventional multiplex nested PCR. Lane M 100 bp marker, lane 1 467bp for dengue genotype 1, lane 2 204 bp for dengue genotype 2, lane 3 275 bp for dengue genotype 4, lane 5 377 bp for dengue genotype 4, and lane 5 511 bp of first round PCR product.

**2. Detection and typing of dengue virus by multiplex nested RT-PCR**

Multiplex nested RT-PCR assay was developed to reversed transcribe dengue viral RNA and amplify four differently sized of PCR products according to a genotype of dengue virus. Sera from 100 dengue virus infected patients were determined by multiplex RT-PCR. RNA isolated was subjected to the RT-PCR assay according to the studied by Lanciotti et al (19). The size of DNA product was obtained for each of dengue viruses after amplification with primers DEN and DENR. The first round amplification was generated DNA products of 511 bp for all dengue virus genotypes. (Figure.3a) Each of DNA product was types by second round of amplification with the type-specific primers. These primers are positioned such that a differently sized product is generated from each type, dengue genotype 1, 467 bp; dengue genotype 2, 204 bp, dengue genotype 3, 275 bp, and dengue genotype 4, 377 bp. Several modifications were made from the original protocol. The concentration of each primer was adjusted for the optimal amplification in all four PCR products. First PCR products that positive by Nested PCR were used for genotype detection by real-time PCR.

### 3. Primer DENLC

Before conducting the real-time PCR, the synthesized PCR primers DENG and DENLC that used in real-time PCR were first tested in a conventional thermocycler to determine the optimal conditions for generating appropriate PCR products. This is especially important since PCR amplification can occur at a wide variety of conditions. The optimal annealing temperature for primers was chosen based on the maximal yield of PCR product with low or formation of primer-dimers or nonspecific products. Recombinant plasmid, pTT47, contain dengue genotype 2 New Guinea C strain, was used in the PCR assay. The first round amplification was performed by using DEN and DENR. The size of DNA product is 511 bp. The second round amplification with DENG and DENLC was obtained the DNA product size 220 bp (Figure 4). The nested PCR assay was performed by varies the annealing temperature from 50 to 55°C. The 55°C was chosen as annealing temperature in order to reduce nonspecific amplification. Later, these optimal PCR conditions were imported into the LightCycler.

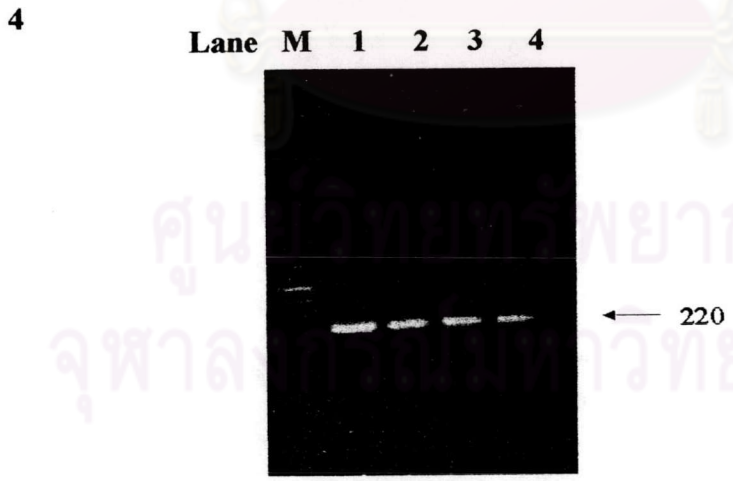



Figure 4. Gel electrophoresis of DEN 1-4 from primer DENG and DENLC. Lane M 100 bp marker, lane 1-4 DEN 1-4 220 bp

## 4. Cation titration

Preliminary nested real-time PCR with melting curve genotyping experiments suggested that hybridization of the FRET probes required optimization for mutation discrimination during this portion of the procedure. $Mg^{2+}$ titration (1-8 mM) experiments were done to determine the optimal cation concentration for detection of dengue genotype-specific melting peak by using recombinant plasmid, pTT47 (Figure 5). The reactions with 3 mM $MgCl_2$ provided the greatest discrimination among the melting peaks for the genotypes tested.

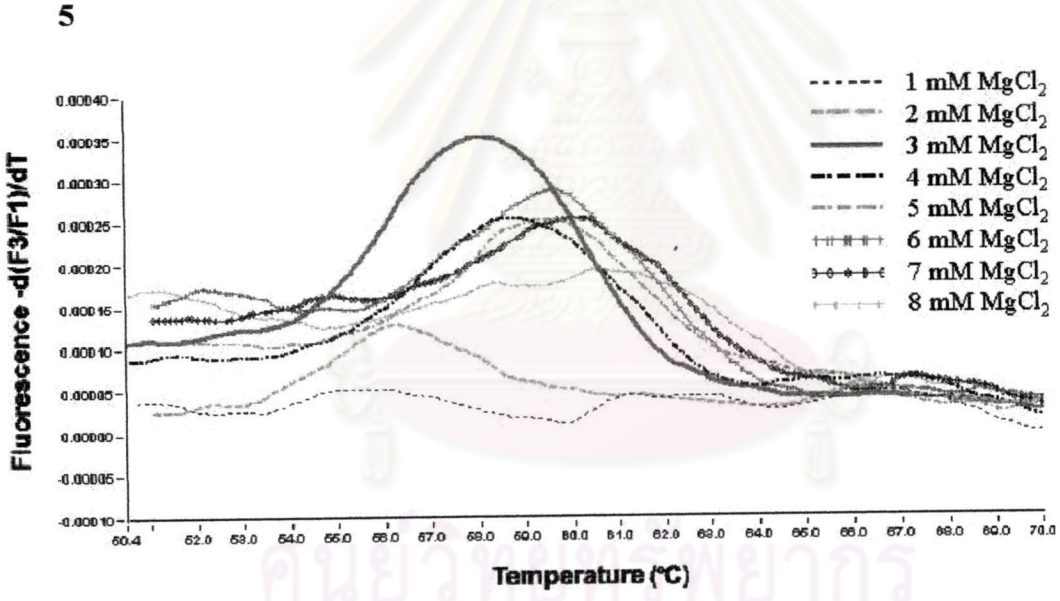
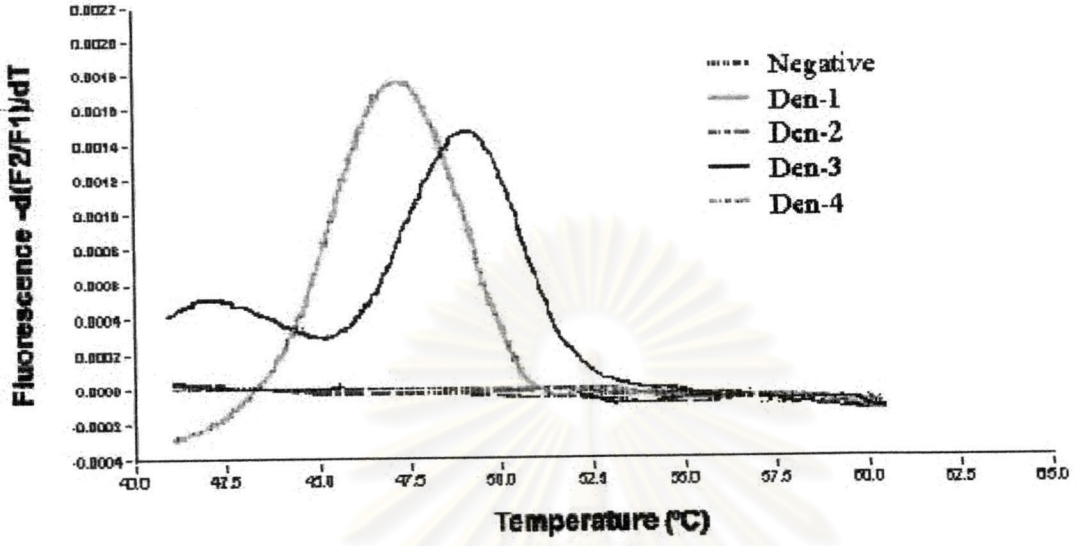



Figure5. $Mg^{2+}$ titration experiment using recombinant plasmid, pTT47, show derivative melting curve plots of LC-Red 705 fluorescence [-d(F3/F1)/dT] vs temperature were obtained with pTT47 in 1, 2, 3, 4, 5, 6, 7, or 8 mM $MgCl_2$

**5. Comparative testing**

The melting temperature of dengue virus genotype 1-4 from the LightCycler — FastStart DNA Master Hybridization Probes Reaction Kit (Roche Diagnosis, Germany) and in-house master mix were evaluated whether in-house master mix can be used instead of the commercial kit. The same samples were done in both reactions. Figure 6 (A-B) showed the melting peak of dengue genotype 1-4 by using in-house master mix and Figure 7 (A-B) using the commercial kit. Dengue genotype 1 and 3 in the in-house master mix was gave a fluorescence signal better than commercial kit. The fluorescence signal of dengue genotype 2 and 4 of both reactions was similar. This result demonstrates that the commercial reaction mix can be substitute by in-house master mix without any deterioration of the result. In order to save the budget in this study, we used the in-house master mix for diagnosis and genotyping the dengue virus.

6A




6B

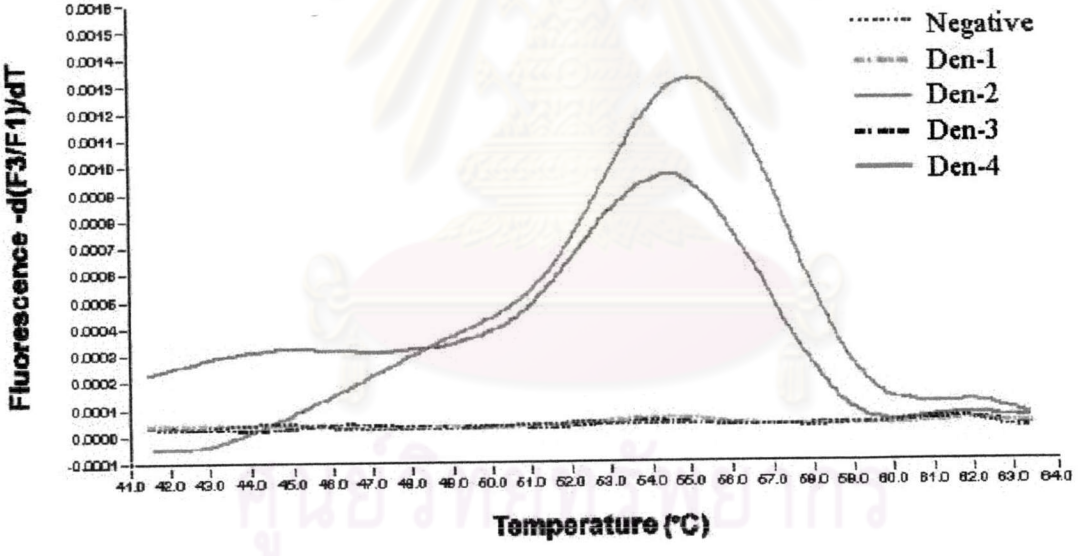
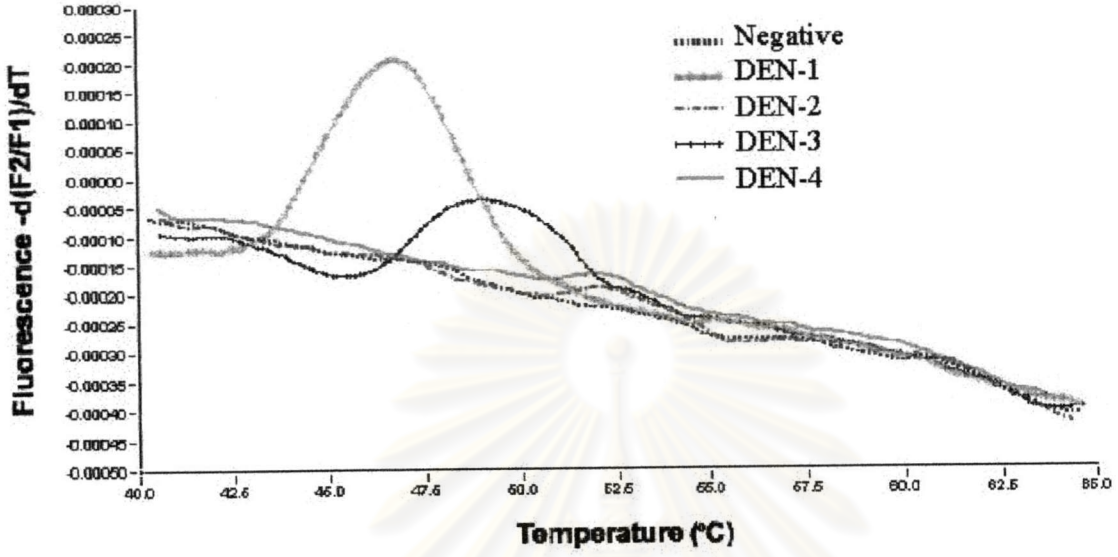



Figure 6(A-B). Genotype-specific melting transitions for four samples in 3 mM $MgCl_2$ by using the in-house master mix. Data were obtained by monitoring the fluorescence of FRET detection probe during heating from 40 to 70°C. The dengue genotypes are distinguished by their different $T_m$s. (A) $T_m$ analysis performed, and fluorescence signal were first measured in F2 for LC-Red 640 emission. Dengue genotype 1 and 3 isolates can easily be determined by different $T_m$ values. (B) For detection the dengue genotype 2 and 4, $T_m$s are determined using measurement of LC-Red705 emission in F3. Genotype 2 and 4 display close $T_m$s.

7A




7B

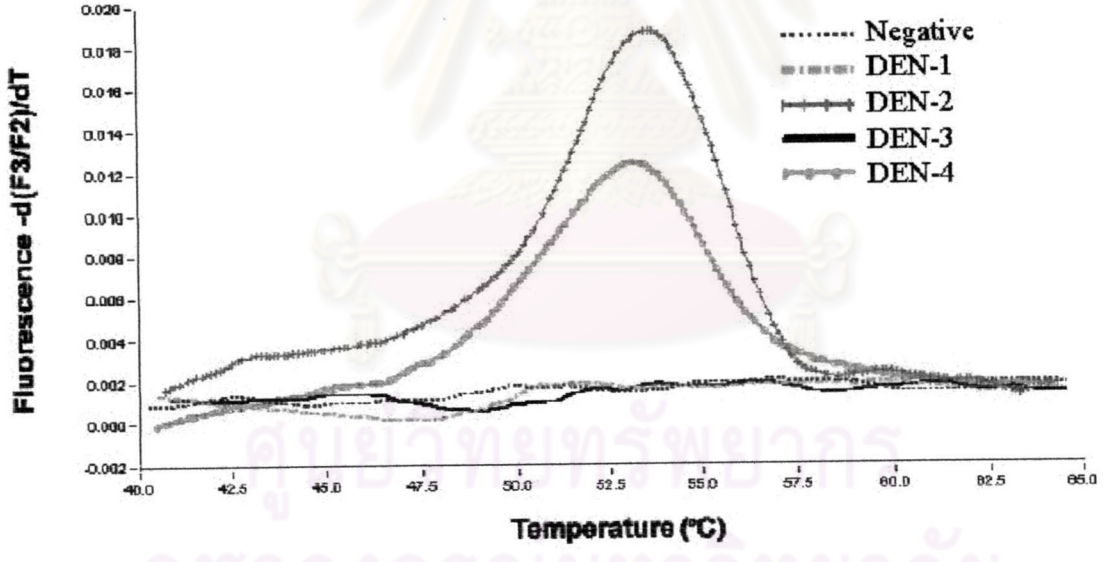



Figure 7(A-B) Using the LightCycler—FastStart Master DNA Hybridization Probes Kit. The respective dengue genotypes are distinguished by their different $T_m$s. (A) $T_m$ analysis performed, and fluorescence signal from dengue genotype 1 and 3 were first measured in F2 for LC-Red 640 emission. (B) For detection the dengue genotype 2 and 4, $T_m$s are determined using measurement of LC-Red705 emission in F3. Genotype 2 and 4 display narrow discrimination $T_m$s.

**6. Melting curve analysis for genotyping dengue virus**

Sera from dengue virus infected patients were determined by dual-color detection in real-time PCR. Forty cycles of amplification were performed with target DNA with different genotypes using fluorescent resonance energy transfer (FRET) detection system. Two color multiplexing and analysis of dengue genotype 1, 2, 3, and 4 was accomplished on the LightCycler instrument. The fluorescence signal was measured in channel F2 and F3 at the end of each annealing phase and increase as product accumulated. The process of hybridization and melting of detection probes to the target was monitored by melting curve analysis. By plotting the negative derivative of the fluorescence signal with temperature (-dF/dT), peaks are obtained at the respective melting temperature ($T_m$).

The DEN-1 and DEN-3 were detected by the PDEN2 LC-Red 640 detection probe and identified in channel F2 based on melting temperature. The DEN-1 product had a melting temperature of about 47.12°C while the DEN-3 product had a melting temperature of 48.59°C. (Figure.8 A-B) The 1.47°C difference in melting temperature was used to differentiate these two genotypes of dengue virus. On the other hand, the DEN-2 and DEN-4 were detected by the PDEN4 LC-Red 705 detection probe and seen in channel F3. The melting temperature of the DEN-2 product appeared to be approximately 54.21°C while the DEN-4 product had a melting temperature of 54.80°C (Figure.8 C-D). The distribution of melting temperature and melting temperature ranges for each genotype are shown in table 4. Therefore, the melting curve clearly demonstrates its genotype as the difference in melting temperature ($T_m$).

Table 4. $T_m$s observed for the respective genotypes in this study[a]

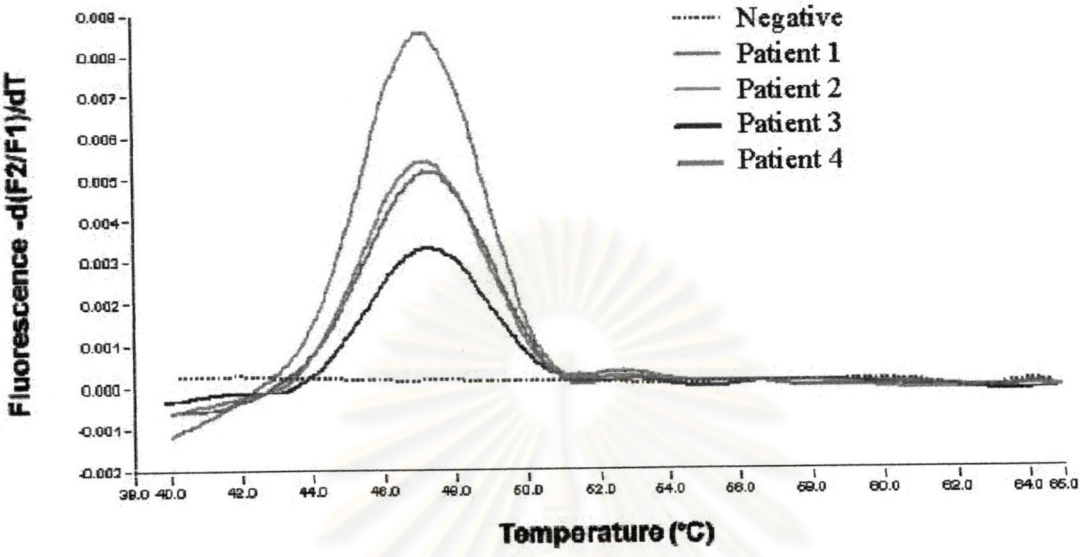
| Dengue genotype | Melting Temperature(°C)[b] | |
|---|---|---|
| | F2 | F3 |
| 1 | 47.12(46.58-47.51) | |
| 2 | | 54.21(53.90-54.52) |
| 3 | 48.59(48.36-48.96) | |
| 4 | | 54.80(54.59-55.05) |

[a] Using F2, genotype 1 and 3 can be identified unequivocally.

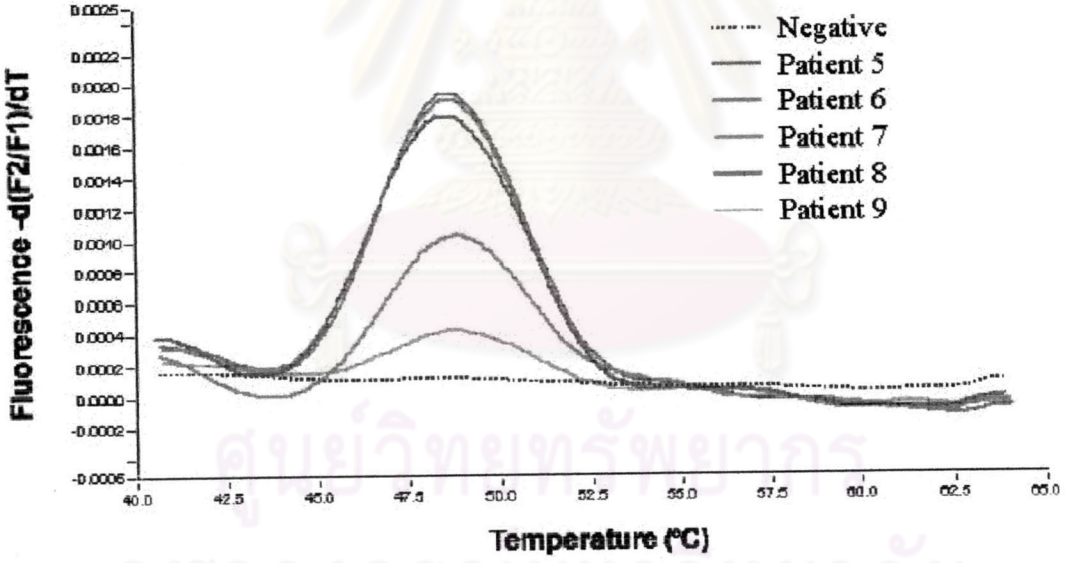
In contrast, genotype 2 and 4 have a nearly identical $T_m$s by using F3.

[b] Values are means. Values in parenthesis are interval

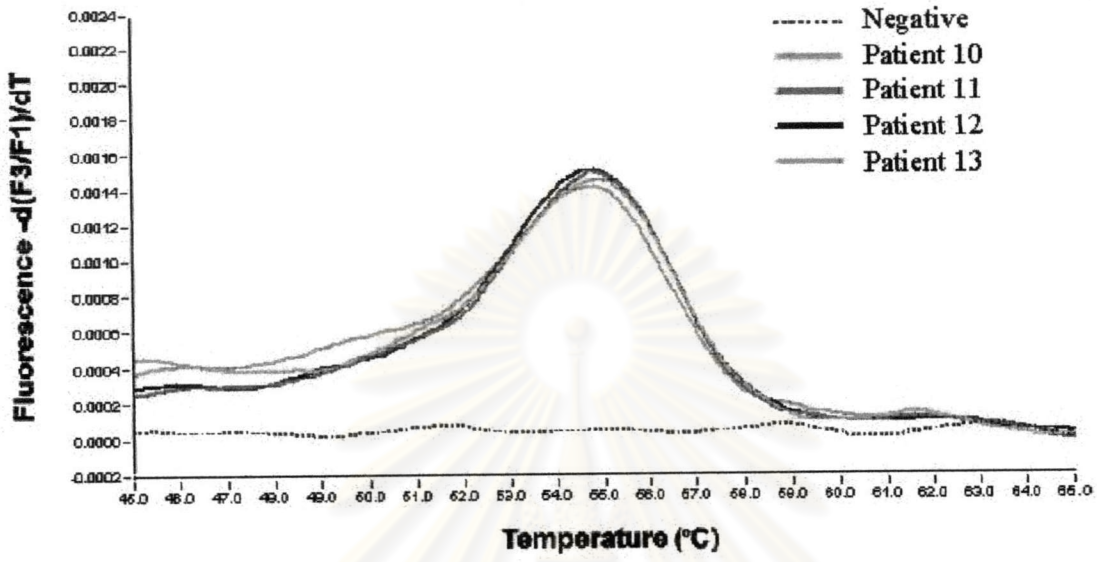
**8A. DEN-1**




**8B. DEN-3**



**8C DEN-2**




**8D. DEN-4**

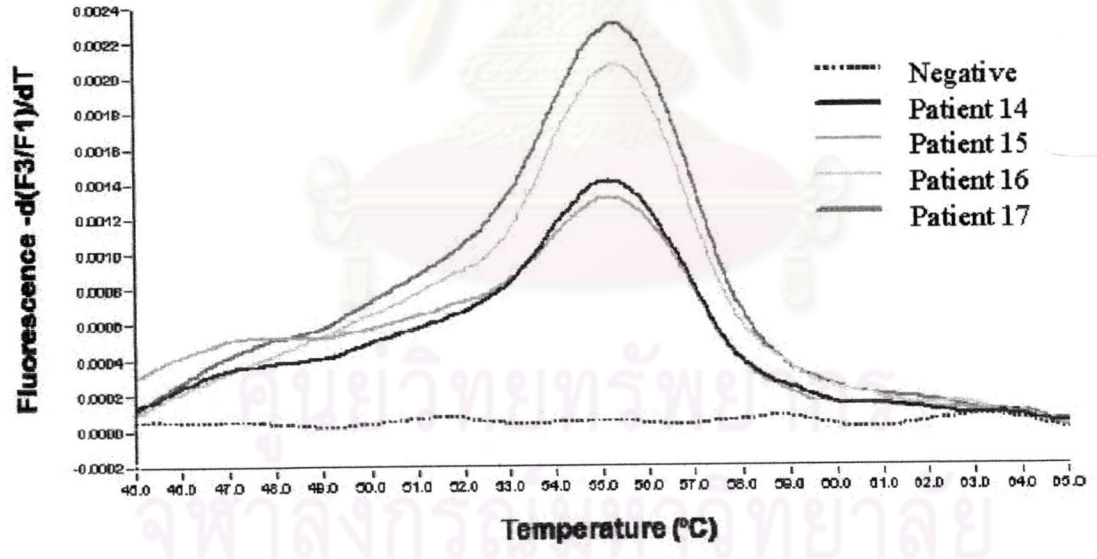



Figure 8(A-D). Representative serotyping experiment using real-time PCR and melting curve analysis. Patient samples were genotyped with 3 mM $MgCl_2$. Derivative melting curve plots are shown. Water blank was included to control for reagent contamination. (A) Four patient samples with DEN-1 and (B) 5 patient samples with DEN-3 were measured LC-Red 640 emission at F2. (C, D) Four patient samples with DEN-2 and Den-4 were measured LC-Red 705 emission.

**7. Reproducibility of $T_m$ for dengue virus serotyping**

To analyze the variance of repeated runs, isolates of all four dengue genotypes were tested two times on different days. The observed range of $T_m$ found for each dengue genotype test is indicated in table 5. The maximum within run variation was determined from runs with more than one sample of a specific genotype; this analysis included all samples with concordant results by the two assays. Figure 8 is demonstrated the ability of FRET detection probe to discriminate genotype of dengue virus in patient samples.

Table 5. Within- and between-run variation of $T_m$s for samples of indicated dengue genotypes by melting curve analysis.

| | Variation of $T_m$s (°C) | | | | |
|---|---|---|---|---|---|
| | Between runs | | | Within runs[a] | |
| Dengue genotype | interval | range | mean(SD) | interval | range |
| 1 | 46.58-47.51 | 0.93 | 47.12(0.19) | 47.07-47.51 | 0.44 |
| 2 | 53.90-54.52 | 0.62 | 54.21(0.25) | 53.90-54.51 | 0.61 |
| 3 | 48.36-48.96 | 0.60 | 48.59(0.14) | 48.45-48.80 | 0.35 |
| 4 | 54.59-55.05 | 0.46 | 54.80(0.16) | 54.59-55.05 | 0.46 |

[a] Data only from runs with more than one samples of specific genotype

**8. The sensitivity and specificity of real-time PCR**

The molecular sensitivity of detection by the LightCycler assay was compared to that of the conventional thermal cycler assay by use of the recombinant plasmid. The tenfold dilutions of plasmid pTT47 were tested by real-time PCR on the LightCycler instrument, the detection limit was found to be 75 molecules. Sensitivity of real-time PCR is similar to the result from nested PCR (table 6). The result of plasmid dilution from LightCycler assay and conventional thermal cycler assay are shown in figure 9.

Each sample was correctly typed by real-time PCR (Figure 8 A-D). The specificity was also verified by performing DNA sequencing. A total of 16 samples (four from each genotype) were subjected to nucleotide sequencing for validation of the typing. The DNA product was correctly typed when assay by nucleotide sequencing (data not shows). The probes and primers were blasted with NCBI four dengue virus-related flaviviruses (West Nile virus, Japanese encephalitis virus, St. Louis encephalitis virus, and Yellow fever virus). Primer DEN and DENR were matched with virus of Japanese encephalitis complex but not react with dengue virus type specific oligonucleotides or dengue type specific probes. The result showed that real-time PCR is specific for dengue virus. There is no PCR product form normal human serums which serve as negative control in every run.

Table 6. Comparison of the sensitivity of detection of tenfold dilution of plasmid pTT47 by the conventional thermal cycler assay and the LightCycler system assay

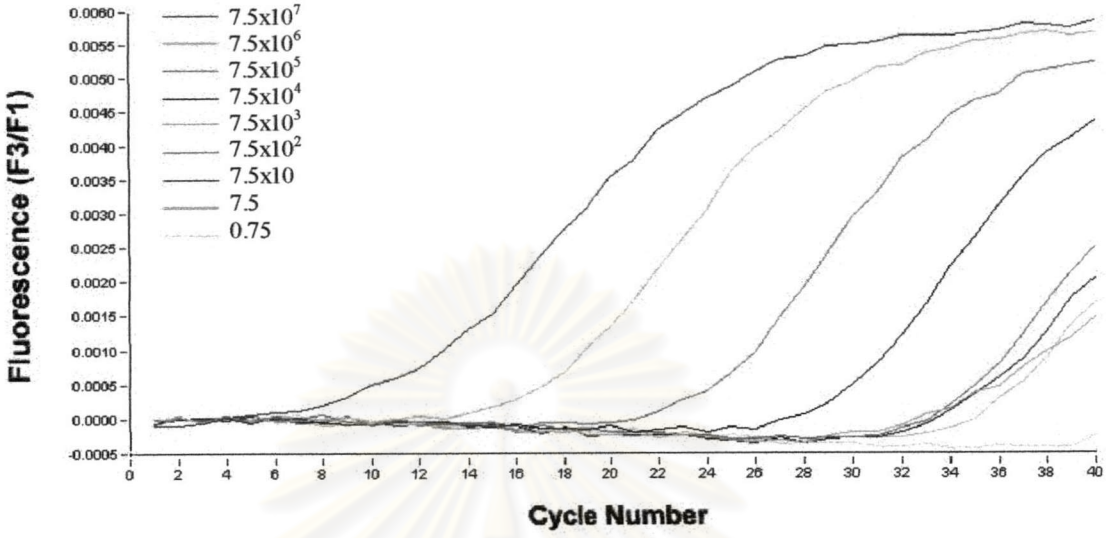
| Vial No. | Plasmid concentration[a] (molecule) | results of the following assay[b]: Conventional thermal cycler | LightCycler system assay |
|---|---|---|---|
| 1 | None | Neg | Neg |
| 2 | $7.5x10^8$ | Pos | Pos |
| 3 | $7.5x10^7$ | Pos | Pos |
| 4 | $7.5x10^6$ | Pos | Pos |
| 5 | $7.5x10^5$ | Pos | Pos |
| 6 | $7.5x10^4$ | Pos | Pos |
| 7 | $7.5x10^3$ | Pos | Pos |
| 8 | $7.5x10^2$ | Pos | Pos |
| 9 | 7.5x10 | Pos | Pos |
| 10 | 7.5 | Equiv[c] | Equiv[c] |
| 11 | 0.75 | Neg | Neg |

[a] The Plasmid concentration was determined by measuring the absorbance at 260 nm and calculate by concentration of DNA = OD value x dilution factor x conversion factor (50).

[b] Pos, Positive; Neg, Negative

[c] All samples were tested as replicates ten. These samples were inconsistently positive and so were classified as equivocal (Equiv).

**9A**




**9B**

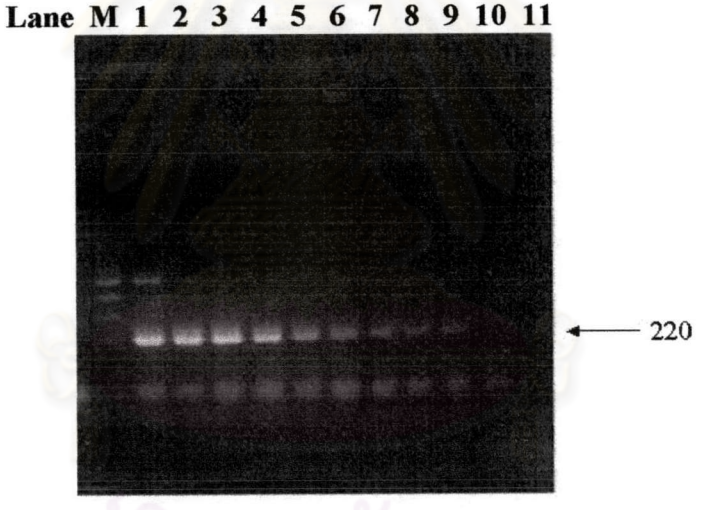



Figure9 (A-B). (A) Representative result of real-time PCR with LightCycler instrument. The amplification of tenfold dilution of plasmid at 7.5 x $10^3$, 7.5 x $10^2$, 7.5 x 10, 7.5 and 0.75 molecules. (B) Agarose gel electrophoresis of PCR products from tenfold dilution of plasmid and negative control by conventional thermal cycler. Lane M 100 bp marker, lane 1-11 tenfold plasmid dilution 7.5 x$10^8$, 7.5 x $10^7$, 7.5 x$10^6$, 7.5 x $10^5$, 7.5 x $10^4$, 7.5 x $10^3$, 7.5 x $10^2$, 7.5 x 10, 7.5, 0.75 molecules, respectively.

**9. The sequencing and phylogenetic analysis**

In order to validate the dengue genotyping by the LightCycler assay, the direct DNA sequencing assay was used to genotype the samples from the LightCycler assay. A phylogenetic analysis of the reference dengue virus that retrieved from GeneBank (URL: http://www.ncbi.nih.gov): genotype 1, NC_00477; genotype 2, NC_001474; genotype 3, NC_001475; genotype 4, NC_002640. the reference sequences were loaded to clustal W program to construct the phulogenetic tree.

The four LightCycler product of each dengue virus genotype were loaded to ABI PRISM sequencer. Alter the sequences were analyzed, the data were copy into FASTA format with Chromas program before loaded the sequences to the Clustal W program. The dengue virus genotypes were identified by grouping with the referenced dengue virus sequences. The results from the LightCycler genotyping by melting curve analysis and phylogenetic analysis showed the concordance dengue virus genotype.