IV. RESULTS

1. Construction of dengue mutant cDNA clones

To investigate the effect of point mutation at the positions P1 and P2 of the prM-M cleavage junction of dengue virus polyprotein, five mutant sequences each were introduced into the codon positions 204 and 205 of the dengue cDNA. The pBK (S1SP6-1547) Δ402 *Pst* I (-) plasmid, 4.5 kb in size, was used as a template in the PCR-based site-directed mutagenesis in which the mutant oligonucleotide primers were extended during temperature cycling by *Pfu* DNA polymerase, employing cycling parameters shown in Table 4. Following mutagenesis, the mutant plasmids were transformed into *E. coli*, selected with low level of ampicillin (25 μg/mL), and propagated at room temperature (20-25°C). In the 10 mutagenesis experiments, from 16 to 187 ampicillin-resistant transformant *E. coli* colonies/plate were observed at about 2 days after transformation (Table 7). The lowest number of transformants was found following the introduction of the R205H sequence, possibly due to the variation in transformation efficiency. The presence of intended mutations was then determined in 2 to 18 colonies for each mutation by digesting small-scale plasmid DNA preparations, or PCR products (nt 134-1518) derived from amplification of the small-scale plasmid preparation, with appropriate restriction enzymes. Screening with restriction enzyme digestion revealed that the mutant sequences were successfully introduced in all cases, but with varying efficiency. The lowest levels of efficiency, 11% and 17%, were detected in the introduction of the K204D and K204H sequences, respectively, whereas the highest efficiency, 100%, was found in the R205H mutagenesis (Table 7). However, the latter may represent an over-estimate because only 2 transformant colonies were selected for screening. Following screening with restriction enzyme digestion, two colonies containing each of the desired mutations were grown in a 10-mL scale for the preparation of plasmid DNA.

Nucleotide sequence analysis of the prM-M junction was performed on one plasmid preparation for each intended mutation; the desired mutation was detected in every case (Figure 5 and Appendix H). Moreover, no additional mutation was detected in the region nt 212-1535 of the dengue sequence.

The mutant pBK (S1SP6-1547) Δ402 *Pst* I plasmids were then digested with *Pst* I and the 1.3-kb fragment (nt 214-1531) was purified from agarose gel. This fragment was used for the successive construction of the mutant 5'half-genome and full-length mutant cDNA clones (Table 7).

Table 7. PCR-based mutagenesis of the codons 204 and 205 within pBK S1SP6-1547 Δ402 *Pst* I subclone

Desired mutation	Ampicillin resistant *E. coli* (colony/plate)	Mutation screening method	Colony with intended mutation/ colony selected for screening (%)	Plasmid preparation (clone)	Clone with confirmed sequence
K204A	187	*Bss*H II digestion	3/8 (38)	2	1
K204D	117	Amplification and *Cla* I digestion	2/18 (11)	2	1
K204H	25	Amplification and *Cla* I digestion	4/8 (50)	2	1
K204R	35	*Bsr*B I digestion	3/7 (43)	2	1
K204S	135	Amplification and *Nru* I digestion	3/18 (17)	2	1
R205A	66	*Hind* III digestion	5/8 (63)	2	1
R205D	45	*Pfl*F I digestion	6/8 (75)	2	1
R205H	16	*Dra* III digestion	2/2 (100)	2	1
R205K	55	Sequence analysis	1/2 (50)	2	1
R205S	32	*Eco*ICR I digestion	2/8 (25)	2	1

Nucleotide sequence

```
DEN-2(16681)              214    GGACGAG GACCATTAAA ACTGTTCATG GCCCTGGTGG  250
K204A                            ....... .......... .......... ..........
K204D                            ....... .......... .......... ..........
K204H                            ....... .......... .......... ..........
K204R                            ....... .......... .......... ..........
K204S                            ....... .......... .......... ..........
R205A                            ....... .......... .......... ..........
R205D                            ....... .......... .......... ..........
R205H                            ....... .......... .......... ..........
R205K                            ....... .......... .......... ..........
R205S                            ....... .......... .......... ..........

DEN-2(16681)     251 CGTTCCTTCG TTTCCTAACA ATCCCACCAA CAGCAGGGAT ATTGAAGAGA 300
K204A                ..........  .......... .......... .......... ..........
K204D                ..........  .......... .......... .......... ..........
K204H                ..........  .......... .......... .......... ..........
K204R                ..........  .......... .......... .......... ..........
K204S                ..........  .......... .......... .......... ..........
R205A                ..........  .......... .......... .......... ..........
R205D                ..........  .......... .......... .......... ..........
R205H                ..........  .......... .......... .......... ..........
R205K                ..........  .......... .......... .......... ..........
R205S                ..........  .......... .......... .......... ..........

DEN-2(16681)     301 TGGGGAACAA TTAAAAAATC AAAAGCTATT AATGTTTTGA GAGGGTTCAG 350
K204A                ..........  .......... .......... .......... ..........
K204D                ..........  .......... .......... .......... ..........
K204H                ..........  .......... .......... .......... ..........
K204R                ..........  .......... .......... .......... ..........
K204S                ..........  .......... .......... .......... ..........
R205A                ..........  .......... .......... .......... ..........
R205D                ..........  .......... .......... .......... ..........
R205H                ..........  .......... .......... .......... ..........
R205K                ..........  .......... .......... .......... ..........
R205S                ..........  .......... .......... .......... ..........
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Figure 5. Nucleotide sequence of the 1.3-kb *Pst* I fragments (nt 214-1531) which were digested from mutant pBK S1SP6-1547 Δ402 *Pst* I plasmid clones containing each of 10 different intended mutations of the prM-M cleavage junction. Dots represent bases identical to the dengue serotype 2 (16681) parent strain. The change at position 402 resulted from previous intended mutation of the *Pst* I site. The final 2 bases at the 5'end and 4 bases at the 3'end were generated by *Pst* I digestion and were not resolved by capillary electrophoresis.

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Nucleotide sequence

DEN-2(16681)     351 GAAAGAGATT GGAAGGATGC TGAACATCTT GAATAGGAGA CGCAGATCTG 400
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     401 CAGGCATGAT CATTATGCTG ATTCCAACAG TGATGGCGTT CCATTTAACC 450
K204A                .C........  ..........  ..........  ..........  ..........
K204D                .C........  ..........  ..........  ..........  ..........
K204H                .C........  ..........  ..........  ..........  ..........
K204R                .C........  ..........  ..........  ..........  ..........
K204S                .C........  ..........  ..........  ..........  ..........
R205A                .C........  ..........  ..........  ..........  ..........
R205D                .C........  ..........  ..........  ..........  ..........
R205H                .C........  ..........  ..........  ..........  ..........
R205K                .C........  ..........  ..........  ..........  ..........
R205S                .C........  ..........  ..........  ..........  ..........


DEN-2(16681)     451 ACACGTAACG GAGAACCACA CATGATCGTC AGCAGACAAG AGAAAGGGAA 500
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     501 AAGTCTTCTG TTTAAAACAG AGGATGGCGT GAACATGTGT ACCCTCATGG 550
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........
```

Figure 5. (continued)

Nucleotide sequence

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DEN-2(16681)     551 CCATGGACCT TGGTGAATTG TGTGAATACA CAATCACGTA CAAGTGTCCC 600
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     601 CTTCTCAGGC AGAATGAGCC AGAAGACATA GACTGTTGGT GCAACTCTAC 650
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     651 GTCCACGTGG GTAACTTATG GGACGTGTAC CACCATGGGA GAACATAGAA 700
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     701 GAGAAAAAAG ATCAGTGGCA CTCGTTCCAC ATGTGGGAAT GGGACTGGAG 750
K204A                .....GCGC. C.....................  ..........  ..........
K204D                .....G.TC. ..........  ..........  ..........  ..........
K204H                .....C.TC. ..........  ..........  ..........  ..........
K204R                ....GCGG.. ..........  ..........  ..........  ..........
K204S                .....TCGC. ..........  ..........  ..........  ..........
R205A                .........GC T.........  ..........  ..........  ..........
R205D                .........GA C.....C..  ..........  ..........  ..........
R205H                .........CA C.........  ..........  ..........  ..........
R205K                .......G.A GAGC......  ..........  ..........  ..........
R205S                .......G.. C.........  ..........  ..........  ..........
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Figure 5. (continued)

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Nucleotide sequence

DEN-2(16681)     751 ACACGAACTG AAACATGGAT GTCATCAGAA GGGGCCTGGA AACATGTCCA 800
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     801 GAGAATTGAA ACTTGGATCT TGAGACATCC AGGCTTCACC ATGATGGCAG 850
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     851 CAATCCTGGC ATACACCATC GGAACGACAC ATTTCCAAAG AGCCCTGATT 900
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........


DEN-2(16681)     901 TTCATCTTAC TGACAGCTGT CACTCCTTCA ATGACAATGC GTTGCATAGG 950
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........
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Figure 5. (continued)

Nucleotide sequence

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DEN-2(16681)     951 AATGTCAAAT AGAGACTTTG TGGAAGGGGT TTCAGGAGGA AGCTGGGTTG 1000
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1001 ACATAGTCTT AGAACATGGA AGCTGTGTGA CGACGATGGC AAAAAACAAA 1050
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1051 CCAACATTGG ATTTTGAACT GATAAAAACA GAAGCCAAAC AGCCTGCCAC 1100
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1101 CCTAAGGAAG TACTGTATAG AGGCAAAGCT AACCAACACA ACAACAGAAT 1150
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........
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Figure 5. (continued)

Nucleotide sequence

```
DEN-2(16681)    1151 CTCGCTGCCC AACACAAGGG GAACCCAGCC TAAATGAAGA GCAGGACAAA 1200
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1201 AGGTTCGTCT GCAAACACTC CATGGTAGAC AGAGGATGGG GAAATGGATG 1250
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1251 TGGACTATTT GGAAAGGGAG GCATTGTGAC CTGTGCTATG TTCAGATGCA 1300
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1301 AAAAGAACAT GGAAGGAAAA GTTGTGCAAC CAGAAAACTT GGAATACACC 1350
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........
```

Figure 5. (continued)

Nucleotide sequence

```
DEN-2(16681)    1351 ATTGTGATAA CACCTCACTC AGGGGAAGAG CATGCAGTCG GAAATGACAC 1400
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1401 AGGAAAACAT GGCAAGGAAA TCAAAATAAC ACCACAGAGT TCCATCACAG 1450
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1451 AGGTTCGTCT GCAAACACTC CATGGTAGAC AGAGGATGGG GAAATGGATG 1500
K204A                ..........  ..........  ..........  ..........  ..........
K204D                ..........  ..........  ..........  ..........  ..........
K204H                ..........  ..........  ..........  ..........  ..........
K204R                ..........  ..........  ..........  ..........  ..........
K204S                ..........  ..........  ..........  ..........  ..........
R205A                ..........  ..........  ..........  ..........  ..........
R205D                ..........  ..........  ..........  ..........  ..........
R205H                ..........  ..........  ..........  ..........  ..........
R205K                ..........  ..........  ..........  ..........  ..........
R205S                ..........  ..........  ..........  ..........  ..........

DEN-2(16681)    1501 AGGTTCGTCT GCAAACACTC CATGGTAGAC A          1531
K204A                ..........  ..........  ..........  .
K204D                ..........  ..........  ..........  .
K204H                ..........  ..........  ..........  .
K204R                ..........  ..........  ..........  .
K204S                ..........  ..........  ..........  .
R205A                ..........  ..........  ..........  .
R205D                ..........  ..........  ..........  .
R205H                ..........  ..........  ..........  .
R205K                ..........  ..........  ..........  .
R205S                ..........  ..........  ..........  .
```

Figure 5. (continued)

To construct the mutant 5'half-genome, the 1.3-kb *Pst* I fragment with intended mutation was ligated with dephosphorylated 6.0-kb *Pst* I fragment of the 5'half-genome cDNA. Ligation mixtures were then transformed into competent *E. coli*, and transformants were propagated at room temperature (25°C) with low level of ampicillin (25 μg/mL) selection. Eleven to 313 colonies of ampicillin-resistant transformant *E. coli* were detected in each transformation plate and two sizes of bacterial colonies, large and small, were observed (Table 8). Only small colonies were picked and grown in 2-mL scale for preparation of the plasmid. Screening by *Pst* I digestion of the small-scale plasmid preparation revealed two *Pst* I fragments, 1.3 and 6.0 kb, in all cases, indicating successful ligation of the mutant fragment. The orientation of the ligated mutant *Pst* I fragment (nt 212-1535) in the resultant 5'half-genome was determined next by digesting the mutant 5'half-genome with *Sph* I (nt 1380-1384) and *Eco*R I (nt 2340-2344 and 2871-2875). With the two enzymes cutting together, the correct orientation of the ligated 1.3-kb *Pst* I fragment yielded the 5.8, 0.9 and 0.5 kb fragments, whereas the incorrect orientation resulted in the 4.8, 1.9 and 0.5 kb fragments (Figure 6A and 6B). The efficiency of ligation varied from 45% to 100% and the proportion of colonies containing the correct orientation of the mutant 1.3-kb *Pst* I fragment varied from 25% to 83% (Table 8). From 2 to 4 of the 5'half-genome clones, which contained the correct insertion of mutant prM-M junction sequence, were grown in the 50-mL scale for the preparation of plasmid DNA. The size, general integrity, and the presence of key restriction enzyme sites were determined by digesting the resultant 5'half-genome plasmid DNA with *Eco*R I, *Eco*R V, *Kpn* I, *Nco* I and *Pst* I (Figures 7 and 8). Also, the presence of intended mutation at the prM-M cleavage junction was confirmed in at least two plasmid clones by digesting with appropriate restriction enzymes.

Table 8. Construction of mutant 5'half-genomes

Desired mutation	Ampicillin resistant *E. coli* (colony/plate)	Colony with 1.3-kb *Pst* I insert/ colony screened (%)	Colony with correct orientation/ colony selected for checking (%)	Plasmid preparation (clone)	Plasmid clone with confirmed sequence
K204A	11	8/11 (73)	4/8 (50)	4	2
K204D	152	10/10 (100)	4/8 (50)	4	4
K204H	81	9/10 (90)	2/6 (33)	2	2
K204R	81	10/10 (100)	4/6 (67)	4	2
K204S	33	9/20 (45)	4/8 (50)	2	2
R205A	313	13/24 (54)	4/9 (44)	3	2
R205D	163	10/10 (100)	2/8 (25)	2	2
R205H	25	10/10 (100)	8/3 (38)	3	2
R205K	126	8/8 (100)	5/6 (83)	2	2
R205S	130	9/10 (90)	7/9 (78)	2	2

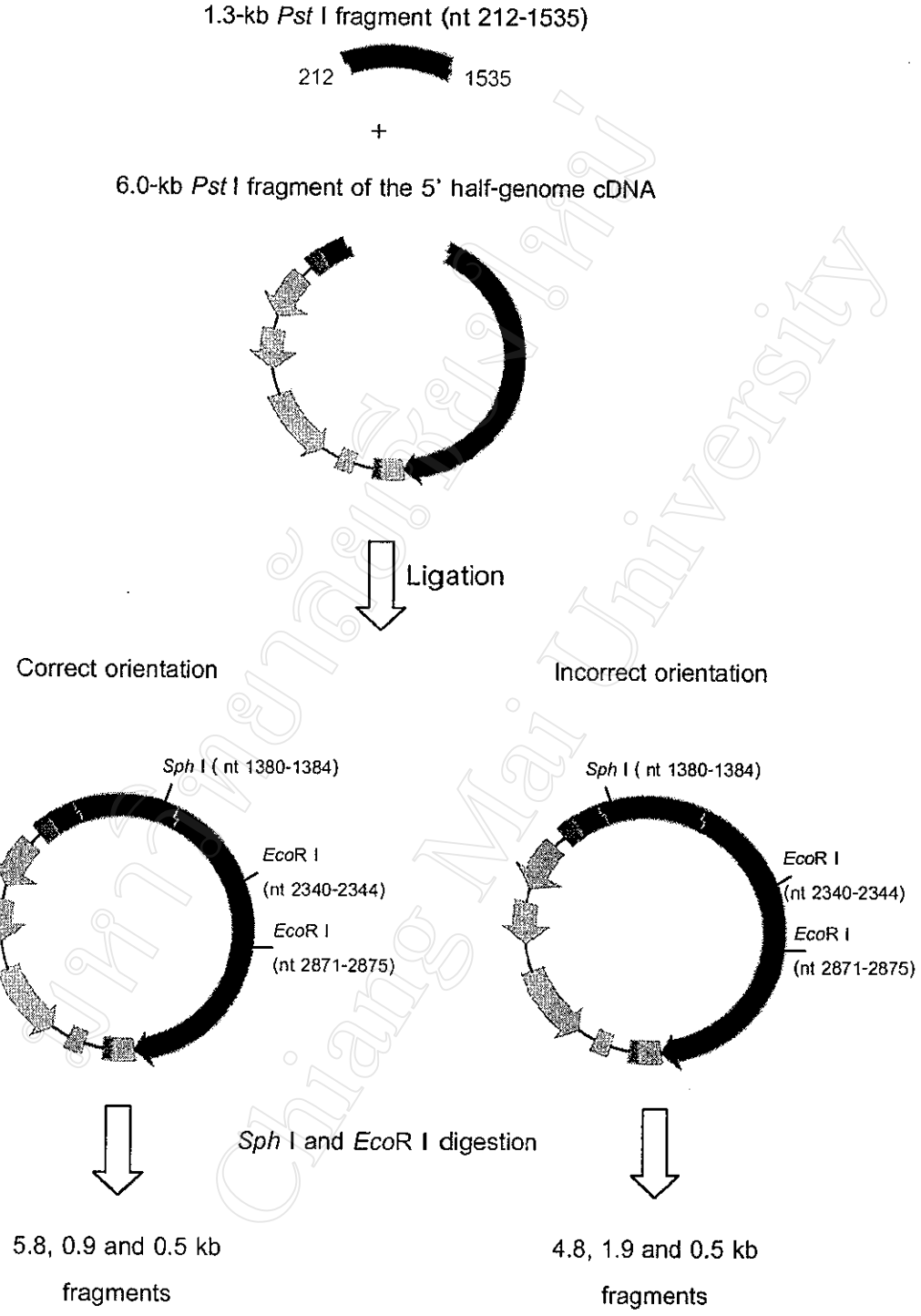


Figure 6A. Two insertion patterns of the mutant *Pst* I fragment (nt 212-1535) within the 5'half-genome.

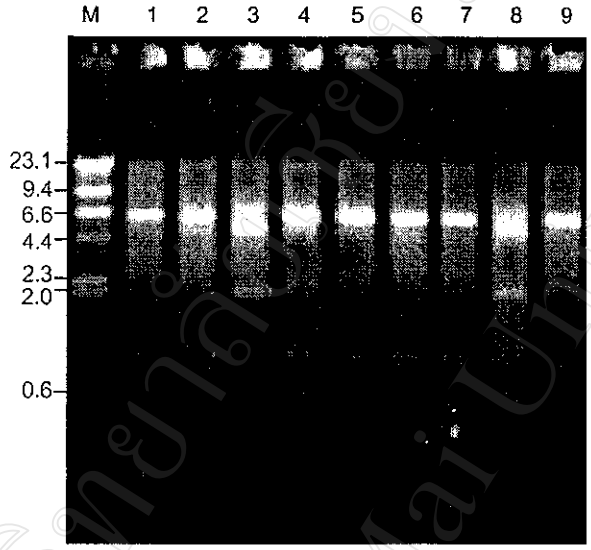


Figure 6B. Determination of the orientation of the ligated mutant *Pst* I fragment (nt 212-1535) in 5'half-genomes by digesting 5'half genomes with by *Sph* I and *Eco*R I. Lane 1: phage lambda DNA *Hind* III fragments. Lanes 2, 3, 5, 6, 7, 8 and 10: The three *Pst* I fragments, 5.8, 0.9 and 0.5 kb, which indicated the correct orientation of the 1.3 kb mutant *Pst* I fragment within the 5'half-genome. Lanes 4 and 9: an incorrect orientation that resulted in the 4.8, 1.9 and 0.5 kb fragments.

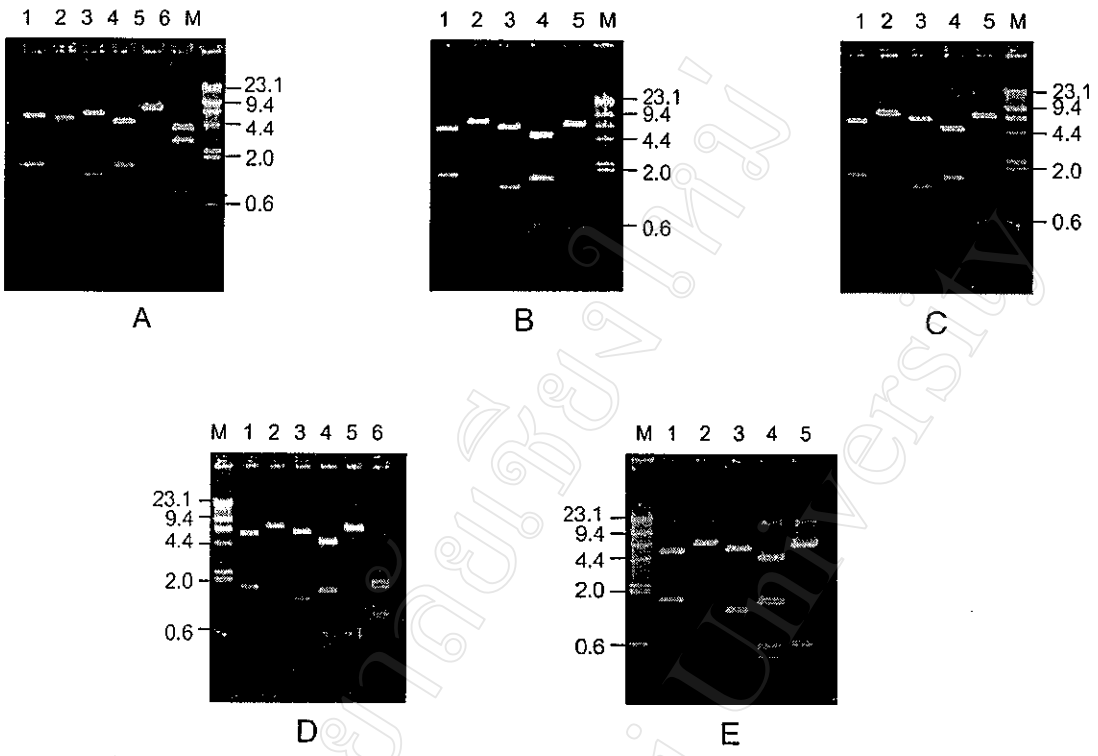


Figure 7. Analysis of mutant 5'half-genomes (pBK S1S6-4497Δ402 *Pst* I) by digesting with restriction enzymes to determine size, general integrity, presence of key restriction enzyme sites and the intended prM-M mutation. Set A: K204A mutation, set B: K204D mutation, set C: K204H mutation, set D: K204R mutation, set E: K204S mutation. Lane M shows phage lambda DNA *Hind* III fragments; lane 1 shows *Hind* III digestion (5.6 and 1.7 kb); lane 2 shows *Kpn* I digestion (7.3 kb); lane 3 shows *Pst* I digestion (6.0 and 1.3 kb); lane 4 shows *Nco* I digestion (5.3, 1.6, 0.7, 0.5 and 0.1 kb); lane 5 shows *Eco*R I digestion (6.8 and 0.5 kb). In lane 6, two mutant 5'half-genomes (containing the K204A mutation and K204R mutation) were digested, respectively, with appropriate enzymes that were specific for the intended mutation of the prM-M junction; A, *Bss*H II (5.8, 2.8 and 0.8 kb); D, *Bsr*B I (2.1, 1.8, 1.0, 0.99, 0.90, 0.45 and 0.23 kb).

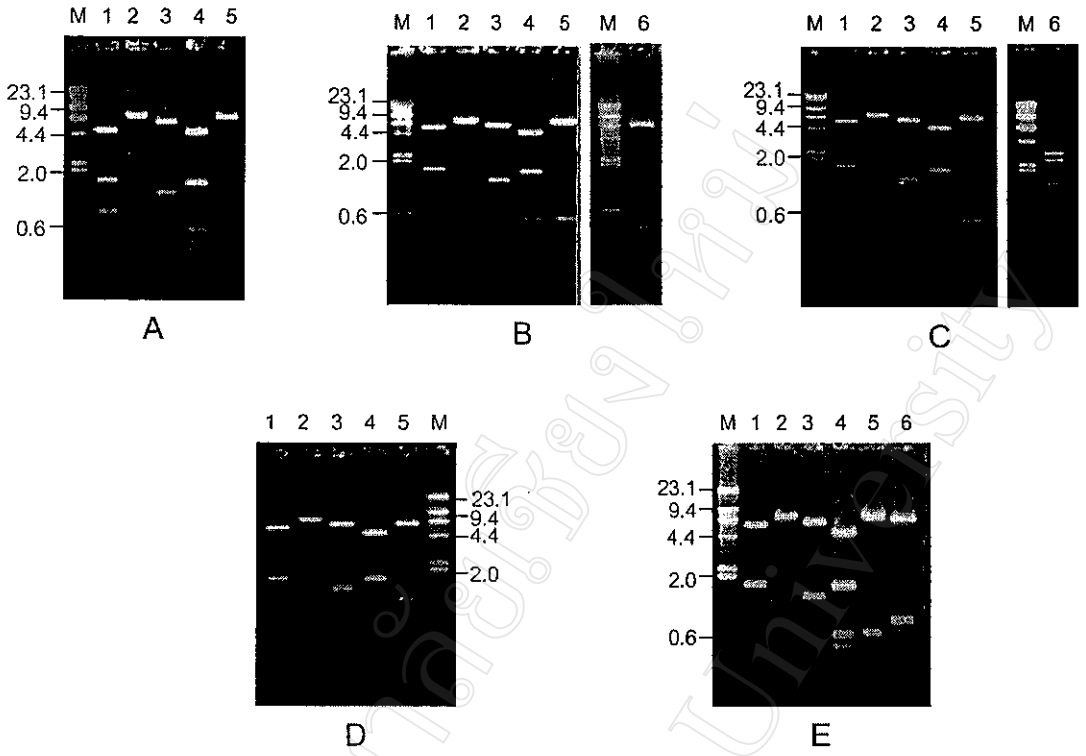


Figure 8. Analysis of mutant 5'half-genomes (pBK S1S6-4497Δ402 *Pst* I) by digesting with restriction enzymes to determine size, general integrity, presence of key restriction enzyme sites and the intended prM-M mutation. Set A: R205A mutation, set B: R205D mutation, set C: R205H mutation, set D: R205K mutation, set E: R205S mutation. Lane M shows phage lambda DNA *Hind* III-digested fragments; lane 1 shows *Hind* III digestion (5.6 and 1.7 kb); lane 2 shows *Kpn* I digestion (7.3 kb); lane 3 shows *Pst* I digestion (6.0 and 1.3 kb); lane 4 shows *Nco* I digestion (5.3, 1.6, 0.7, 0.5 and 0.1 kb); lane 5 shows *Eco*R I digestion (6.8 and 0.5 kb). In lane 6, three mutant 5'half-genomes (containing the R205D mutation, R205H mutation and R205S mutation) were digested, respectively, with appropriate enzymes that were specific for the intended mutation of the prM-M junction; B, *Pfl*F I (7.0 and 0.3 kb); C, *Dra* III (3.5, 2.5 and 1.2 kb); E, *Eco*ICR I (6.5 and 0.7 kb). The R205A mutation was confirmed by *Hind* III digestion (set A, lane 2; 4.8, 1.7 and 0.9 kb), whereas, the R205K mutation required nucleotide sequence analysis.

To construct the full-length cDNA plasmid containing each mutation, the 6.2-kb *Kpn* I fragment that was separated from the 3'half-genome plasmid and contained nt 4497-10723 of the dengue sequence was ligated to the *Kpn* I-digested mutant 5'half-genome. Two of mutant full-length cDNA clones, which contained the correct insertion of the 6.2-kb fragment, were grown in the 50-mL scale for the preparation of plasmid DNA. The size and the presence of key restriction enzyme sites were determined by digesting the resultant full-length cDNA plasmid with *Eco*R I, *EcoR* V, *Kpn* I, *Nco* I and *Pst* I (Figures 9 and 10).

The full-length cDNA plasmids that contained a mutant prM-M junction sequence were linearized by digestion with *Xba* I. The expected size of the plasmid was 13.6 kb (Figure 11). The linearized full-length cDNA was used as the template for producing of genome-length capped RNA by *in vitro* transcription. The yield and approximate size of the products were analyzed by electrophoresis in 0.7% agarose gel containing 3.7% formaldehyde in MOPS-EDTA-Sodium acetate running buffer. After staining with 10-μg/mL ethidium bromide, destaining and visualizing with an ultraviolet light source, unpurified *in vitro* transcripts RNA appeared a sharp band representing full-length transcript together with smeared tail of incomplete transcripts (Figure 12).

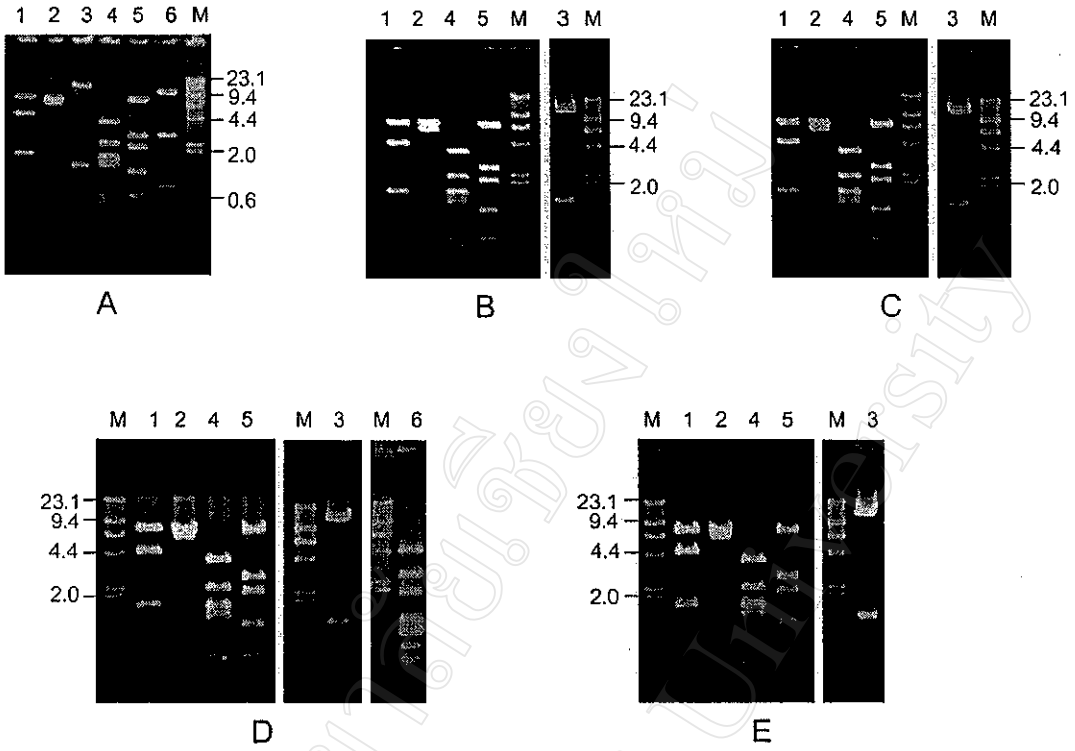


Figure 9. Checking mutant dengue full-length cDNA clones (pBK S1SP6-10723Δ402 *Pst* I) by restriction enzyme digestion. Set A: K204A mutation, set B: K204D mutation; set C: K204H mutation; set D: K204R mutation; set E: K204S mutation. Lane M shows phage lambda DNA *Hind* III-digested fragments. Lane 1 shows *Xba* I and *Hind* III digestion (7.5, 4.5 and 1.7 kb); lane 2 shows *Kpn* I digestion (7.8 and 5.8 kb); lane 3 shows *Pst I* digestion (12.3 and 1.3 kb); lane 4 shows *Nco* I digestion (3.6, 2.3, 1.7, 1.3, 0.5, 0.3 and 0.1 kb); lane 5 shows *Eco*R I digestion (6.9, 2.7, 2.1, 1.2, 0.5, 0.3 and 0.2 kb). In lane 6 shows two mutant full-length clones (containing the K204A mutation and K204R mutation) were digested, respectively, with appropriate enzymes that were specific for the intended mutation of the prM-M junction, A: *Bss*H II digestion (10.0, 2.8, 0.8 kb), D: *Bsr*B I digestion (4.2, 2.5, 1.8, 1.2,1.0, 0.9, 0.75, 0.72, 0.2 and 0.1 kb).

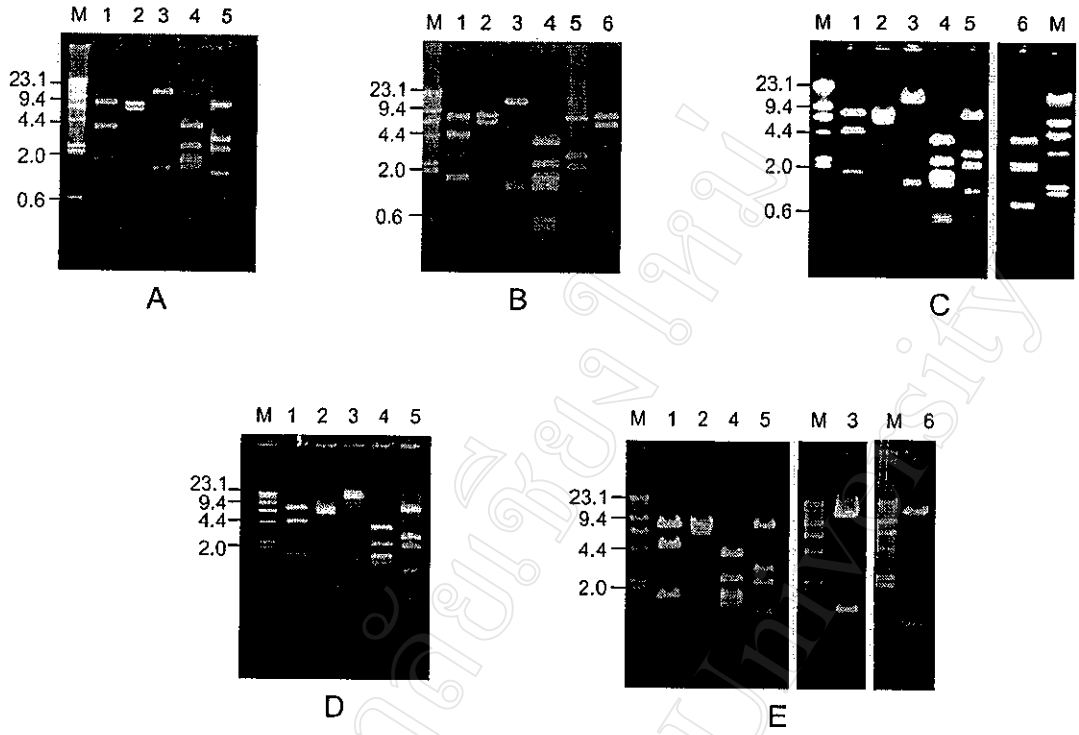


Figure 10. Checking mutant dengue full-length cDNA (pBK S1SP6-10723Δ402 *Pst* I) by restriction enzyme digestion. Set A: R205A mutation, set B: R205D mutation, set C: R205H mutation, set D: R205K mutation, set E: R205S mutation. Lane M shows phage lambda DNA *Hind* III-digested fragments. Lane 1 shows *Xba* I and *Hind* III digestion (7.5, 4.5 and 1.7 kb); lane 2 shows *Kpn* I digestion (7.8 and 5.8 kb); lane 3 shows *Pst* I digestion (12.3 and 1.3 kb); lane 4 shows *Nco* I digestion (3.6, 2.3, 1.7, 1.3, 0.5, 0.3 and 0.1 kb); lane 5 shows *Eco*R I digestion (6.9, 2.7, 2.1, 1.2, 0.5, 0.3 and 0.2 kb). In lane 6, three mutant full-length clones (containing the R205D, R205H, R205S mutations) were digested, respectively, with appropriate enzyme that was specific for the intended mutation of the prM-M junction, B: *Pfl*F I digestion (7.6, 5.7 and 0.3 kb), C: *Dra* III digestion (5.3, 3.6, 3.2 and 0.3 kb), E: *Eco*ICR I digestion (13.0 and 0.7 kb). The R205A mutation was confirmed by *Hind* III digestion (set A; lane 2, 7.5, 3.6, 1.7, 0.8 kb) whereas the R205K mutation required nucleotide sequence analysis.

Figure 11. A full-length cDNA clone, pBK S1SP6-10723Δ402 *Pst* I R205S, linearized by digesting with *Xba* I. The expected size of the plasmid was 13.6 kb. Lane M shows phage lambda DNA *Hind* III-digested fragments and lane 1 shows pBK S1SP6-10723Δ402 *Pst* I R205S, *Xba* I digestion.

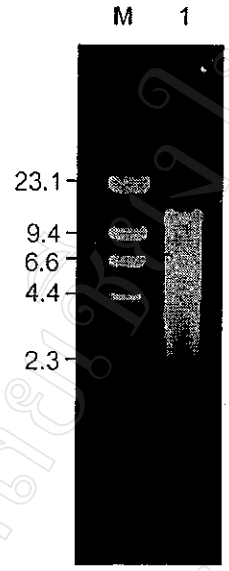


Figure 12. Analysis of the capped *in vitro* transcripts of a mutant full-length cDNA clone containing the R205S mutation at the prM-M cleavage junction. The *in vitro* transcription products were heated and electrophoresed in 1% formaldehyde agarose gel employing MOPS-EDTA-sodium acetate buffer. Lane M shows phage lambda DNA *Hind* III-digested fragments (unheated) and lane 1 shows heated *in vitro* transcripts of pBK S1SP6-10723Δ402 *Pst* I R205S. The phage lambda DNA *Hind* III-digested fragments were not the usual size markers for RNA, but were included in this gel just to indicate gel-to-gel variation of electrophoresis. Also, appropriate RNA marker as large as 10.7 kb was not commercially available.

2. Generation of prM-M junction mutant dengue viruses

To generate the prM-M junction mutant dengue viruses, 5-10 μL of unpurified *in vitro* RNA transcripts were transfected into C6/36 cell monolayers by employing lipofectin. In the first set of transfection experiments, *in vitro* transcripts of full-length cDNA clones, containing each of the five mutations of the position P1 (polyprotein position 204), were transfected into C6/36 cells in parallel with transcripts from an unmutated full-length cDNA clone. As a negative control, lipofectin without added RNA transcripts was used. Following transfection, the release of mutant viruses from transfected cells into culture medium was monitored on days 4, 7, 11, 14, 21, 28, 35, 42, 49 and 56 after transfection by dot immunoassay employing pooled convalescent human sera (Figure 13).

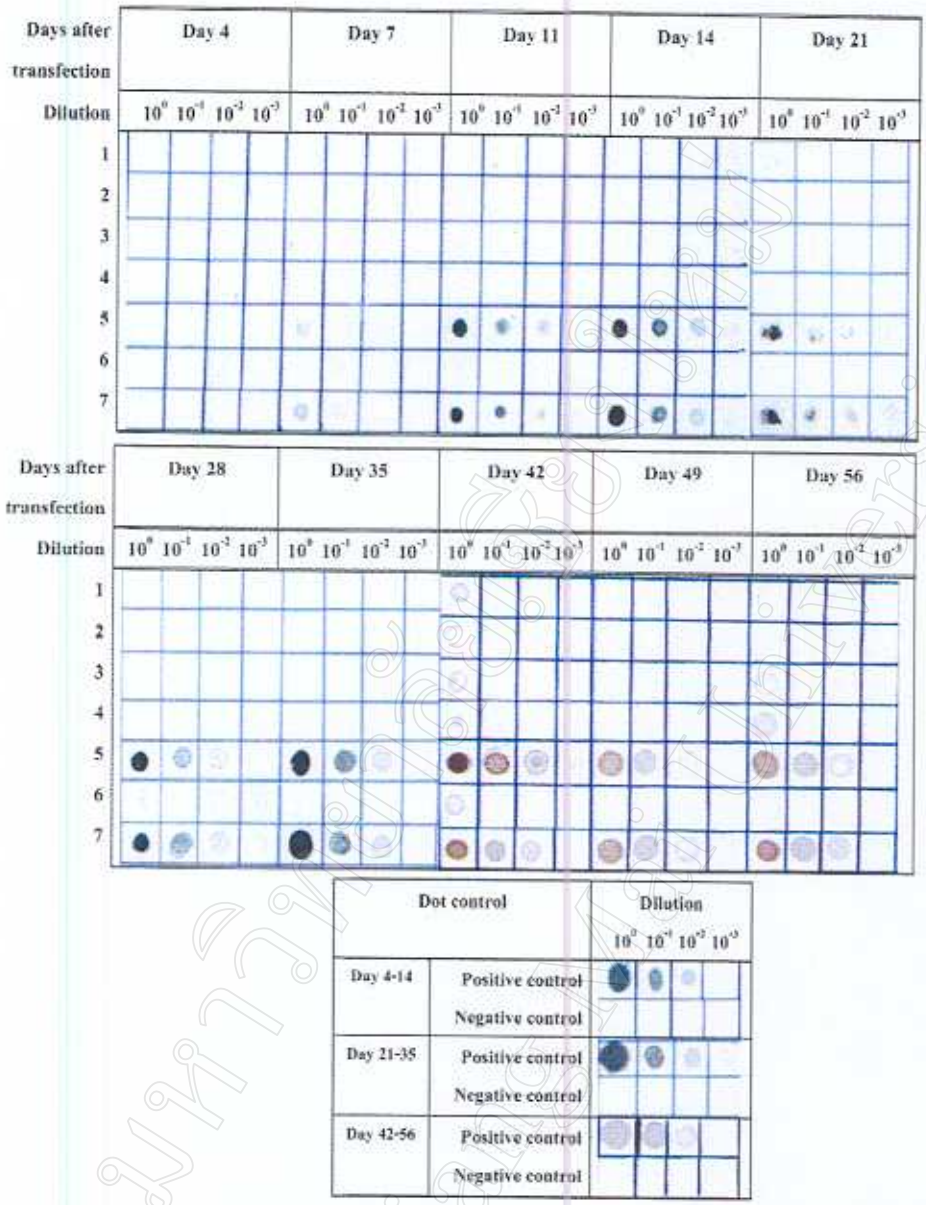


Figure 13. Release of K204 mutant dengue virus set following transfection. Culture media from indicated post-transfection days were diluted serially, dotted onto nitrocellulose membrane and reacted with pooled convalescent human sera. Row 1: lipofectin control; row 2: K204A mutant; row 3: K204D mutant; row 4: K204H mutant; row 5: K204R mutant; row 6: K204S mutant; and row 7: parental RNA transcript. The positive control was C6/36 culture media containing 7.0×10^{7} ffu/mL of 16681*Nde*(+) virus whereas the negative control was culture media of uninfected C6/36 cells.

In the transfection of K204 mutation set, a detectable level of dengue virus released into culture medium from C6/36 transfected with parental capped RNA transcript was observed as early as day 7 after transfection and appeared to reach the plateau level on days 11-14 after transfection (Figure 13, row 7). As expected, no or very low level of signal was observed from the culture media of C6/36 transfected with lipofectin alone all through 56 days of the transfection experiment (Figure 13, row 1). From C6/36 monolayers receiving capped RNA transcripts of mutant full-length cDNA clones, virus production was detected by dot immunoassay only from cells transfected with the K204R mutant transcript (Figure 13, row 5). Weak signal observed from transfection with mutant transcripts containing the K204A, K204D and K204H mutation at the lowest dilution of culture medium on days 42 and 56 post-transfection was likely to be enhanced background because similarly weak signal was also observed in the lipofectin-alone transfected cells. Interestingly, the kinetic of virus releasing in C6/36 cells transfected with K204R mutant transcript closely resembled that of the parent transcript.

To determine the level of infectious virus released from transfected C6/36 cells, culture media were subjected to virus titration using PS clone D cells and focus immunoassay (Figures 14 and 15). As expected, detectable level of viruses were observed only from C6/36 cells transfected with the parental transcript and the K204R mutant transcript. The parental virus was first detected on day 4 after transfection with titer of 70 ffu/mL, and then the titer rapidly increased to the peak level of 2.45×10^7 ffu/mL on day 14 after transfection. Subsequently, the titer remained at high level throughout the remaining 6 weeks of observation. The K204R mutant virus was first detected on day 11 after transfection and the titer rapidly increased to a peak of 3.26×10^7 ffu/mL on day 14 after transfection. Afterwards, the level of the K204R mutant virus remained consistently high until day 56 after transfection. No infectious virus was detected in the culture media of C6/36 cells receiving the K204A, K204D, K204H and K204H mutant transcripts when assessed

on days 7, 28 and 56 after transfection. This result suggests that positively charged amino acid residue is required at the position 204 of the polyprotein (P2 of the prM-M junction) for efficient dengue virus replication. However, lysine is not absolutely required at this position; either arginine or lysine, both of which contained positively charged amino acid side chain, is sufficient.

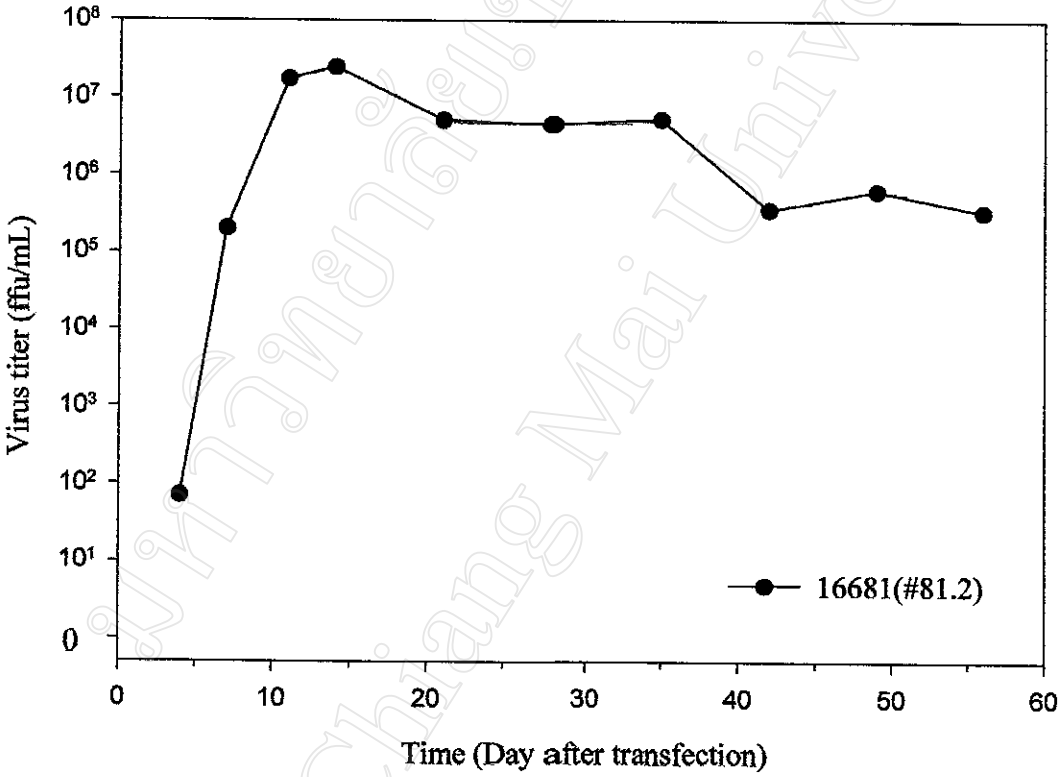


Figure 14. Kinetics of virus production following transfection by transcripts of parental dengue strain 16681 (#81.2) into C6/36 cells. (Data are shown in appendix A.)

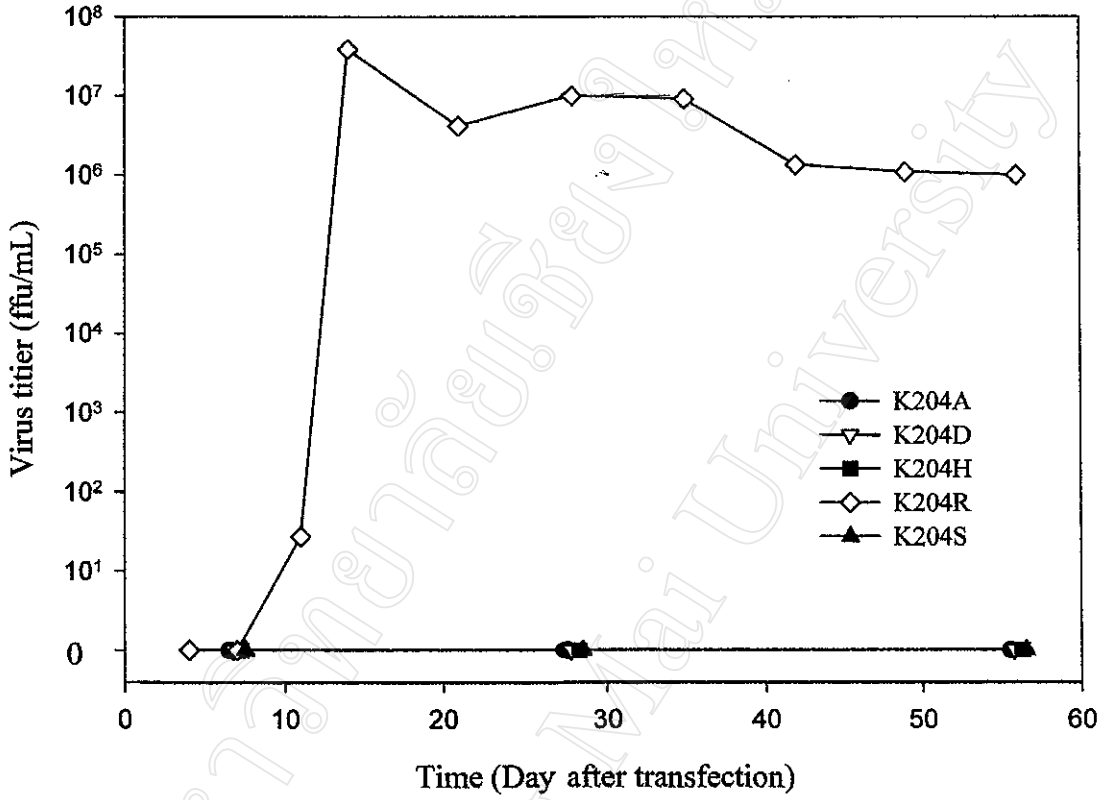


Figure 15. Kinetics of virus production following transfection of five different K204 mutant transcripts into C6/36 cells. For the transfection of K204A, K204D, K204H and K204S transcripts, virus titration was performed only on days 7, 28 and 56. (Data are shown in appendix A.)

In the transfection of R205 mutation set, *in vitro* transcripts of the full-length cDNA clones containing the R205 mutation set was transfected into C6/36 cells. As a negative control, lipofectin without added RNA transcripts was used. As it is expected that mutation at this position would be deleterious for virus replication, the positive control for transfection was intentionally omitted in order to eliminate the chance of cross-contamination. Following transfection, release of mutant viruses from transfected cells into culture medium was monitored on days 4, 7, 11, 14, 21, 28, 35, 42, 49 and 56 after transfection by dot immunoassay employing pooled convalescent human sera (Figure 16A and 16B).

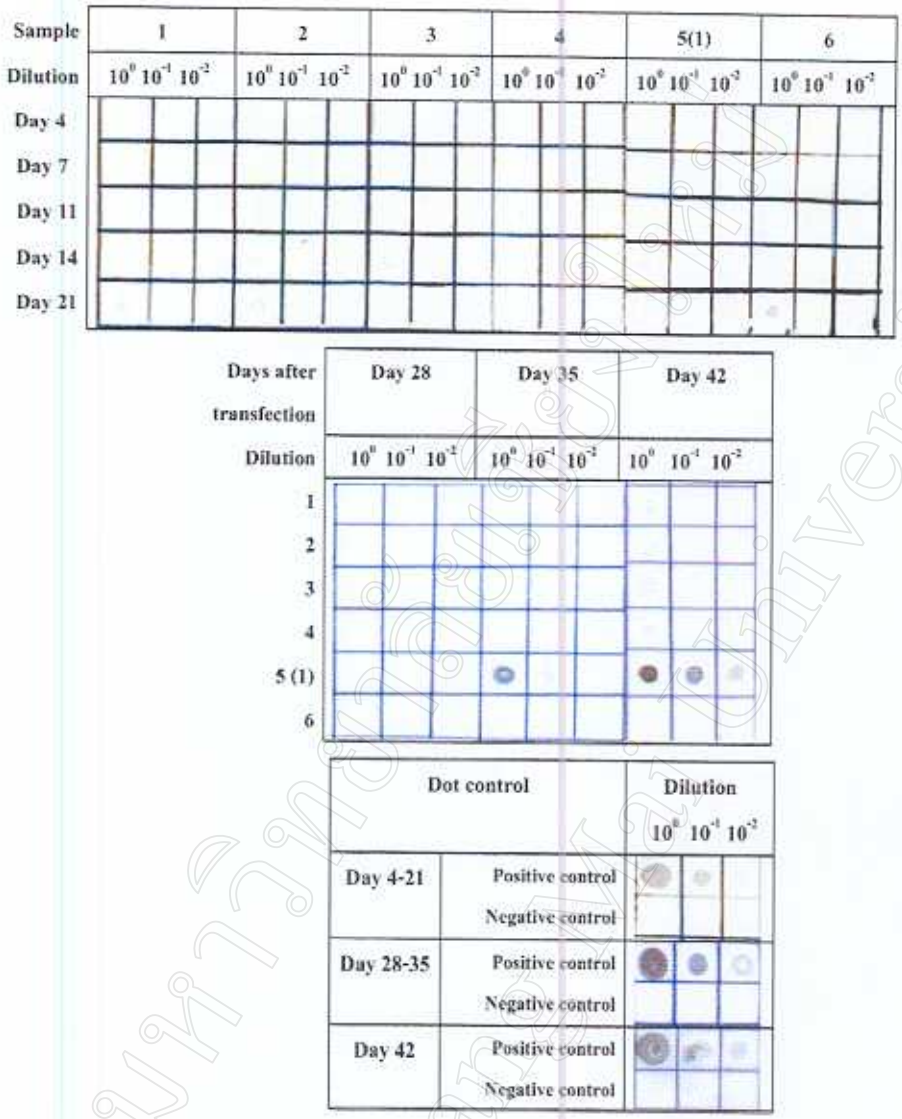
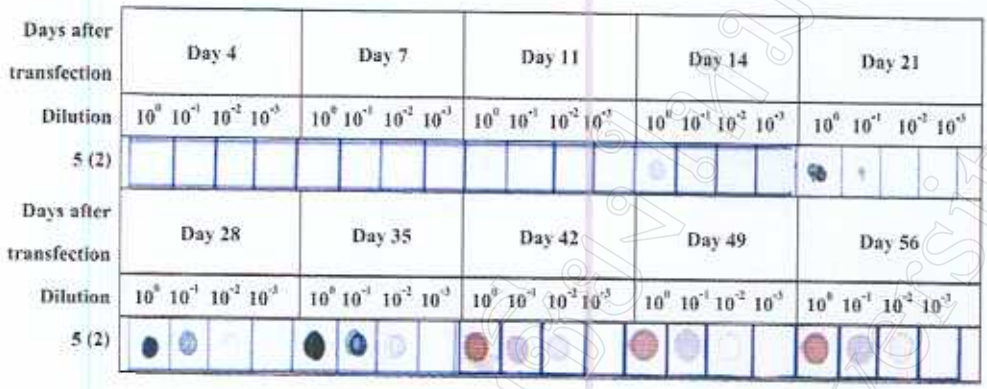


Figure 16A. Release of mutant dengue viruses from C6/36 monolayers following transfection with five different R205 mutant transcripts. Culture media from indicated post-transfection days were diluted serially, dotted onto nitrocellulose membrane and reacted with pooled convalescent human sera. Set 1: lipofectin control, set 2: R205A mutant, set 3: R205D mutant, set 4: R205H mutant, set 5 (1): R205K mutant (experiment I), and set 6: R205S mutant. The positive control was C6/36 culture media containing 7.0 x 10^7 ffu/mL of 16681*Nde*(+) virus whereas the negative control was culture media of uninfected C6/36 cells.



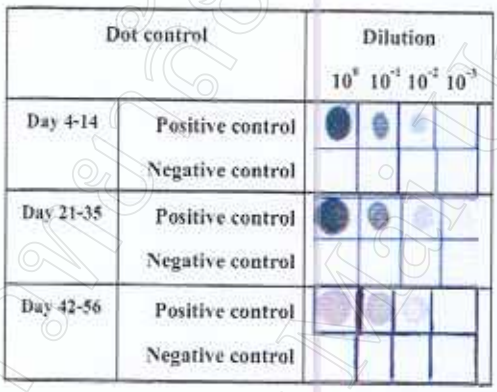


Figure 16B. Release of R205K mutant dengue virus, set 5 (2), from transfected C6/36 monolayers in the second experiment. Culture media from indicated post-transfection days were diluted serially, dotted onto nitrocellulose membrane and reacted with pooled convalescent human sera. The positive control was C6/36 culture media containing 7.0 x 10^{7} ffu/mL of 16681*Nde*(+) virus whereas the negative control was culture media of uninfected C6/36 cells.

In first transfection experiments on the R205K mutant, a detectable level of dengue virus released into culture medium from C6/36 was observed day 35 after transfection [Figure 16A, set 5(1)]. Whereas a detectable level of dengue virus released in the second transfection was detected earlier on day 14 after transfection [Figure 16B, set 5(2)]. As expected, no or very low level of signal was observed from the culture media of C6/36 transfected with lipofectin alone all through 56 days of the transfection experiment (Figure 16A, set 1). In both transfection experiments on the R205K mutant, virus production from C6/36 monolayers receiving capped RNA transcripts was detected by dot immunoassay only from cells transfected with the R205K mutant transcript [Figure 16A, set 5(1) and Figure 16B, set 5(2)]. Weak signal observed from transfection with mutant transcripts containing the R205A and R205D mutation at the lowest dilution of culture medium on days 21 post-transfection was likely to be enhanced background because similarly weak signal was also observed in the lipofectin-alone transfected cells.

In both transfection experiments on the R205K mutant, infectious viruses were detected at first by the focus immunoperoxidase assay on day 14 after transfection. In the first transfection, low titer of the R205K mutant viruses (4.00 x 10^1 to 1.36 x 10^2 ffu/mL) were detected on days 14, 21 and 28 after transfection. On day 49 and day 56 after first transfection the virus titer rose to 6.00 x 10^6 and 1.27 x 10^7, respectively (Figure 17). Similar results were obtained in the second transfection of the R205K mutant transcript: infectious viruses were produced at low titer (10 ffu/mL) on day 14 post-transfection, followed by a higher plateau range (2.82 x 10^4 to 4.82 x 10^4 ffu/mL) on days 21-56 after transfection (Figure 17). No infectious virus was detected in the culture media of C6/36 cells receiving the R205A, R205D, R205H and R205H mutant transcripts when assessed on days 7, 28 and 56 after transfection.

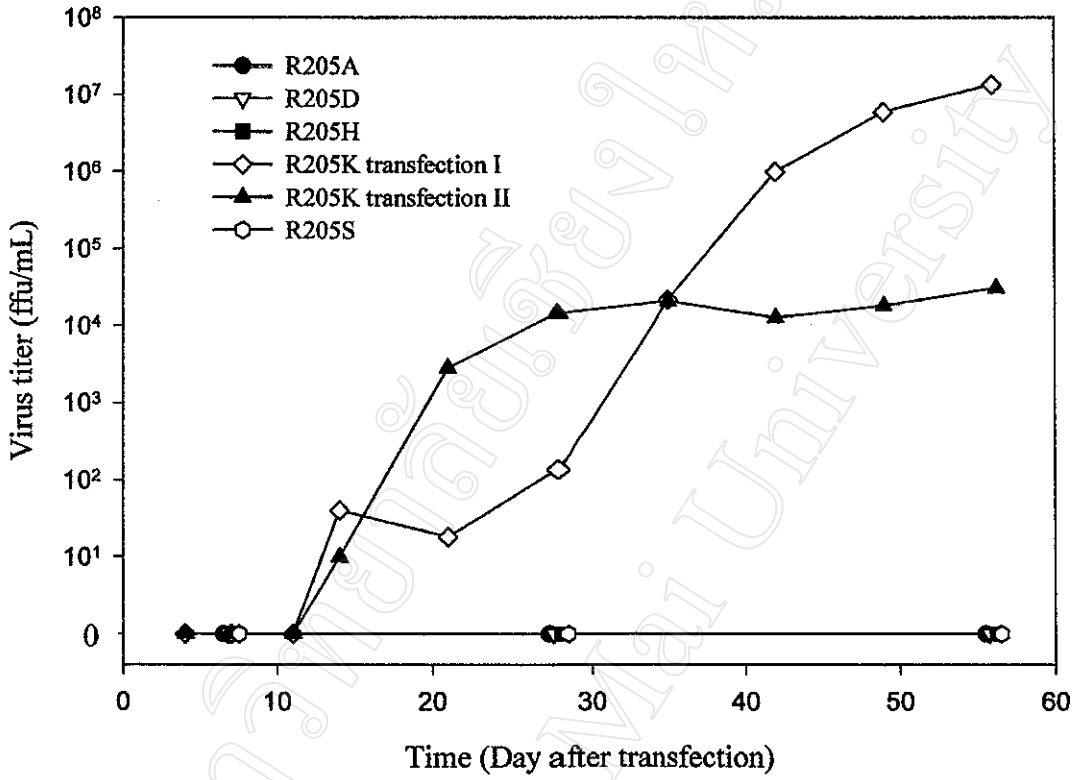


Figure 17. Kinetics of virus release into culture media following transfection of C6/36 cells with five different R205 mutant transcripts. The culture media were taken at indicated day following transfection and the infectious virus titer was determined by focus immunoassay. For the transfection of R205A, R205D, R205H and R205S transcripts, virus titration was performed only on days 7, 28 and 56. (Data are shown in appendix B.)

During the titration of the mutant viruses by focus immunoassay, it was observed that the K204R mutant viruses generated the larger foci of infected PS clone D cells, when assessed by a 4-step immunoperoxidase staining method, than the R205K mutant dengue virus. When 56 K204R-infected PS foci from days 11, 14 and 56 after transfection were photographed and infected cells in each focus counted manually, most K204R foci contained more than 130 cells/focus with the mean value of 163.6 cells/focus (Appendix C). The burst size (number of infected cells/focus) of the K204R mutant was quite comparable with that of 16681 parent (mean, 209.91 and 178.82 cells/focus; N. Sittisombut and P. Keelapang, unpublished results). By visual inspection, the large size of the K204R foci was observed throughout 56 days following the transfection (Figure 18, 21A and 21B). When the genomic RNA was extracted from K204R mutant viruses collected on days 11 after transfection, reverse transcribed, amplified and sequence analyzed. No other nucleotide sequence changes were detected in the region nt 501-800 surrounding the prM-M junction in any of the mutant viruses. (Figure 19 and Appendix I)

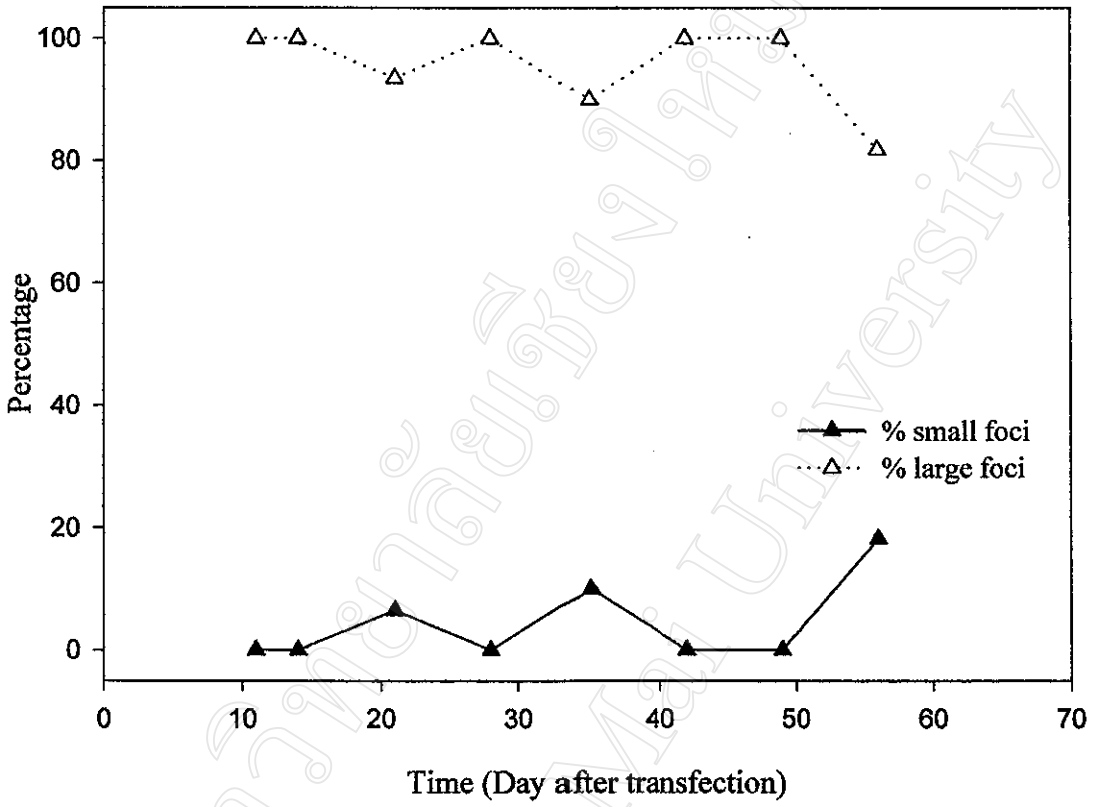


Figure 18. Proportion of small and large foci of the K204R mutant virus following transfection into C6/36 cells. Small foci contained 20 or less infected cell/focus, whereas, large foci contained more than 20 infected cells/focus. (Data shows in appendix C.)

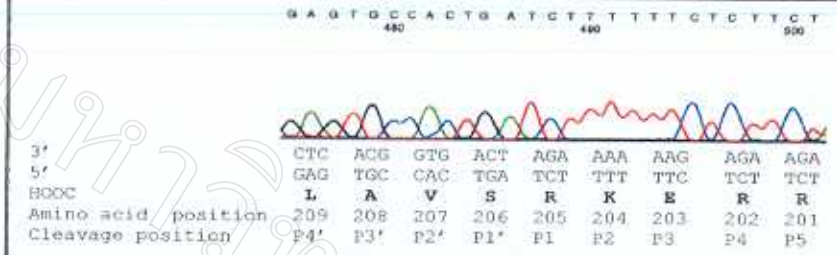
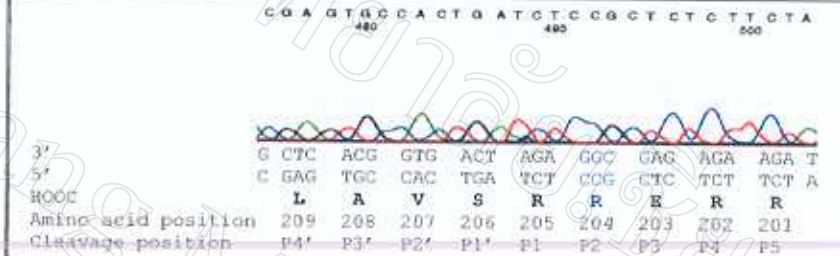
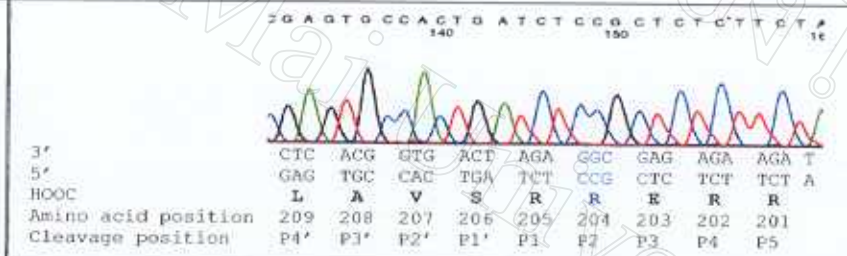
Sample	Sequence at prM/M junction
pBK S1SP6-10723Δ402 *Pst* I full-length cDNA clone of dengue serotype 2 strain 16681	
pBK S1SP6-1547Δ402 *Pst* I-K204R cDNA clone	
Nested RT-PCR product of K204R virus, day 11 after transfection	

Figure 19. Nucleotide sequences surrounding the prM-M cleavage site of K204R cDNA and K204R virus as compared with the parent cDNA clone. Sequence analysis was performed by the primer C859, which allowed the determination of the non-coding strand of the plasmid sequence or the RT-PCR product of the mutant virus. The blue letters indicate intended mutation.

In contrast to the large size of infected Ps clone D foci observed with the K204R mutant, the R205K mutant virus exhibited distinct small foci on days 14-28 after transfection (range 2 to 19 infected cells/focus; mean 6.3 infected cells/focus; n = 19 foci) (Figure 20 and 21C). Interestingly, on day 28 after transfection a number of larger foci was observed and the proportion of the large focus rapidly increased in such a way that most of the foci from days 42-56 after transfection were of large size (range 76 to 563 infected cells/focus, mean 227.4 infected cells/focus, n = 17 foci) (Figure 20 and 21D). The mean burst size of R205K viruses from late transfection days increased by 36 folds when compared with viruses from the early post-transfection period (Appendix D).

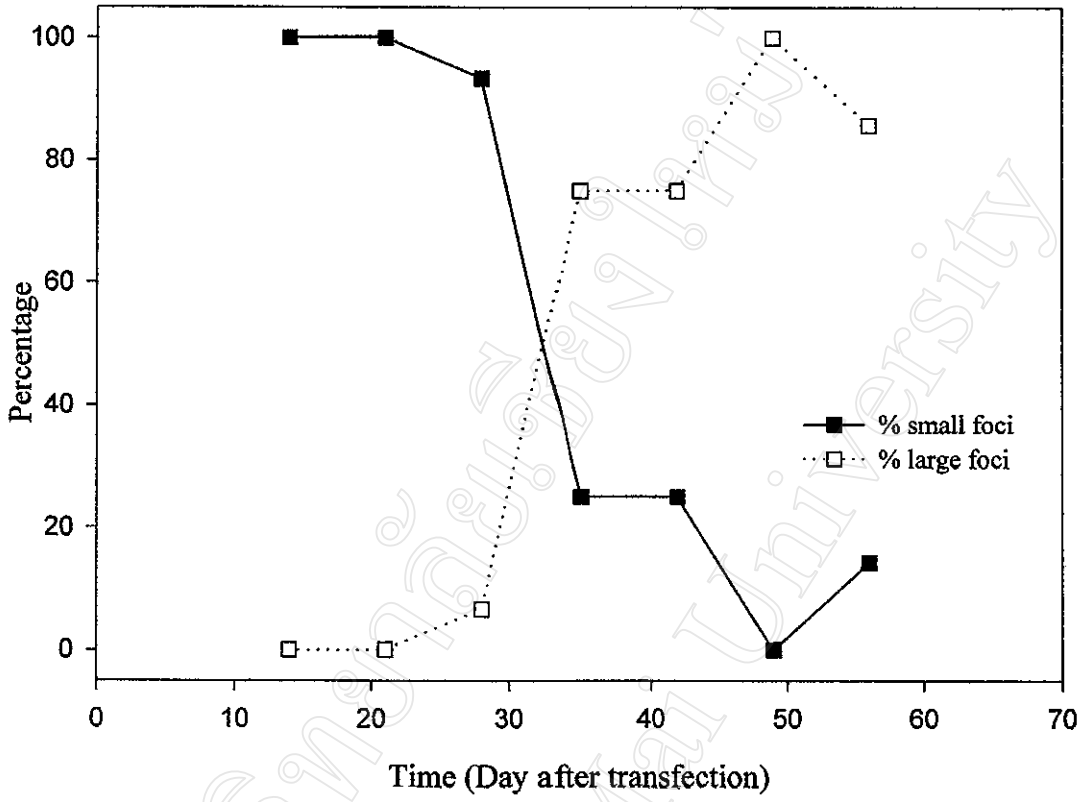


Figure 20. Proportion of R205K mutant viruses generating small and large infected PS foci during 56 days of transfection into C6/36 cells. The R205K mutant virus was generated by transfecting capped mutant RNA transcripts into C6/36 cells. At indicated days, virus titration was performed in PS clone D cells and the number of infected cell per focus was determined manually. Small foci contained 20 or less infected cell/focus, whereas large foci contained more than 20 infected cells/focus. (Data are shown in appendix D.)

Table 9. Comparison of the dengue K204R and R205K mutant viruses.

Characteristics	K204R mutant		R205K mutant (transfection I)		R205K mutant (transfection II)	
	Early (Days 11-21)	**Late** (Days 56)	**Early** (Days 14-28)	**Late** (Day 56)	**Early** (Days 14-21)	**Late** (Day 56)
Peak titer (ffu/mL)	$3.87x10^7$	$1.00x10^6$	$1.36x10^2$	$1.36x10^7$	$2.82x10^3$	$3.90x10^4$
Mean burst size, expressed as infected cells/focus (n)	185.2 (31)	136.8 (25)	6.3 (19)	227.4 (17)	ND	ND
Proportion of large foci[A] (n)	96.2% (78)	85.7% (14)	4.8% (21)	85.8% (14)	0% (32)	54.7% (53)
Proportion of small foci[B] (n)	3.9% (78)	14.3% (14)	95.2% (21)	14.3% (14)	100% (32)	45.3% (53)

A, large foci contained more than 20 infected cells/focus by visual inspection.

B, small foci contained 20 or less infected cells/focus by visual inspection.

ND, not done.

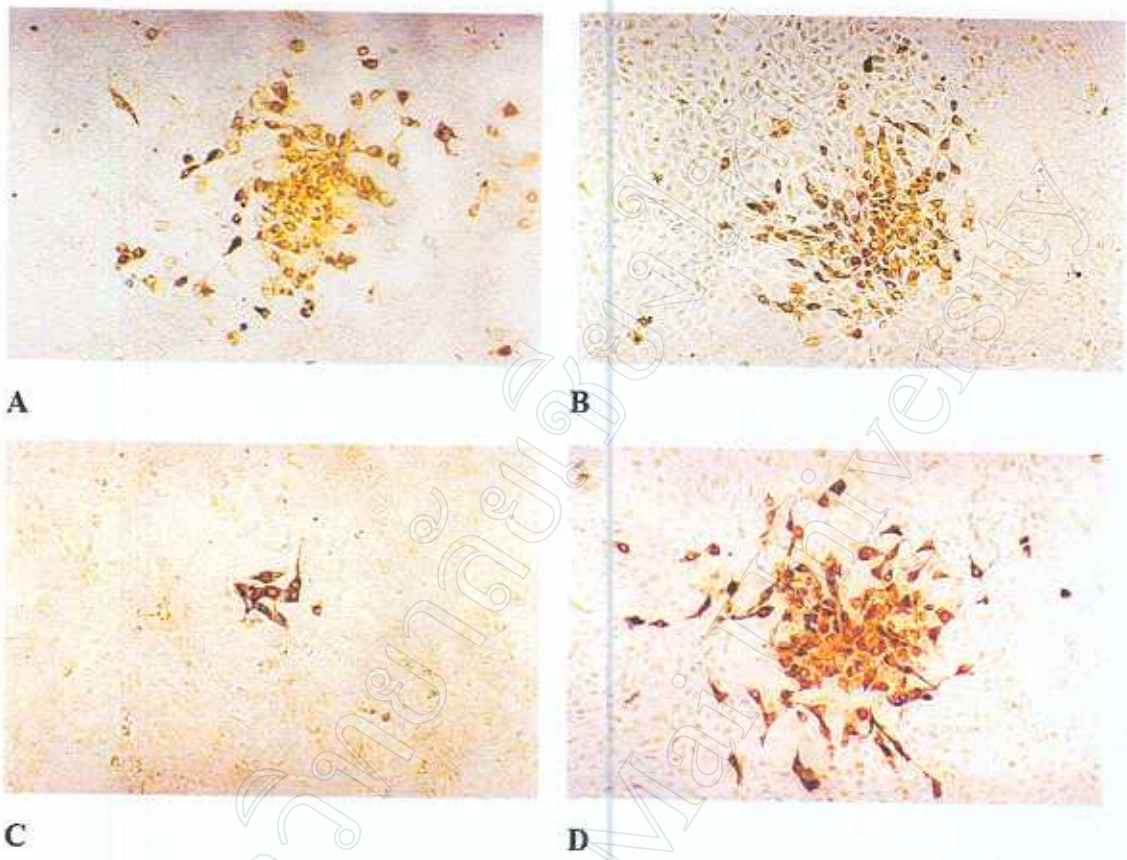


Figure 21. Representative foci of the K204R and R205K mutant viruses, as observed in infected PS clone D cells following immunoperoxidase staining. **A**: K204R mutant at day14 after transfection. **B**: K204R mutant at day 56 after transfection. **C**: R205K mutant at day 21 after transfection. **D**: R205K mutant at day 56 after transfection.

To explore the underlying cause of the changes in virus titer in the culture media and the change in the burst size of infected foci observed in the second month of the transfection of C6/36 cells with the R205K mutant transcript, genomic RNA was extracted from R205K mutant viruses collected on days 14, 28, 35 and 42 only after the first R205K transfection, reverse transcribed and amplified. The PCR products were subjected to nucleotide sequence analyses and the prM-M junction sequences obtained compared with the parent 16681 virus and the corresponding mutated full-length cDNA clone. When compared with the wild type 16681 sequence, the full-length cDNA clone with the R205K mutation contained 6 substituted bases: A708G, G710A, A711G, T712A, C713G and A714C. The prM-M junction sequence of the mutant R205K virus collected from transfected C6/36 medium on days 14 post-transfection, which generated small PS foci, was identical to the mutant full-length cDNA clone. Mutant R205K virus collected on days 28 and 35 post-transfection, however, displayed a mixture of intended sequence and a reversion of the nt 710 (A to G, Figure 22), which resulted in the amino acid arginine at the P1 position (position 205 of the polyprotein) instead of the intended lysine. The presence of the A710G reversion observed on days 28 - 35 after transfection corresponded with the appearance of infectious viruses causing large PS foci when assessed by focus immunoassay. On day 42 after transfection, the A710 intended base disappeared completely (Figure 22) and only the reverted sequence G710 was observed. On this day, the percentage of viruses causing large PS foci reached a submaximal level of 70%. No other nucleotide sequence changes were detected in the region nt 501-800 surrounding the prM-M junction in any of the mutant viruses. (Figure 21 and Appendix I). Thus, from this transfection experiment, mutant dengue virus with lysine at the position P1 of the prM-M cleavage junction was viable, but replicated to lower titer and displayed small focus phenotype. In contrast, its reverted mutant progeny grew more efficiently as detected by higher virus titer and large focus

phenotype. This result suggested that efficient dengue virus replication required the arginine side chain at the P1 position of the prM-M cleavage junction; lysine side chain, although is also positively charged, is not optimal for virus multiplication.

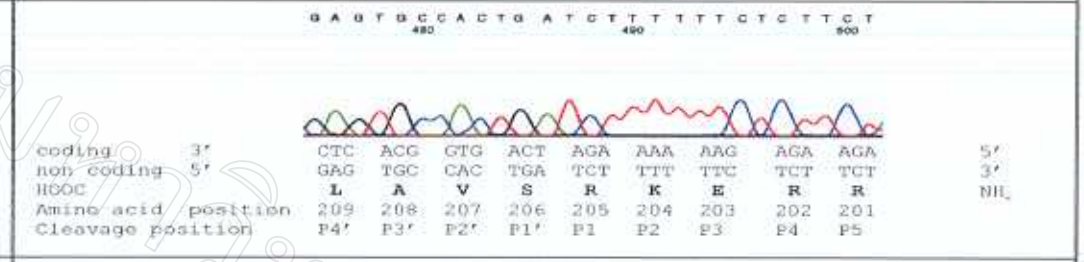
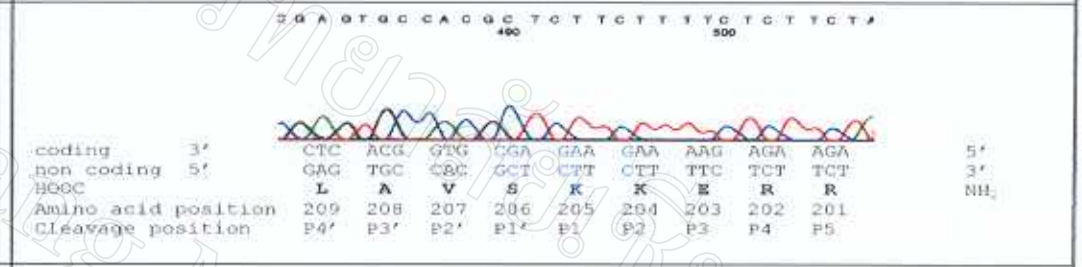
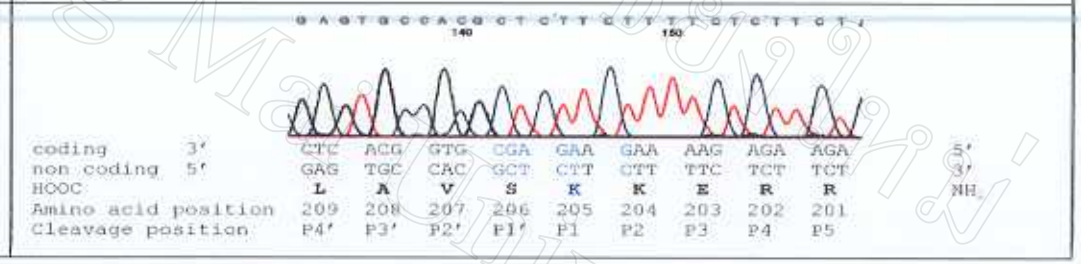
Sample	Sequence at prM/M junction
pBK S1SP6-10723Δ402 *Pst* I full-length cDNA clone of dengue serotype 2 strain 16681	
pBK S1SP6-10723Δ402 *Pst* I-R205K full-lenght cDNA clone	
Nested RT-PCR product of R205K virus, day 14 after transfection	

Figure 22. Nucleotide sequence surrounding the prM-M cleavage site of K204R cDNA and K204R virus compared with the wild type by using primer C859. Sequence analysis was performed by the primer C859, which allowed the determination of the non-coding strand of the plasmid sequence or the RT-PCR product of the mutant virus. The blue letters indicate intended mutation and the red letters indicate the reverting sequence.

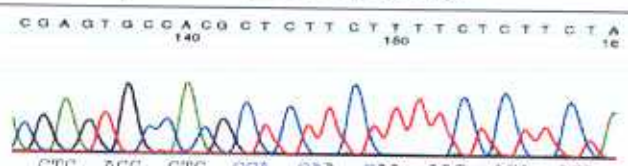
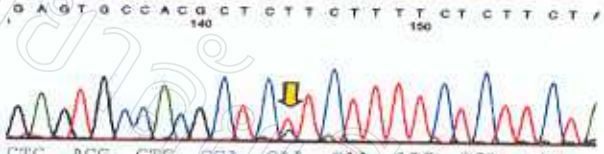
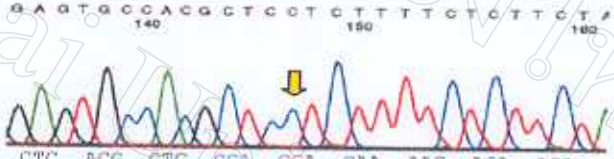
Sample	Sequence at prM/M junction
Nested RT-PCR product of R205K virus, day 21 post transfection	 coding 3′ CTC ACG GTG CGA GAA GAA AAG AGA AGA 5′ non coding 5′ GAG TGC CAC GCT CTT CTT TTC TCT TCT 3′ HOOC L A V S K K E R R NH2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5
Nested RT-PCR product of R205K virus, day 35 post transfection	 coding 3′ CTC ACG GTG CGA GAA GAA AAG AGA AGA 5′ non coding 5′ GAG TGC CAC GCT CTT CTT TTC TCT TCT 3′ HOOC L A V S K K E R R NH2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5
Nested RT-PCR product of R205K virus, day 42 after transfection	 coding 3′ CTC ACG GTG CGA GGA GAA AAG AGA AGA 5′ non coding 5′ GAG TGC CAC GCT CCT CTT TTC TCT TCT 3′ HOOC L A V S R K E R R NH2 Amino acid 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5

Figure 22. (Continue)