III. MATERIALS AND METHODS

1. *E. coli*

The bacteria *E. coli*, strain DH5$_\alpha$ F', was used to propagate and prepare recombinant plasmid DNA containing dengue virus complementary DNA sequence. The genotype of *E. coli* strain DH5$_\alpha$F' is F', ϕ80*dlac*ZΔM15, Δ(*lac*ZYA -*arg*F)U169, *end*A1, *rec*A1, *hsd*R17(rK$^-$mK$^+$), *deo*R, *thi*-1, *sup*E44 λ^-, *gyr*A96, *rel*A1. The strain DH5$_\alpha$ F' allows cloning of large plasmids due to the lack of endogenous restriction endonuclease and a recombination enzyme. This strain also provides α-complementation of β-galactosidase activity.

2. Cell lines

C6/36 *Aedes albopictus* mosquito cell line (Igarashi, 1978), maintained in Libovitz's L15 medium (GIBCO-BRL, Grand Island, New York, USA) containing 10% fetal bovine serum, was used as a target of the dengue full genome-length capped RNA-transfection and for propagating virus.

PS clone D, a porcine kidney cell line, maintained in Libovitz's L15 medium (GIBCO-BRL) containing 10% fetal bovine serum, was used for the titration of dengue wild-type and its mutants by focus immunoassay (Westaway, 1966; Sriburi *et al*, 2001).

3. Recombinant plasmids containing dengue virus genome

The 5' and 3'half-genomes and the full-length cDNA clones of a dengue serotype 2, strain 16681, are recombinant plasmids containing the 5'region, 3'region and the whole complementary DNA sequence, respectively, of the virus genome.

They had been constructed by using a high copy number plasmid, pBluescript II KS (Stratagene, California, USA)(Sriburi *et al.*, 2001).

3.1 pBK(S1SP6-4497)Δ402 *Pst* I(-), a 5'half-genome, was 7.3 kb in size. It contains one SP6 RNA polymerase promoter sequence followed by the region nt 1-4497 of dengue sequence. In addition, it also contains a unique *Kpn* I site (nt 4493-4498) and two *Pst* I sites at nt 208-213 and 1531-1536 within the viral sequence (Figure 3A). This plasmid was employed in the construction of the full-length mutant cDNA clone in two steps. Upon digesting with *Pst* I and getting rid of the 1.3-kb small *Pst* I fragment, the remaining 6.0-kb large fragment could be ligated with the 1.3-kb *Pst* I fragment containing mutated viral sequence derived from a subclone. In the second step, the mutant 5'half-genome was digested with *Kpn* I, dephosphorylated, and ligated to the *Kpn* I-digested fragment containing the region nt 4497-10723 from a 3'half-genome to form a full-length cDNA clone with mutated prM-M sequence.

3.2 pBK D2V4200-10723, a 3'half-genome, is 9.1 kb in size. It contains the region nt 4200-10723 of the dengue sequence and an introduced *Xba* I restriction site just distal to the 3'end of the dengue genome (Figure 3B). This plasmid also has two *Kpn* I restriction sites: a unique site at the nt 4493-4498 of the viral sequence and an introduced site distal to the 3'end of dengue and the *Xba* I site. Upon digesting with *Kpn* I, the 6.2-kb fragment (containing nt 4497-10723 of the dengue sequence, the *Xba* I site, and two *Kpn* I ends) could be ligated to the *Kpn* I-digested 5'half-genome to generate the full-length cDNA clone.

3.3 pBK(S1SP6-1547)Δ402 *Pst* I(-) is 4.5 kb in size. This plasmid is a subclone of the 5'half-genome as it contains only the region nt 1–1547 of the dengue sequence (Figure 3C). Within the viral sequence, a *Pst* I restriction site at the nt 398-403 of the dengue sequence had been abolished by introducing a silent mutation, A402C, at the nt 402 (Sriburi *et al.*, 2001). This mutation was useful in the cloning of the DNA fragment containing the prM-M junction into the 5'half-genome, and also in

differentiating between plasmid-derived recombinant dengue virus and the wild type strain. This plasmid was used as the template for mutation of the prM-M cleavage site employing PCR-based site-directed mutagenesis reactions. Following the mutagenesis, the mutant sequence within this plasmid was excised by digesting with *Pst* I, the resultant 1.3-kb fragment, which contains the region nt 212-1535 was then used for the construction of mutant 5'half-genome.

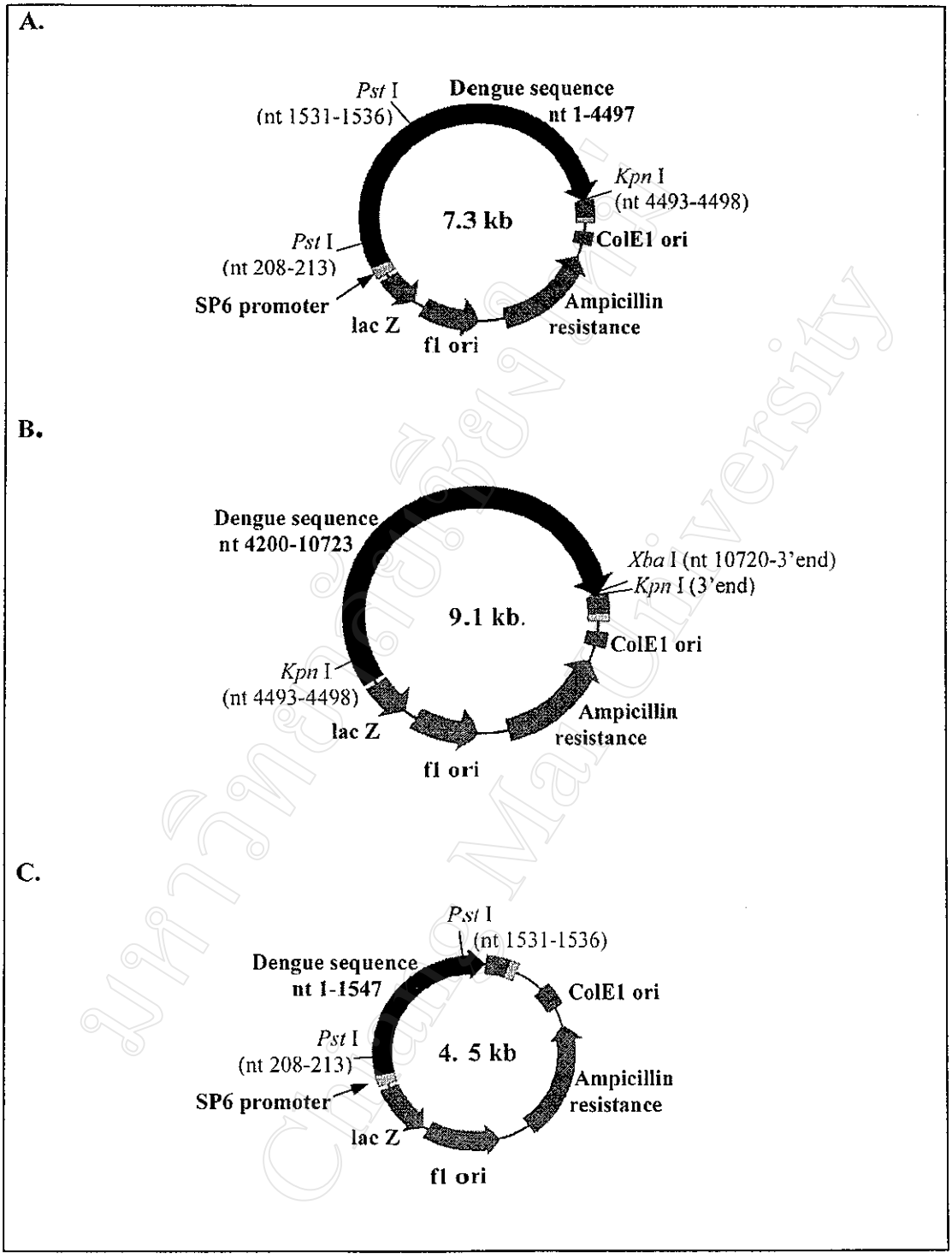

Figure 3. Three plasmids for the construction of the full-length dengue cDNA clone.

A: pBK(S1SP6-4497)Δ402 *Pst* I(-) plasmid. B: pBK D2V4200-10723 plasmid.

C: pBK(S1SP6-1547)Δ402 *Pst* I(-) plasmid.

4. Oligonucleotide primers for PCR-based site-directed mutagenesis

Ten pairs of complementary oligonucleotide primers were designed for PCR-based site-directed mutagenesis of the prM-M cleavage junction (Tables 2 and 3). Each pair of complementary primers contains from one to five altered bases, which would be introduced into the dengue sequence during the amplification of the plasmid DNA template. Also present in the mutated sequence was a new restriction enzyme recognition site, which was useful in the rapid detection/screening of the mutant plasmid clone. The oligonucleotide pairs were not exactly complementary, but overlapping; the intended mutant sequence was placed in the middle of complementary region. By this design, the entire length of both strands of the plasmid DNA template were amplified in linear fashion during several rounds of thermal cycling, generating mutated products containing staggered complementary ends on opposite strands (Hemsley *et al.*, 1989). Products of the linear amplification reaction were double-stranded DNA that contained the desired mutation and whose size was equal to the length of the plasmid DNA template. To get rid of the wild type plasmid DNA template from the mixture of templates and amplified mutated products, the mixture was treated with *Dpn* I, which specifically cleaved fully methylated $G^{m6}ATC$ sequences, present in the wild type, bacterially-derived plasmid DNA template. *Dpn* I digested only the bacterially generated plasmid DNA template, but it did not digest newly-synthesized DNA from in vitro amplification reactions. *Dpn* I-resistant DNA molecules, which were rich in desired mutants, were recovered by transforming *E. coli* and selected by ampicillin resistance.

To mutate the residue 204 (P2) at the prM-M cleavage junction, five pairs of oligonucleotide primers were designed in order to change the lysine residue to alanine (K204A), arginine (K204R), aspartic acid (K204D), histidine (K204H) and serine (K204S), respectively. Similarly, five pairs of oligonucleotide primers were designed

for the mutation of arginine at the position 205 (P1) to alanine (R204A), lysine (R204K), aspartic acid (R204D), histidine (R204H) and serine (R204S), respectively.

Table 2. Oligonucleotide primers for site-directed mutagenesis employed to change at amino acid residue 204

Desired mutation	Primer name	Primer sequence[A]	Nucleotide position[B]	Marker for screening/ selection
1. K204A	SM204K/A	5′ GAGAA**GCGCGC**TCAGTGGCACTCGTTCCA 3′	700-729	*Bss*H II Digestion
	RM204K/A	3′ ACCCTCTTGTATCTTCTCTT**CGCGCG**AGTC 5′	686-705	
2. K204D	SM204K/D	5′ GAGAA**GATC**GATCAGTGGCACTCGTTCCA 3′	700-729	PCR amplified and
	RM204K/D	3′ ACCCTCTTGTATCTTCTCTT**CTAG**CTAGTC 5′	686-705	*Cla* I digestion
3. K204H	SM204K/H	5′ GAGAA**CATC**GATCAGTGGCACTCGTTCCA 3′	700-729	PCR amplified and
	RM204K/H	3′ ACCCTCTTGTATCTTCTCTT**GTAG**CTAGTC 5′	686-705	*Cla* I digestion
4. K204R	SM204K/R	5′ GAGA**GCGG**AGATCAGTGGCACTCGTTCCA 3′	700-729	*Bsr*B I digestion
	RM204K/R	3′ GTACCCTCTTGTATCTTCT**CTCGCC**TCTAG 5′	684-703	
5. K204S	SM204K/S	5′ GAGAA**TCGC**GATCAGTGGCACTCGTTCCA 3′	700-729	PCR amplified and
	RM204K/S	3′ ACCCTCTTGTATCTTCTCTT**AGCG**CTAGTC 5′	686-705	*Nru* I digestion

A, bold letters indicate mutated nucleotide sequences. Underlined letters indicate the restriction enzyme recognition site.

B, numbers represent base position in the genome of strain 16681 according to Kinney *et al.* (1997).

Table 3. Oligonucleotide primers for site-directed mutagenesis employed to change at amino acid residue 205

Desired mutation	Primer name	Primer sequence[A]	Nucleotide position[B]	Marker for screening/ selection
1. R205A	SM205R/A	5′ AAAAA**GCT**TCAGTGGCACTCGTTCCACATG 3′	704-733	*Hind* III
	RM205R/A	3′ CTCTTGTATCTTCTCTTTTT**TCGA**AGTCACC 5′	688-718	digestion
2. R205D	SM205R/D	5′ AAAAA**GAC**TCAGT**C**GCACTCGTTCCACATG 3′	704-733	*Pfl*F I digestion
	RM205R/D	3′ CTCTTGTATCTTCTCTTTTT**CTG**AGTCA**G**CGTGA 5′	688-722	
3. R205H	SM205R/H	5′ AAAAA**CAC**TCAGTGGCACTCGTTCCACATG 3′	704-733	*Dra* III digestion
	RM205R/H	3′ CTCTTGTATCTTCTCTTTTT**GTG**AGTCACC 5′	688-718	
4. R205K	SM205R/K	5′ GAAAA**GAAGAGC**GTGGCACTCGTTCCACATGT 3′	703-734	Sequence analysis
	RM205R/K	3′ CTCTTGTATCTTCTCTTTT**CTTCTCG**CACCG 5′	688-719	
5. R205S	SM205R/S	5′ GAAAA**G**AG**C**TCAGTGGCACTCGTTCCACATG 3′	703-733	*Eco*ICR I or *Sac* I
	RM205R/S	3′ CTCTTGTATCTTCTCTTTT**CTCG**AGTCACC 5′	688-718	digestion

A, bold letters indicate mutated nucleotide sequences. Underlined letters indicate the restriction enzyme recognition site.

B, numbers represent base position in the genome of strain 16681 according to Kinney *et al.* (1997).

5. PCR-based site-directed mutagenesis

The plasmid pBK(S1SP6-1549)Δ402 *Pst* I(-) containing the region nt 1-1547 of the dengue serotype 2 virus 16681 strain was prepared from *E. coli* strain $DH5_{\alpha}$ F' and used as a template in PCR-based site-directed mutagenesis (QuickChange, Stratagene). The oligonucleotide primers containing desired mutation of the prM-M junction were extended during temperature cycling by *Pfu* DNA polymerase (Promega Corporation, Wisconsin, USA). On incorporation of oligonucleotide primers, the mutated plasmid containing staggered ends was generated. The polymerase chain reaction-based mutagenesis was performed in 100 µL volume of mixture containing 10 mM Tris-HCl, pH 8.3 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 200 µM each of four dNTPs, 100 pmol of each oligonucleotide, and 2 units of *Pfu* DNA polymerase. After overlayering the mixture with two drops of mineral oil, the amplification was carried out in the thermal cycler (Model 480, Perkin Elmer, Foster city, California, USA) for 30 cycles as follows: denaturation, 94°C for 45 sec, annealing, 50-55°C for 45 sec, and extension, 72°C for 10 min. The last extension step was at 72°C for 20 min (Table 4). Five µL of the product were analyzed by electrophoresis through a 0.8% agarose gel (Promega) in Tris-acetate-EDTA (TAE) buffer at 50 volts for 1 hour. The gel was stained with 10 µg/mL ethidium bromide for 10 minutes, destained and DNA bands visualized with an ultraviolet light source.

Table 4. Parameter of PCR based site-directed mutagenesis for change at residues 204 and 205.

Mutation Designation	Primer pair	PCR parameter			
		Denaturation	Annealing	Extension	Cycles
1. K204A	SM204K/A, RM204K/A	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30
2. K204D	SM204K/D, RM204K/D	94°C, 45 sec.	50°C, 45 sec	72°C, 10 min.	30
3. K204H	SM204K/H, RM204K/H	94°C, 45 sec.	50°C, 45 sec	72°C, 10 min.	30
4. K204R	SM204K/R, RM204K/R	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30
5. K204S	SM204K/S, RM204K/S	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30
6. R205A	SM205R/A, RM205R/A	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30
7. R205D	SM205R/D, RM205R/D	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30
8. R205H	SM205R/H, RM205R/H	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30
9. R205K	SM205R/K, RM205R/K	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30
10. R205S	SM205R/S, RM205R/S	94°C, 45 sec.	55°C, 45 sec	72°C, 10 min.	30

The PCR products were purified by QIAquick PCR purification kit (QIAGEN, Chatsworth, California, USA) and eluted with 30-50 μL of elution buffer. The purified products were treated with *Dpn* I (New England Biolabs, Beverly, Massachusetts, USA) to digest the parental DNA template and select for the synthesized DNA containing mutation on both DNA strands, as *Dpn* I was active on both methylated and hemimethylated DNA. Two-5 μL of *Dpn* I-digested PCR products were introduced into *E. coli* by transformation and selected by ampicillin. Single colonies of transformant *E. coli* were picked and grown in 2-mL culture for small-scale preparation of plasmid DNA. Plasmids containing the desired mutation were selected based on digestion with corresponding restriction enzymes and by nucleotide sequence analysis.

6. Preparation of mutant plasmid

Competent *E. coli* strain DH5$_\alpha$ F' was prepared by the calcium chloride method for use in the chemical transformation method (Sambrook *et al*, 1989). One hundred μL of competent cells were transferred to a chilled 17x100-mm polypropylene tubes, and *Dpn* I-digested PCR products (~50 ng in volume of 10 μL) were added to each tube. The content of the tubes was mixed by swirling gently, and the tubes were stored on ice for 30 minutes. The tubes were swirled in preheated water at 42°C for exactly 90 seconds. The tubes were transferred to an ice bath and the cells were allowed to chill for 2 minutes. Nine hundred μL of SOC medium were added to each tube and incubated at room temperature for 2 hours to allow for the phenotypic lag. The transforming *E. coli* was selected in L-agar plate with 25 μg/mL of ampicillin and the plates were incubated at 25°C for two days. Isolated bacterial colonies were picked up and grown in LB broth that contained 25 μg/mL of ampicillin for two days at 25°C.

In the alternative method, the electroporation transformation (Sambrook *et al*, 1989): 100 μL of freezing *E. coli* strain DH5$_\alpha$ F' were thawed on-ice and mixed with 10 μL of DNA plasmid or ligation reaction. The bacteria-DNA mixtures were transferred to 0.2-cm gapped electroporation cuvette and incubated on ice for 30 minutes. The mixtures were electroporated by the Gene pulser (Bio-Rad) at 1.5 kV, 200 μF, 25Ω and then 1 mL of SOC medium was added in the cuvette. The *E. coli* was incubated at 25°C in shacking incubator for 1 hr, and then, the transformant *E. coli* was selected in the L-agar plate with 25 μg/mL of ampicillin; the plate was incubated further at 25°C for two days.

6.1 Preparation of plasmid DNA by small-scale boiling lysis

The plasmid DNA for screening by electrophoresis or restriction endonuclease digestion was prepared from small-scale (2 mL) bacterial cultures. Firstly, a 2-mL culture from a single colony was grown overnight in LB broth plus ampicillin (25 μg/mL) at 23-25°C, and 0.5-1 mL of the culture was pelleted by centrifugation at 5,000 X g for 5 minutes in 1.5 mL tubes. The supernatant was removed by aspiration. Bacterial pellet was resuspended in 25 μL of STET buffer and 2 microliters of 10 mg/mL lysozyme. The tubes were placed in boiling water for 45 seconds. The reaction was immediately spun in a microcentrifuge at 13,000 X g for 12 minutes at room temperature. The pellets were removed and discarded with a sterile toothpick and 30 μL of isopropanol were added to the supernatant. After centrifugation for 15 minutes, the DNA pellet was washed with 200-400 μL of 70%(v/v) ethanol and dried. The DNA pellet was resuspended in 10-15 μL of TE buffer. Finally, the plasmid DNA was analyzed by endonuclease digestion and electrophoresis.

6.2 Midi-preparation of plasmid DNA by the alkaline lysis method

Briefly, isolated bacterial colonies were grown for two days in 100 mL of LB broth in the presence of 25-μg/mL ampicillin. The bacterial cultures in 250-mL flasks were shaken at 230 rpm in orbital shaker bath (Model 360, Precision Scientific, USA), at 23-25°C, until slightly turbid. The bacterial cells were harvested by centrifugation at 6,000 X g for 15 min at 4°C. The bacterial pellet was resuspended in 9 mL of resuspension buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA) and 9 μL of 100 μg/mL RNase A was added. Nine mL of lysis buffer (200 mM NaOH; 1% SDS) were added, mixed gently by inverting 4-6 times, and incubated at room temperature for 5 min. An equal volume of chilled neutralizing buffer (3.0 M Potassium acetate, pH 5.0) was added and mixed immediately but gently. After addition of neutralizing buffer, the lysate was incubated on ice for 15 min and passed though a filter cartridge. The filtrate was applied to 4 mL of equilibration buffer (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% TritonX-100) equilibrated the anion-exchange resin in a column and allow the column to empty by gravity flow. Ten mL of washing buffer (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanal) were applied twice and plasmid DNA was eluted by 5 mL of elution buffer (1.25 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanal). Plasmid DNA was precipitated by adding 3.5 mL of isopropanol, centrifuged immediately at 15,000 X g for 30 min at 4°C, and air-dried. The plasmid pellet was dissolved in suitable volume of 10 mM Tris-HCl (pH 8.0) or TE buffer (pH 8.0). The plasmid DNA was analyzed by endonuclease digestion and agarose gel electrophoresis to determine the presence of the mutated sequence and the key restriction enzyme sites, such as *Xba* I site, which was necessary for linearization of plasmid. The resulting DNA preparations were then used for nucleotide sequence analysis and as the template of *in vitro* transcription reaction.

7. Nucleotide sequence analysis for the confirmation of mutated sequence

The 1.3-kb *Pst* I fragments derived from the pBK(S1SP6-1547)Δ402 *Pst* I(-) mutant plasmids were purified by agarose gel-electrophoresis in low melting point agarose gel followed by extraction and elution from gel. The *Pst* I fragments were electrophoresed through a 0.8% low melting agarose gel (Promega) containing 0.01 mg/mL ethidium bromide in TAE buffer at 50 volts for 1 hour. The gels were visualized under an ultraviolet light source and a 1.3-kb DNA band was excised with a sterile scalpel. Elution was accomplished by using the silica-gel membrane binding in spin column (QIAGEN). Briefly, the gel was weighed and 3 volumes of dissolving buffer were added to dissolve it. After incubation at 50°C for 10 min, 1 gel volume of isopropanol was added. Next, the sample was loaded into a spin column placed inside a 2-mL collection tube and centrifuged at 13,000 rpm (~10,000 X g) for 1 min. The flow-through was discarded and 0.75 mL of washing buffer was added successively. The 1.3-kb *Pst* I fragment DNA, which bound to the column, was eluted by 30-50 μL of 10 mM Tris-HCl (pH 8.0). The concentration of 1.3-kb *Pst* I fragment DNA was calculated from the absorbance at 260 nm, as measured in a spectrophotometer (Spectronic Genesysz, U.K.), and estimated by comparing with a known amount of phage lambda DNA *Hind* III -digested fragments (New England Biolabs) in ethidium bromide-stained agarose gel.

Nucleotide sequence analysis was performed by using the Sanger dideoxy-mediated chain-terminator method. The dye terminator cycle sequencing ready reaction kit (Perkin Elmer) was employed according to the manufacturer's protocol with some modifications. Briefly, the cycle sequencing reaction was performed in a 20 μL volume of mixture containing 8 μL of ready reaction mix, 450 ng of 1.3-kb *Pst* I fragments DNA and 3.2 pmol of individual primer. After overlaying the mixture with 40 μL of mineral oil, the cycle sequencing reaction was carried out in a DNA thermal cycler (Model 480, Perkin Elmer) for 25 cycles according to the following

parameters: 94°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 min. The sample was purified by ethanol precipitation. For loading the sample, the sample was resuspended in 25 μL of template suspension reagent and denatured by heating at 95 °C for 2 min. The sample was then analyzed by using an automated DNA sequencer (ABI PRISM 310, Genetic Analyzer, Perkin Elmer). Four oligonucleotide primers; C 616, C 859, C 1204 and S 1151 were employed in the analysis of the region nt 1-1547 of the dengue genome (Table 5).

Table 5. Oligonucleotide primers for polymerase chain reaction and nucleotide sequence analysis

Designation	Sequence (5′ to 3′)	Position[C]
D_2J 134	TCAATATGCTGAAACGCGAGAGAAACCG	134-162
C 1518	CATCTGCAGCAACACCATCTC	1539-1519
C 616	AACAGTCTATGTCTTCTGGCTC	637-616
C 859[A]	TGTCGTTCCTATGGTGTATGC	879-859
C 1204[AB]	GGAGTGTTTGCAGACGAA	1221-1204
S 1151[B]	CGCGCTGCCCAACACAAGGG	1151-1170

A, primer for nucleotide sequence analysis at the prM-M cleavage site.

B, primer for polymerase chain reaction of Sistayanarain *et al* (1996).

C, number represents base position in the genome of strain 16681 according to Kinney *et al.* (1997).

8. Construction of full-length mutant cDNA clones

The plasmid pBK(S1SP6-1547)Δ402 *Pst* I(-), which contained designed mutation, was digested by *Pst* I endonuclease. The 1.3-kb fragment was separated from the other larger fragments by electrophoresis in low-melting temperature agarose gel and extracted from the excised gel. Before using in the construction of full-length cDNA clone, the presence of intended mutation of the prM-M junction within the 1.3-kb fragments was determined by restriction enzyme digestion and nucleotide sequence analysis. Then, the 1.3-kb *Pst* I fragment with intended mutation was ligated to the dephosphorylated 6.0-kb large *Pst* I fragment of the 5'half-genome cDNA (Figure4).

The following reaction conditions are for ligation of DNA inserts with plasmid vector in a 0.5-mL microcentrifuge tube. A molar ratio of 3:1 (insert: vector) is recommended (Sambrook *et al.*, 1989). The reaction components were added as follows: 2 μL of 5X ligase buffer [330 mM Tris (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP], 50 ng of dephosphorylated vector DNA, 150 ng of the 1.3-kb *Pst* I fragment and autoclaved distilled water to 9 μL. One μL (2 units) of T4 DNA ligase (Promega) was added to the reaction, mixed gently, and centrifuged briefly to bring the contents to the bottom of the tube. The mixture was incubated at 14°C for 12 hours. An aliquot of the ligation reaction was diluted fivefold in autoclaved distilled water and used to transform competent *E. coli* cells.

To screen the mutant 5'half-genome clone by restriction enzyme digestion electrophoresis, isolated bacterial colonies were picked and grown in LB broth containing 25 μg/mL of ampicillin for two days at 25°C and the plasmid was prepared by small-scale boiling method. Two 5'half-genome clones were propagated in bacterial culture to prepare adequate quantities of DNA. The resultant 5'half-genome plasmid DNA was checked for the presence of five restriction enzyme sites, *Eco*R I, *Eco*R V, *Kpn* I, *Nco* I and *Pst* I. The clones were expanded for midi-scale DNA

preparation. The mutant 5'half-genome plasmid was then digested by *Kpn* I to linearize and then dephosphorylated by using calf intestine alkaline phosphatase enzyme.

Full-length cDNA clones containing mutant prM-M junction sequence were constructed by ligating the 6.2-kb *Kpn* I fragment from a 3'half-genome, encoding the region of the nt 4497 to 3'end, into the *Kpn* I site (nt 4493-4498) of a mutant 5'half-genome (Figure 4). The ligated DNA was transformed into *E. coli*. The full-length cDNA clones were propagated in *E. coli* culture, to prepare the adequate useful quantities of DNA.

Briefly, isolated bacterial colonies were grown for two days in 100 mL of LB broth in the presence of 25-μg/mL ampicillin. The bacterial cultures in 250-mL flasks were shaken at 230 rpm, at 21-23°C in orbital shaker bath (Precision Scientific) until slightly turbid. The bacterial cells were harvested by centrifugation at 6,000 X g for 15 min at 4°C. The bacterial pellet was used for plasmid preparation by the alkaline lysis method. The full-length clones were checked by endonuclease and/or nucleotide sequencing analysis.

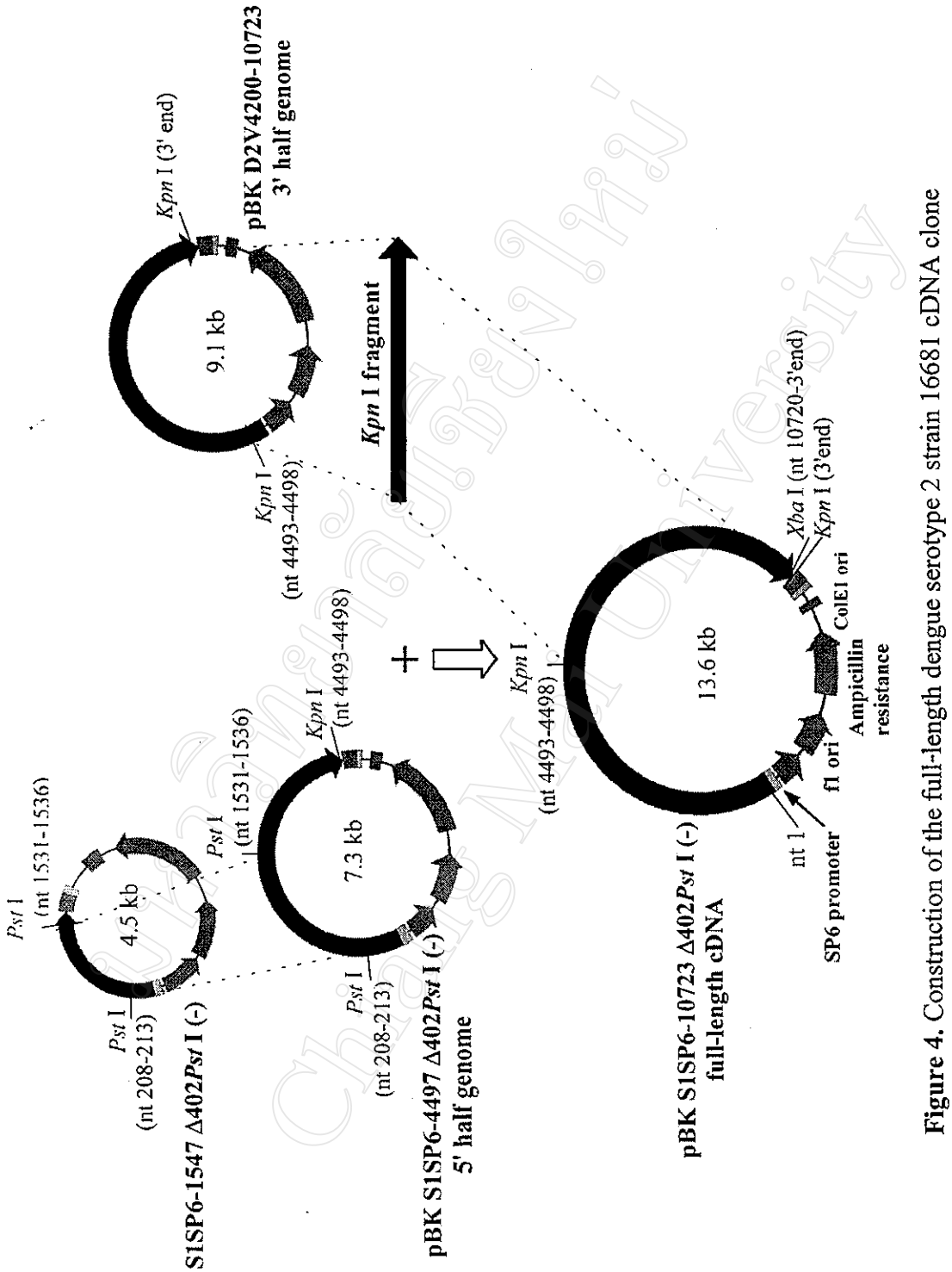

Figure 4. Construction of the full-length dengue serotype 2 strain 16681 cDNA clone

9. Generation of mutant viruses

9. 1 Production of genome-length capped RNA by *in vitro* transcription

The full-length cDNA plasmids that contain a mutant prM-M junction sequence were linearized by digestion with *Xba* I (Promega), followed by phenol/chloroform extraction and ethanol precipitation. The *in vitro* transcription was performed in a 20-μL reaction containing 1 μg of linearized cDNA template, 80 mM HEPES-KOH buffer (pH 7.5), 32 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiotheritol, 5 mM each of rATP, rCTP and rUTP, 1.25 mM of rGTP, 3.25 mM of $m^7G(5')ppp(5')G$ cap structure analogue (New England Biolabs) and 2 μL of SP6 RNA polymerase-pyrophosphatase mix (SP6 Ribomax kit, Promega) (Sriburi *et al.*, 2001). The reaction was incubated at 37°C for four hours. The yield and approximate size of the products were analyzed by electrophoresis throughout the 0.7% agarose gel containing 3.7% formaldehyde in MOPS-EDTA-Sodium acetate running buffer (0.2 M MOPS, pH 7.0; 80 mM sodium acetate; 10 mM EDTA, pH 8.0). The gel was stained with 10-μg/mL ethidium bromide for 30 seconds, destained and visualized with an ultraviolet light source.

9. 2 Introduction of capped RNA transcripts into cells by transfection

The C6/36 mosquito cell line, $3x10^5$ cells in 2 mL of Leibovitz's L-15 medium (GIBCO-BRL) supplemented with 10% fetal bovine serum, was incubated in 35-mm tissue culture plates at 29°C until they were 80-90% confluent. This usually took 24-48 hours. The C6/36 cells were washed three times with 1 mL of serum-free medium and allowed to stand at room temperature during the preparation of transfection mixture. The transfection reaction was prepared in 50-mL sterile tubes. For each transfection, 5-10 μL of capped RNA transcript and 5 μL of lipofectin (GIBCO-BRL) were added to 1 mL of L15 medium. The mixture was mixed gently and incubated at room temperature for 10 min. For each transfection, 1 mL of the

lipofectin-RNA mixture was overlaid onto the cell monolayer. The monolayer was incubated for 4 hours at room temperature. The mixture of RNA-lipofectin was then removed and replaced with 2 mL of Leibovitz's L15 medium containing 1.5% fetal bovine serum and the cells were incubated at 29°C for a total of 56 days. The culture media of RNA transfected-C6/36 cells were collected at days 4, 7, 11, 14, 21, 28, 35, 42, 49 and 56 after transfection. The culture media were screened for the production of dengue E protein by dot blot immunoassay. In the transfection, the parental capped RNA that transcribed from the linearized pBK(S1SP6-10723)Δ402 *Pst* I(-) #81.2 plasmid was transfected parallel.

9. 3 Detection of mutant dengue viruses

Three methods: dot blot immunoassay, reverse transcriptase-polymerase chain reaction (RT-PCR), and focus immunoperoxidase assay staining, were employed to detect mutant dengue viruses generated from transfected C6/36 cells.

9. 3.1 Dot blot immunoassay

The culture media of dengue RNA transfected-C6/36 were ten-fold serially diluted. Two μL of each dilution were dotted on the nitrocellulose membrane BA85: 0.45 μm (Schleicher & Schuell Inc., Germany) and air-dried. The membrane, which was subsequently blocked with 5% skimmed milk in PBS, was incubated with a 1:1,000 diluted solution of pooled convalescent human sera for 1 hour and washed with PBS three times. The membrane was incubated with anti-human Ig conjugated with a horseradish peroxidase (1:1,000 dilution) in 5% skimmed milk in PBS for 1 hour, and washed with PBS three times. The bound E protein was visualized by adding H_2O_2 substrate, 1% $CoCl_2$ and 3, 3' diaminobenzidine chromogen (Sigma, St. Louis, USA) for 15 minutes. Finally, the membranes were washed with sterile water to stop the reaction.

9. 3.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

Viral RNA, present in 140 µL of dengue virus infected-culture fluid, was extracted by using guanidine-isothiocyanate lysis in conjunction with silica gel–membrane purification (RNeasy, QIAGEN). Viral RNA was eluted with 30 µL of nuclease free water.

The viral genomic RNA was reverse transcribed into complementary DNA by using the reverse transcriptase enzyme, as follows: viral genomic RNA was heated to 70°C for 10 min, then quickly chilled on ice. Reverse transcription was carried out in a reaction mixture containing 10 mM Tris-HCl, pH 8.3 at 25°C; 1.5 mM $MgCl_2$; 50 mM KCl; 0.001% gelatin; 10 mM dithiothrietol; 0.5 mM each of the four dNTPs; 100 pmol of antisense primer; and 20 units of Superscript II RNase H^- reverse transcriptase (GIBCO-BRL). Reverse transcription was allowed to proceed at 56°C for 1 hour and stopped by heating at 94°C for 4 min. The cDNA products were used directly in amplification reactions.

The cDNA was amplified in 100 µL volume of mixture containing 10 mM Tris-HCl, pH 8.3 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 200 µM each of four dNTPs, 100 pmol of sense primer, and 2.5 units of Taq DNA polymerase (Promega). After overlayering the mixture with 40 µL of mineral oil, the amplification was carried out in the thermal cycler (Perkin Elmer), as follows:

For the primer pair D_2J-134 and cD_2J-2504 (Table 6): denaturation was at 94°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 10 minutes. The last extension step was at 72°C for 20 min. The reaction was carried out in the thermal cycles for 30 cycles.

Besides, the primer pair D_2J-134 and C1518 (Table 6) was used for semi-nested PCR: denaturation was at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 3 minutes. The reaction was carried out in the thermal cycles for 30 cycles.

To detect the contamination of the extracted RNA by the PCR product, each RNA sample was tested by amplifying the E gene without the synthesis of cDNA.

Five μL of the PCR products were analyzed by electrophoresis through a 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer at 50 volts for 1 hour. The gel was stained with 10-μg/mL ethidium bromide for 10 minutes; destained with water for 5 min. DNA bands were visualized with an ultraviolet light source.

PCR products were analyzed mutation by nucleotide sequence analysis. The nucleotide sequence analysis reactions were 3.2 pmol of C859 primer and 450 μg of PCR products and they were performed by the Sanger dideoxy-mediated chain-termination method.

Table 6. Oligonucleotide primers for reverse transcriptase-polymerase chain reaction

Designation	Sequence (5′ to 3′)	Position[C]
D_2J-134[AB]	TCAATATGCTGAAACGAGAGAGAAACCG	134-162
cD_2J-2504[A]	GGGGATTCTGGTTGGAACTTATATTGTTCTGTCC	2504-2471
C 1518[B]	CATCTGCAGCAACACCATCTC	1539-1519

A, primer pair for reverse transcriptase-polymerase chain reaction of Lewis *et al* (1993).

B, primer pair for semi-nested polymerase chain reaction.

C, number represents base position in the genome of strain 16681 according to Kinney *et al.* (1997).

9.3.3 Virus titration by focus immunoassay

The supernatant from transfected C6/36 was serially diluted (10-fold) in L-15 medium. Fifty µL of each dilution were added to the PS clone D monolayer in a 96-well plate and incubated at 37°C for 2 hr. The 30 µL of 1.5% carboxy-methylcellulose were overlayered on infectious cells and incubated at 37°C in a humidified chamber for 72 hours. Sterile PBS was used for excrete the overlayer carboxy-methylcellulose. The cells at the bottom of the 96-well plate were fixed by 3.7% formaldehyde for 5 min and permeablilized by 0.1% Triton X-100 in PBS for 5 min. The cells were washed in PBS and reacted with 4G2, an anti-Flavivirus E protein monoclonal antibody, at 1:500 dilutions in PBS for 1 hour. The cells were washed in PBS and incubated with rabbit anti-mouse IgG (Cappel Laboratories, USA) at dilution of 1:750 in PBS-Tween-fetal bovine serum for 1 hour. The cells were washed in PBS and goat anti-rabbit IgG (Cappel Laboratories, USA) at 1:1,000 dilutions in PBS-Tween-fetal bovine serum was applied for 1 hour. The cells were washed again in PBS and reacted with peroxidase-rabbit antiperoxidase complex (Cappel Laboratories, USA) at 1:1,000 dilutions in PBS-Tween-fetal bovine serum for 1 hour. This was followed by cell washing and peroxidase reaction, using 0.2 mg/mL of 3, 3' diaminobenzidine (Sigma, USA) and 0.02% of H_2O_2 as the substrate. Sterile water washing stopped the reactions. Focus of infected cells was observed directly using regular microscope as cluster of cells containing brown deposit in the cytoplasm. Each focus contained from 2 to 340 cells. To determine the number of infected cells in each focus, the stained focus was photographed under an inverted microscope using 100 ASA color film (Kodak) and counted manually.