CHAPTER IV

MATERIALS AND METHODS

1. Cell lines

An *Aedes albopictus*-derived cell line, C6/36, (Igarashi, 1978) was provided by Drs. Bruce Innis and Ananda Nisalak, Department of Virology, Armed Forces Research Institute of Medical Science, Bangkok, Thailand. These cells were cultivated in Leibovitz's L-15 medium (GIBCO, BRL) containing 10% (v/v) fetal bovine serum (FBS) (GIBCO, BRL), 1X (0.26 gm %) tryptose phosphate broth (GIBCO, BRL), 1X glutamine-penicillin-streptomycin solution (GIBCO, BRL) and they were incubated at 29ºC. C6/36 cells were adherent to the surface of culture vessels.

C6/36 was routinely maintained in T25 cm^2 flask and subcultured every 4 days when their growth reached 90-100% confluency. Cells were washed once with phosphate buffer saline (PBS), pH 7.4. The adherent cells were scraped off by a plastic cell scraper and 2 ml of new L-15 medium containing 10% FBS were added. Cells were resuspended by repeated pipetting and about one tenth of cells were used for seeding.

The porcine stable kidney (PS) cell line (Westaway, 1966) was maintained in L-15 medium containing 10% FBS, 1X (0.26 gm %) tryptose phosphate broth, 1X glutamine-penicillin-streptomycin solution, in T25 cm^2 and they were incubated at 37ºC in a moist chamber. These cells showed epithelial-like morphology. PS cells were passaged when they reached 90-100 % confluency. Cells were washed once with 5 ml PBS, pH 7.4. Then, 2 ml of 0.25% trypsin-EDTA (2.5 g/L trypsin, 0.38 g/L EDTA.4Na) were added, and the trypsin-treated cells were incubated at 37ºC for 1 minute. Cells were observed under light microscope and the culture vessel was tapped until cells detached completely. Then, 5 ml of L-15 containing 10% FBS were added into the culture, and cells were resuspended. The cell suspension was centrifuged at

3,000 rounds per minute (rpm) for 5 minutes, and supernatant was discarded. Five ml of L-15 containing 10% FBS were added to the pellet, and cells were resuspended. Then, 4.5×10^5 cells were used for seeding.

2. Viruses

A dengue serotype 2 virus, strain 16681Nde(+), and mutant viruses including JEVpr/16681 chimeric virus, 16681prE203A, 16681pr(+4,-0)HS, 16681pr(+7,-0), 16681pr(+7,-2) were generated from several previous studies (Sriburi et al., 2001, Keelapang et al., 2004, Songjaeng, 2004). The strain 16681(+) was described previously (Sriburi et al., 2001). The chimeric virus JEVpr/16681 was generated by substituting the 13-amino acid residue segment just proximal to the pr-M junction of dengue virus prM with the homologous segment of JEV (Keelapang et al., 2004). A mutant virus, 16681prE203A, was constructed by substituting glutamic acid at the position 203 of dengue prM protein with alanine (J. Junjhon and N. Sittisombut, unpublished results). Three multiple point mutant viruses, 16681pr(+4,-0)HS, 16681pr(+7,-0) and 16681pr(+7,-2), were constructed by Mr. Adisak Songjaeng (Songjaeng, 2004). Three other dengue virus mutants, including 16681pr(+6,-2)P8,10+, 16681pr(+6,-2)P8,13+, 16681pr(+6,-2)P10,13+, were generated in this study. The 13-amino acid sequences just proximal to the pr-M junction of strains 16681(+), JEVpr/16681 and other mutant viruses were shown in table 2.

3. Antibodies

A mouse monoclonal antibody, 4G2, specifically recognizing the flavivirus E protein (Henchal et al., 1982) was provided by Dr. Chunya Puttikhunt. An alkaline phosphatase-conjugated goat antibody specific for mouse IgG(H+L) was purchased from Jackson ImmunoResearch, a rabbit anti-mouse IgG antibody from Cappel, Organon Teknika, Turnhout, Belgium, a goat anti-rabbit IgG antibody and a peroxidase-rabbit anti-peroxidase complex from DAKO, Denmark.

Table 2. The amino acid sequences adjacent to the pr-M cleavage site of the dengue viruses used in this study.

Viruses	13 amino acid sequences just proximal to the pr-M cleavage site	References
position #	15 10 5 1 ↓	
DENV-2 strain 16681	YGTCT TMGEH RREKR S	Sriburi et al., 2001
16681prE203A	YGTCT TMGEH RRAKR S	Sittisombut, personal communication
JEVpr/16681	YGRCT RTRHS KASRR S	Keelapang et al., 2004
16681pr(+4,-0)HS	YGTCT TMGHH RRSKR S	Songjaeng, 2004
16681pr(+7,-0)	YGRCT RMRHH RRSKR S	
16681pr(+7,-2)	YGRCT RMREH RREKR S	
16681pr(+6,-2)P8,10+	YGTCT RMREH RREKR S	this study
16681pr(+6,-2)P8,13+	YGRCT TMREH RREKR S	
16681pr(+6,-2)P10,13+	YGRCT RMGEH RREKR S	

↓ : cleavage site

4. Preparation of competent *E. coli* cells and transformation

4.1 Competent cell preparation

Escherichia coli (*E. coli*), strain DH5αF′ (GIBCO, BRL), was used to prepare competent cells. The genotype of this strain was F′ϕ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R(r_k^-, m_k^+) *gal*$^-$ *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1. This strain was used for construction of infectious dengue virus cDNA clones (Sriburi et al., 2001). Competent *E. coli* was prepared as follows.

E. coli from a stock culture were streaked on Luria-Bertani (LB) agar plate, and incubated at 37ºC for 18-24 hours. On the next day, an isolated colony was picked and inoculated into 2 ml of LB broth. The culture was incubated at 37ºC for 16 hours with 200 rpm shaking. A portion (100 µl) of the culture was inoculated into 100 ml of LB broth, and the culture was incubated at 37ºC for 2-3 hours with vigorous shaking. The bacteria were harvested by centrifugation at 5,000 rpm at 4ºC for 10 minutes, and the supernatant was discarded. The cell pellet was resuspended gently with 30 ml of ice-cold 0.1 M $CaCl_2$ and the suspension incubated at 4ºC for 30 minutes. The cell suspension was centrifuged at 5,000 rpm at 4ºC for 5 minutes. The cell pellet was resuspended with 3 ml of ice-cold 0.1 M $CaCl_2$ and sterile glycerol was added into the cell suspension to make a final concentration of 20% (v/v). The competent cells were stored in small aliquots at -70ºC. The transformation efficiency of these frozen competent cells as evaluated by transforming with the pUC18 (Promega) by the heat shock method (Sambrook et al., 1989) was 3.0×10^5 colonies forming unit per µg of plasmid DNA.

4.2. Transformation

The transformation of plasmid DNA into *E. coli* competent cells was performed by employing the heat shock method (Sambrook et al., 1989). A ligation mixture was added into 100 µl of thawed competent cells, mixed gently and the transformation mixture was incubated on ice for 30 minutes. During the incubation period, the mixture was gently mixed at every 10 minutes. Then, the mixture was

heated at 37ºC for 2 minutes and chilled on ice for 5 minutes. One ml of SOC medium was added into the transformation mixture. The mixture was incubated at room temperature with shaking at 200 rpm for 90 minutes to allow for the phenotypic lag. The bacteria were harvested by centrifugation at 3,000 rpm for 5 minutes. The bacteria pellet was resuspended in 50-200 µl of SOC medium and cells were spread on LB agar containing 25 µg/ml of ampicillin. The culture plate was incubated at 22.5ºC for 2-3 days.

5. Construction of full-length cDNA clones containing mutations of the pr-M junction

5.1 Plasmid vectors

Three plasmid vectors, i.e. the subclone plasmid [pBK(S1SP6-1547)Δ402 Pst I], the 5′ half-genome [pBK(S1SP6-4497)Δ402 Pst I], and the 3′ half-genome [pBK(4166-10723)] plasmids were used in the constructions of the full-length cDNA dengue mutant viruses. These vectors were based on a high copy number plasmid, pBluescript II KS (Stratagene, California, USA), with different regions of dengue serotype 2 cDNA sequences (Sriburi et al., 2001, Keelapang et al., 2004). The subclone plasmid contains nucleotides 1-1547 with a silent point mutation at the nucleotide position 402 to abrogate a Pst I site. The 5′ half-genome contains nucleotides 1-4497 whereas the 3′ half-genome contains nucleotides 4166-10723. Maps of these plasmids are shown in Figure 6.

5.2 Oligonucleotides

Oligonucleotides used to generate the three dengue pr-M junction mutant viruses, 16681pr(+6,-2)P8,10+, 16681pr(+6,-2)P8,13+, 16681pr(+6,-2)P10,13+, are shown in Table 3. The oligonucleotide sequences were designed based on the nucleotide sequence of the 13-amino acid region just proximal to the pr-M cleavage site of 16681pr(+7,-2).

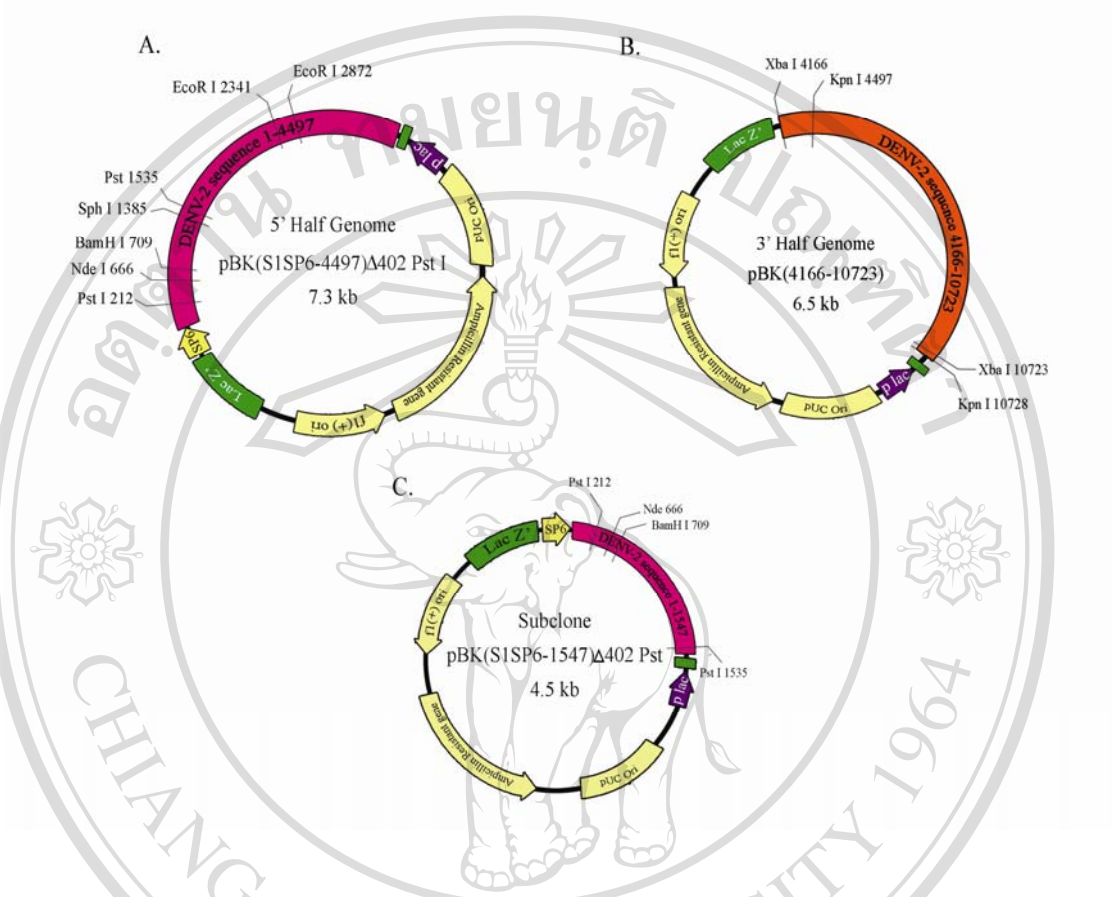

Figure 6. Maps of three plasmid vectors used in the construction of mutant viruses: the subclone plasmid, pBK(S1SP6-1547)Δ402 Pst I; the 5′ half genome, pBK(S1SP6-4497)Δ402 Pst ; and the 3′ half genome, pBK(4166-10723).

Table 3. Sequences of oligonucleotides used for the construction of the full-length cDNA dengue pr-M mutants.

Mutation	Type of Oligonucleotide	Oligonucleotide sequence (5'→ 3')	Restriction site
16681pr(+6,-2)P8,10+	Coding	TATGGGACGTGTACCCGCATGCGAGAGCATAGAAGAGAAAAAA	Sph I
	Noncoding	GATCTTTTTTCTCTTCTATGCTCTCGCATGCGGGTACACGTCCCA	
16681pr(+6,-2)P8,13+	Coding	TATGGGAGGTGTACCACAATGCGAGAGCATAGAAGAGAAAAAA	-
	Noncoding	GATCTTTTTTCTCTTCTATGCTCTCGCATTGTGGTACACCTCCCA	
16681pr(+6,-2)P10,13+	Coding	TATGGGAGGTGTACACGCATGGGAGAGCATAGAAGAGAAAAAA	BsrG I
	Noncoding	TTTTTTTTCTCTTCTATGCTCCCTCATGCGTGTACACCTCCA	

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5.3 Construction strategy

In the generation of dengue mutant viruses, the mutations were firstly introduced into the subclone plasmid vector by oligonucleotide mediated site-directed mutagenesis. The oligonucleotides with the mutations were annealed and inserted into the subclone vector at the Nde I - BamH I sites (Figure. 7A). The mutated subclone plasmid was digested with Pst I to obtain a 1.3 kb fragment containing the mutated pr-M junction region (Figure. 7B). This mutated 1.3 kb DNA fragment was used to replace the homologous region within the 5′ half genome vector (Figure. 7C and 7D). The 5′ half genome vector carrying the designed mutation was linearized by digestion with Kpn I, and dephosphorylated by employing a phosphatase reaction (Figure. 7E). This linearized 5′ half genome and a large fragment of the 3′ half genome were ligated together (Figure. 7F). The large fragment of the 3′ half genome was obtained as a Kpn I-digested fragment from the 3′ half genome. A full-length cDNA of dengue virus with the mutation at the pr-M junction was then obtained (Figure. 7G). More details on the construction are described in the following sections.

Oligonucleotide was resuspended with sterile water to a final concentration of 100 pmol/µl and then phosphorylated in a reaction mixture containing 100 pmol of oligonucleotide, 1X T4 polynucleotide kinase buffer (Fermentas) [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol], 1 mM of ATP and 5 units of T4 polynucleotide kinase (Fermentas). The kinase reaction was carried out at 37ºC for 10 minutes. Following the kinase step, the reaction mixture was heated at 70ºC for 10 minutes. The two oligonucleotide of each pair were mixed and allowed to anneal at 37ºC for 10 minutes. The resulting double-stranded oligonucleotide possessed Nde I compatible ends at the 5′ end and the BamH I compatible end at the 3′ end. This double-stranded oligonucleotide was inserted into the Nde I-BamH I site of the subclone vector which was prepared as followed.

The subclone plasmid vector carrying Nde I and BamH I sites at the nucleotide positions 666 and 709 (Keelapang et al., 2004) was digested with Nde I and BamH I. The digestion products were separated by 0.7% agarose gel electrophoresis and the DNA band containing a large fragment of the subclone vector was excised. The DNA was purified from the agarose gel by a gel extraction kit

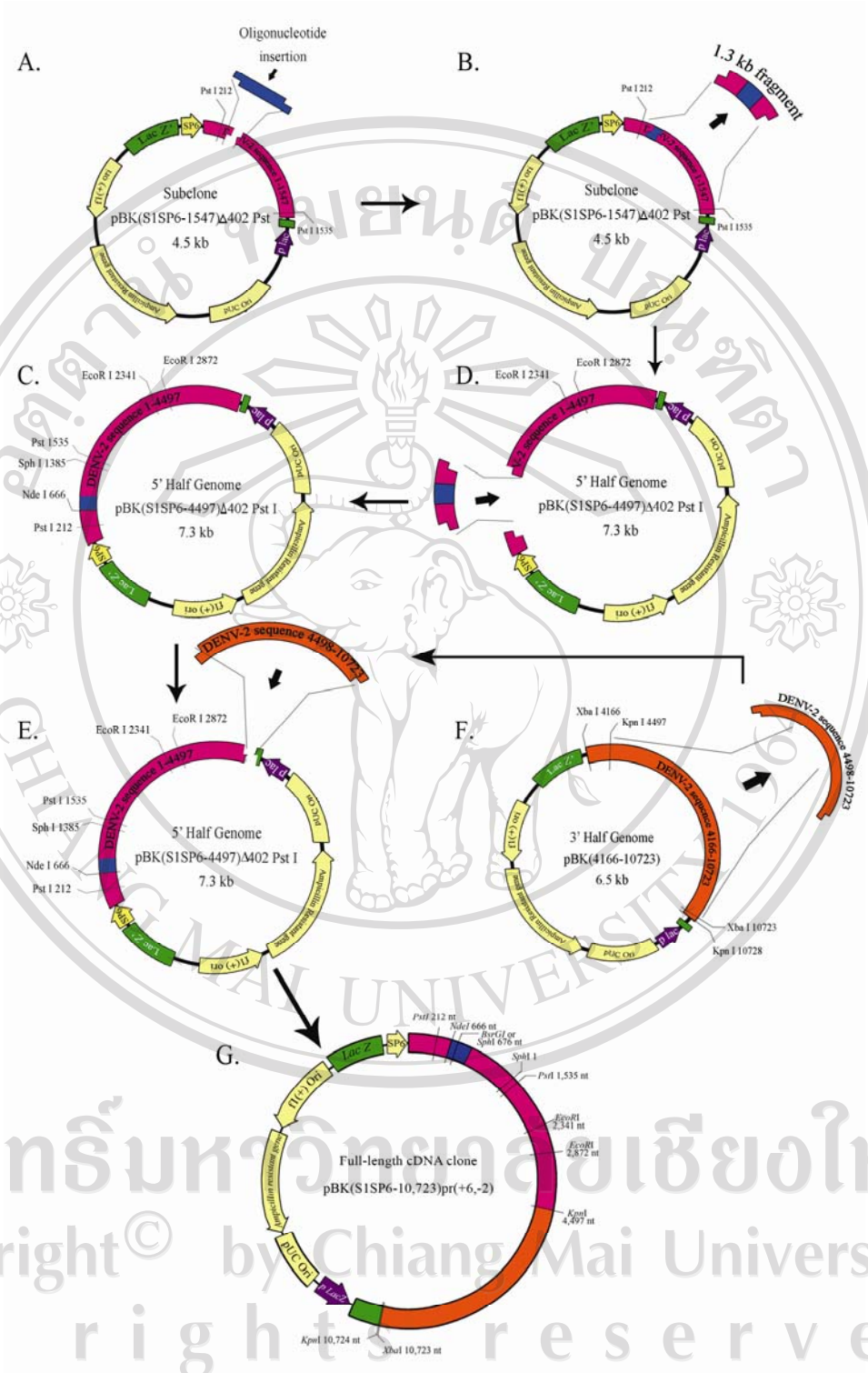

Figure 7. Steps in the construction of the full-length cDNA mutant clone for the generation of the dengue pr-M junction mutant viruses (Adapted from Keelapang et al., 2004; Sriburi et al., 2001; Songjaeng, 2004).

5.4 Mutagenesis of the subclone plasmid

(QIAGEN) according to the manufacturer's protocol. DNA fragment was eluted by applying a 30 µl of elution buffer (EB) onto the membrane at room temperature for 3 minutes. The column was centrifuged at 14,000 rpm for 1 minute. The purified DNA was analyzed with 0.7% (w/v) agarose gel electrophoresis, and DNA concentration was measured by UV spectrophotometer.

In the insertion of the double-stranded oligonucleotide into Nde I-BamH I-digested subclone vector, the ligation reaction contained 0.04 pmol of annealed oligonucleotide, 100 ng of the large fragment of Nde I-BamH I-digested subclone vector, 1X ligation buffer (Fermentas) [40 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol] and 2.5 units of T4 DNA ligase (Fermentas) in the total volume of 20 µl. The reactions were incubated overnight at 14ºC. *E. coli* strain DH5αF′ competent cells was transformed with the ligation mixture by heat shock method. The positive control for the ligation reaction was Hind III-digested lambda DNA. The desired mutant subclone plasmids were ascertained by digesting the mini-prep DNA with appropriate restriction enzymes (Sph I and BsrG I for pBK(S1SP6-1547)pr(+6,-2)P8,10+ and pBK(S1SP6-1547)pr(+6,-2)P10,13+, respectively), or by nucleotide sequence analysis (pBK(S1SP6-1547)pr(+6,-2)P10,13+). Two clones were expanded by inoculating the mini-culture into 100 ml of LB broth containing 25 µg/ml of ampicillin. The cultures were incubated at 22.5ºC with shaking (200 rpm) for 2-3 days. These cultures were harvested and the plasmid DNA was purified. The midi-prep DNA was confirmed by digestion with the corresponding restriction enzymes or by sequencing as stated previously.

5.5 Mini- and midi-preparations of plasmid from cultured *E. coli*

Mini-preparation of plasmid

Isolated colonies of transformants were individually picked and inoculated into 2 ml of LB broth containing 25 µg/ml of ampicillin. The cultures were incubated at 22.5ºC with shaking for 2-3 days. Then, 1 ml of the bacteria culture was

centrifuged at 3,000 rpm at room temperature for 5 minutes, and the supernatant was discarded. The cell pellet was resuspended with 300 µl of a suspension buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA]. Subsequent steps were performed according to the manufacturer's protocol. The plasmid DNA pellet was dissolved with 15 µl of 10 mM Tris-HCl (pH 8.0) and was analyzed by 0.7% (w/v) agarose gel electrophoresis.

Midi-preparation of plasmid

A bacteria culture of 100 ml was harvested by centrifugation at 3,000 rpm for 15 minutes. The supernatant was discarded. The pellet of bacteria was resuspended in 10 ml of a suspension buffer (QIAGEN) and subsequent steps were performed according to the manufacturer's protocol. The cell lysate was loaded into Qiagen-100tip (QIAGEN). These columns were first prepared by loading with 4 ml of the equilibration buffer (QBT) [750 mM NaCl, 50 mM MOPS (pH 7.0), 15 % isopropanol and 0.15 % Triton X-100]. After the QBT flew through the column completely, then the cell lysate was loaded into the column. Following the cell lysate was through the column, the column was washed 2 times with 10 ml of washing buffer (QC) [1.0 M NaCl, 50 mM MOPS (pH 7.0), 15% isopropanol]. Then plasmid DNA was eluted off the column by loading with 5 ml of elution buffer (QF) [1.25 M NaCl, 50 mM Tris-HCl (pH 8.5) and 15% isopropanol]. The eluted DNA in solution was precipitated with 3.5 ml of isopropanol. The precipitate DNA was collected by centrifugation at 14,000 rpm at 4ºC for 30 minutes. Clear supernatant was discarded and DNA pellet was washed with 500 µl of freshly prepared 70% ethanol. The tube was centrifuged at 14,000 rpm at 4ºC for 10 minutes. The DNA pellet was allowed to dry at room temperature. The dry pellet was dissolved with 10 mM Tris-HCl pH 8.0. This DNA was analyzed by 0.7% (w/v) agarose gel electrophoresis, and DNA concentration was measured by UV spectrophotometer at the wave length of 260 nm.

5.6 Preparation of the 1.3 kb DNA fragment of the mutant subclone plasmids

DNA of the mutant subclone plasmid was digested with Pst I, and the 1.3 kb fragments of the mutant subclone plasmid DNA were purified from agarose gel by the

gel extraction kit (QIAGEN) after it was separated by electrophoresis. This 1.3 kb fragment was then used for the construction of the mutant 5′ half genome plasmid.

5.7 Construction of the mutant 5′ half genome plasmid

In the construction of the 5′ half genome containing the desired mutation, 200 ng of purified, dephosphorylated 6.0 kb fragment of Pst I-digested pBK(S1SP6-4497) from Songjeang, (2004) were ligated with 100 ng of the mutated 1.3 kb DNA fragment. The ligation buffer (Fermentas) contained 250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 25% (w/v) polyethylene glycol 8000 and 2 units of T4 DNA ligase (Fermentas) in 10 µl volume. This reaction was incubated overnight at 14ºC. The ligation mixture was transformed into the competent DH5αF′cells as described in the previous section. The desired mutant subclone plasmids were identified by digestion the mini-prep DNA with appropriate restriction enzymes or by nucleotide sequence analysis as described in the previous section. In addition, the three mutant 5′ half genome plasmids were also verified by digesting with a set of six restriction enzymes: Nco I, Hind III, EcoR I, Sph I, Pst I, and Kpn I. Selected mutant 5′ half genome plasmid clones were expanded in 100-ml culture and plasmid DNA was prepared by using a commercial kit (Plasmid midi kit, QIAGEN) .

5.8 Construction of mutant full-length cDNA clones

In the construction of the full-length mutant cDNA clones, a mutant 5′ half genome was first linearized by digesting with Kpn I. The linearized mutant 5′ half genome was dephosphorylated with CIAP, and the DNA was purified from agarose gel by using a gel extraction kit (QIAGEN). The purified, CIAP-treated, Kpn I-digested mutant 5′ half genome was ligated to the large fragment of Kpn I-digested 3′ half genome plasmid, which was obtained by digesting the 3′ half genome plasmid with Kpn I and separating the large fragment of DNA by agarose gel electrophoresis and excision from the gel. The components of ligation reaction included 100 ng of the linearized mutant 5′ half genome, 200 ng of the wild type, large fragment of Kpn I-digested 3′ half genome, 50 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$, 10 mM

dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin, and 2 units of T4 DNA ligase (Fermentas) in the final volume of 20 µl. The reaction was incubated overnight at 14ºC. Then the ligation mixture was transformed into the competent DH5αF´ cells. Following incubation at 22.5°C for 3-4 days, the transformant colonies appeared as shiny, pinpoint colonies. The transformant colonies were grown and the mini plasmid preparations were obtained as described in the previous section. The mutant full-length cDNA plasmid clones with the correct orientation were selected by digesting with Xba I and Hind III. The plasmid DNA was prepared as described in the previous section. The mutant full-length cDNA plasmid clones were verified by digesting with Nco I, EcoR I+Sph I, Xba I-Hind III, Kpn I, and Pst I.

The template for an in vitro transcription reaction was prepared by digesting 5 µg of the full-length cDNA plasmid clones with Xba I, which cut the plasmid at a unique site at nucleotide position 10724. The linearized full-length cDNA was purified from the digestion mixture by using a gel extraction kit (QIAGEN). The concentration of the purified linear full-length DNA was measured by using a UV spectrophotometer at 260 nm.

6. Synthesis of infectious RNA by *in vitro* transcription.

One µg of the linear mutant full-length cDNA clones was used as the template to generate infectious RNA by an *in vitro* transcription reaction. The RNA synthesis was catalyzed by SP6 DNA-dependent RNA polymerase. The *in vitro* transcription reaction contained 1 µg of linearized full-length cDNA, 1X reaction buffer [80 mM Hepes-KOH (pH7.5), 32 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol], 5 mM of each ribonucleoside triphosphates, i.e. ATP, CTP, UTP and 1.5 mM GTP, 3.5 mM cap analog $m^7G(5')ppp(5')GTP$ and 20 units of SP6 RNA polymerase (Promega). The reaction was incubated at 30ºC for 4 hours. In a separate tube, a positive control reaction for the *in vitro* transcription was performed using 1.0 µg of linear DNA control (Promega) together with all the components of the *in vitro* transcription but without the cap analog. The products of the *in vitro* transcription reactions were analyzed by electrophoresis in 0.7% (w/v) agarose – 3.7% formaldehyde gel. The gel was prepared with 1X MOPE-EDTA-sodium acetate

buffer (0.2 M MOPS, 80 mM sodium acetate, 10 mM EDTA, pH 8.0). Two µl of the *in vitro* transcripts were mixed with 8 µl of RNA loading buffer [62.5% deionized formamide, 1.14 M formaldehyde, 200 µg/ml bromophenol blue, 200 µg/ml xylene cyanol, 1.25 X MOPS-EDTA-sodium acetate buffer (0.25 MOPS pH 7.0), 100 mM sodium acetate, 12.5 mM EDTA (pH 8.0)]. The RNA mixture was heated at 65ºC for 10 minutes before loading. The running buffer contained 3.7% formaldehyde in 1X MOPS-EDTA-sodium acetate [0.2 M MOPS (pH 7.0), 80 mM sodium acetate, 10 mM EDTA (pH 8.0)]. The electrophoresis was performed at 50 volts for 1 hour. The gel was stained with 10 µg/ml ethidium bromide for 1 minute and extensively destained with tap water. RNA bands were observed by using the UV transilluminator. Before transfection into C6/36 cells, the reaction mixture was incubated with 1 unit of RNase-free DNase (Promega) at 37ºC for 15 minutes.

7. Lipofectin-mediated transfection of C6/36 cells and detection of dengue virus E protein by dot-blot immunoassay

7.1 Lipofectin-mediated transfection of C6/36 cells

C6/36 cell monolayer at about 90% confluence in a 35-mm^2 culture dish was washed twice with 1X L-15 medium. Five µl of Lipofectin (Invitrogen) were added into 1 ml of 1X L-15 medium. The mixture was mixed and incubated at room temperature for 1 minute. Then, 10 µl of the *in vitro* transcription products were added into the Lipofectin mixture. This mixture was gently mixed by swirling and incubated at room temperature for 1 minute. Before adding the transfection solution onto C6/36 monolayer, the medium in the culture vessel was discarded, and the Lipofectin-RNA mixture was added. The dish was gently mix by rocking back and forth. The dish was incubated at 29ºC for 4 hours. Then, the transfection medium was replaced with 2.5 ml of 1X L-15 medium containing 1.5% FBS. The culture dish was returned to incubate at 29ºC for specified times.

At 0, 4, 7, 11 and 14 day after transfection, 2.5 ml of the culture medium was collected. After additional 2.5 ml of the L-15 medium containing 1.5% FBS were added, the culture was further incubated at 29ºC. The culture supernatant was

spun at 5,000 rpm for 5 minutes to remove cells and debris, and adjusted with FBS to the final concentration of 20% FBS. The supernatant was stored at -70 ºC until use in evaluation of infectious virus titers and subsequent expansion.

7.2 Detection of dengue virus E protein by dot-blot immunoassay

Two µl of culture supernatants were dotted onto the nitrocellulose membrane in duplicate. The membrane was allowed to dry at room temperature. This membrane was soaked in 5 ml of 5% (w/v) skim milk in PBS, and incubated at room temperature for 1 hour with constant shaking. The blocking solution was removed and the blot was reacted with 4G2, an anti-flavivirus E protein antibody, at the final dilution of 1:2,000 in 5% (w/v) skim milk in PBS for 1 hour at room temperature. The membrane was washed 3 times with PBS and then reacted with alkaline phosphatase –conjugated rabbit anti-mouse immunoglobulin G light and heavy chains (IgG H+L) antibody (DAKO Cytomation, Glostrup, Denmark) at the dilution of 1:2,000 in PBS containing 5% (w/v) skim milk for 1 hour at room temperature. After the membrane was washed 3 times with 5 ml of PBS, it was then incubated with a chromogen solution containing 0.335 mg/ml of the NBT and 0.165 mg/ml of BCIP in an alkaline buffer [0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 0.005 M $MgCl_2$]. When blue-purple precipitate was detected at about 45 minutes of incubation at room temperature, the membrane was washed several times with tap water to stop the reaction. The image of the signals was captured by scanning and stored in a digital format.

8. Virus propagation

The parent virus, strain 16681Nde(+), and the mutant viruses were expanded in C6/36 cells for further studies. Two-day-old C6/36 cell monolayer at approximately 90% confluency in T-75 cm^2 flask was used for the infection. Ten ml of the culture medium were removed, leaving about 5 ml of the medium within the culture vessel. Stock viruses were thawed quickly in a 37ºC water bath, and the contents were mixed by vortexing. The virus suspension was added onto the C6/36 cell monolayer. Then cells were incubated at room temperature for at least 4 hours with constant mixing.

Following 4 hours of incubation at room temperature, the culture vessel was further incubated overnight at 29ºC. On the next day, 10 ml of 1X L-15 medium containing 1.5% FBS were added, and the culture was returned to an incubator. At 5 and 7 days after infection, the culture media were collected, and replaced with 10-15 ml of the 1X L-15 medium containing 1.5% FBS. The culture supernatant was centrifuged at 3,000 rpm at room temperature for 5 minutes to remove detached cells and cellular debris. The clear supernatant was collected, adjusted with FBS to the final concentration of 20% and then stored in small aliquots at -70ºC.

9. Characterization of virus

9.1. Quantitation of infectious virus by focus immunoassay titration

The titer of infectious dengue virus was measured by the 2-step focus immunoassay titration using PS cells. PS cells were seeded at 1.4-2.4×10^4 cells/well in a 96-well plate and incubated at 37ºC for 2-3 days. Frozen viral samples were thawed at 37ºC in water bath, and mixed by pulse vortexing. These virus suspension was serially diluted in 10-fold steps with L-15 medium containing 3% FBS. The culture media was aspirated from each well, leaving about 25 µl at the bottom of the well. Then, 50 µl of the undiluted sample or serially diluted viral suspension were added into the wells in duplicates. The 96-well plate was tapped in order to mix the virus suspension and the remaining medium, and then was incubated at 37ºC for 2 hours in a moist chamber. The plate was tapped gently every 15 minutes during the 2-hour incubation period. At the end of the incubation, 125 µl of 1X L-15 medium containing 3% FBS and 1.5% (w/v) carboxymethyl cellulose (Sigma) were added to each well, and the plate was further incubated for 3 more days at 37ºC.

Foci of infected cells were then visualized by an immunostaining method. The cells were gently washed 5 times with 200 µl of PBS, fixed by incubating with 3.7% (v/v) formaldehyde in PBS (100 µl/well) for 10 minutes at room temperature and then permeabilized by incubating with 2.0% (v/v) Triton-X 100 in PBS for 10 minutes at room temperature. These cells were washed for 5 times with 200 µl of PBS to remove Triton-X 100. Fifty µl of 4G2 at the dilution of 1:4,000 in PBS were added

into each well and the plate was incubated for 1 hour at 37ºC to allow the formation of antigen-antibody complex. Unbound and non-specifically bound 4G2 molecules were removed by washing with PBS 200 µl for 5 times. Then, cells were incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (H+L) antibody at the final dilution of 1:5,000 in 0.05% Tween-20, 2% FBS in PBS (pH 7.4) for 1 hour at 37ºC. After unreacted rabbit antibody was removed by washing with PBS 200 µl for 5 times, the cells were then reacted with 100 µl of fresh-prepared NBT-BCIP solution containing 0.335 mg/ml of NBT, 0.165 mg/ml of BCIP in alkaline buffer [0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.005 $MgCl_2$]. The plate was incubated at room temperature in a light-proof chamber for 30-45 minutes. The reaction was stopped by washing the plate several times with tap water. Fine precipitate of dark blue-purple substances could be observed in the cytoplasm of PS cells, and clusters of infected cells (foci) were counted under a light microscope in each well. All foci counted from wells with less than 80-100 foci were included in the calculation of infectious titer of the sample, which was expressed as focus forming units (FFU)/ml.

9.2 Extraction of viral genomic RNA and amplification of the prM coding region by reverse transcription-polymerase chain reaction

Extraction of viral genomic RNA

Viral genomic RNA was extracted by using the QIAamp viral RNA mini spin kit (QIAGEN). Virus particles in 140 µl of culture supernatant were lysed with 560 µl of AVL lysis buffer containing carrier RNA. The tube was mixed by pulse vortexing for 15 seconds. Then, 560 µl of absolute ethanol were added and the mixture was mixed by pulse vortexing for 15 seconds and was incubated at room temperature for 10 minutes. To bind RNA with the membrane in the column, the mixture was added into a spin column and the column was centrifuged at 8,000 rpm at room temperature for 1 minute. The impurities were removed by adding 500 µl of AW1 containing 70% ethanol and the column was again centrifuged at 8,000 rpm for 1 minute. Next, 500 µl of AW2 containing 70% ethanol were added into the column and the column was spun at 14,000 rpm for 3 minutes. The AW2 solution that still remained at the edge of

the column was carefully removed by pipetting. The column was then spun at 14,000 rpm for 1 minute. Finally, RNA was eluted with 30 µl of AVE solution. The elution was carried out twice and the eluted materials were pooled. Filtered tips were employed through out the extraction to minimize carry over and contamination.

Amplification of the prM coding region by RT-PCR and semi-nested PCR

In the reverse transcription (RT) reaction, purified viral genomic RNA served as the templates for the cDNA synthesis. The reaction contained 10 µl of the viral genomic RNA, 1X reverse transcriptase buffer [50mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 50 mM DTT], 0.5 mM of each of the ribodeoxynucleoside triphosphates [dATP, dCTP, dGTP, dTTP], 30 pmol of C2504 primer (Table 4) and 20 units of M-MuLV reverse transcriptase (Fermentas). The reactants were mixed by vortexing, and then the reaction was allowed to proceed by incubating at 42ºC for 1 hour.

After the RT reaction was completed, the viral cDNA was amplified by polymerase chain reaction (PCR). The PCR reaction was composed of 5 µl of the RT product, 1X *Taq* DNA polymerase buffer (Fermentas) [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 50 mM $MgCl_2$, 0.1% Tween 20], 30 pmol of both D_2J 134 (Table 4) and C2504 primers and 2 units of *Taq* DNA polymerase (Fermentas). All components were in a final volume of 100 µl. The amplification protocol was as follows: (1) denaturation at 95ºC for 20 minutes, (2) 35 cycles of denaturation at 95ºC for 30 second, annealing at 65ºC for 30 second, and extension at 72ºC for 3 minutes, (3) final extension at 72ºC for 20 minutes. The PCR products were analyzed by electrophoresis in 0.7% (w/v) agarose gel.

In many cases with low yield of the amplification products, the second round of amplification by semi-nested PCR was attempted. The reaction contained 0.01 µl or 0.001 µl of the product from the RT-PCR step, 1X PCR buffer [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2 SO_4$, 50 mM $MgCl_2$, 0.1% Tween 20], 30 pmol of D_2J 134, C1518 (or C1976) primers (Table.4) and 2 units of *Taq* DNA polymerase (Fermentas). All of the components were in 100 µl of final volume. The same PCR

protocol as the RT-PCR was performed. The PCR products were analyzed by electrophoresis in 0.7% (w/v) agarose gel.

Table 4. Primers for reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing of dengue type 2 viruses.

Primer	Sequence (5'→3')	Priming site (nucleotide positions)
D_2J 134[AB]	TCAATATGCTGAAACGAGAGAGAAACCG	134-162
C 2504[A]	GGGGATTCTGGTTGGAACTTATATTGTTCTGTCC	2504-2471
C 1518[B]	CATCTGCAGCAAGAGGATCTC	1539-1519
C 1976[B]	TGTAATCAGGCGACCTAAGA	1550-1921
S 350[*]	GGAAAGAGATTGGAAGGATGA	350-371

A is primer for RT-PCR (Lewis et al, 1993)
B is primer for semi-nested PCR,
* is a primer for DNA sequencing.
S and C represent for forward and reverse primers.

9.3 Nucleotide sequence analysis

DNA sequencing reaction contained 400-600 ng of DNA of the purified RT-PCR or semi-nested PCR products, 8 µl of reaction mix (BigDye, Applied Biosystem, version 3.10), 3.2 pmol of S350 primer in the final volume of 20 µl. The cycling parameter was: denaturation at 94ºC for 30 seconds, annealing at 50ºC for 15 seconds and extension at 60ºC for 4 minutes. The reaction was performed for 35 cycles. Sequencing products were precipitated with ethanol-sodium acetate precipitation according to the manufacturer's protocol. The DNA pellet was heated to dry at 60ºC for 1 minute. This sequencing product was dissolved in 25 µl of a signal enhancer reagent (HiDi, Applied Biosytem), and the product was resuspended by extensive

vortexing. The sequencing product was denatured by heating at 95ºC for 2 minute. The sequencing product was analyzed in an automated DNA sequencer (ABI prism DNA analyzer, Applied Biosystem).

9.4. Determination of focus size by immunostaining assay

In the quantitation of focus size using PS cells, 1.74×10^5 detached cells were seeded into each well of a 24-well plate and incubated at 37ºC for 3 days. The virus stock was diluted with 1X L-15 medium containing 3% FBS and about 50 FFU were dispensed into a well. After the diluted viral suspensions were added, the plate was incubated at room temperature for 2 hours with periodic tapping to allow adsorption. At the end of the incubation, 250 µl of 0.6% (w/v) of carboxymethyl cellulose in L-15 medium containing 3% FBS were added into each well. The plate was further incubated for 72-75 hours at 37ºC.

The infected cell culture was then fixed with formaldehyde and solubilized with Triton X-100 as described above. For immunostaining, 250 µl of the anti-flavivirus E protein antibody, 4G2, at the dilution of 1:2000 were added into each well and incubated at 37ºC for 1 hour. The solution was removed, and the infected cells were washed with PBS for 5 times. This culture was reacted with the rabbit anti-mouse IgG (Cappel). This antibody was diluted in PBS containing 0.05% Tween-20 and 2.0% FBS at the dilution of 1:2000, and 250 µl of this antibody solution were added into all the wells. The plate was incubated at room temperature on a flip-flop shaker for 1 hour. The antibody solution was discarded, and cells were washed with PBS for five times. 2Then, 50 µl of goat anti-rabbit IgG antibody diluted in PBS containing 0.05% Tween-20 and 2.0% FBS at a dilution of 1:2000 were added into every wells. These cells were incubated at 37ºC for 1 hour. Subsequently, 250 µl of the peroxidase-rabbit anti-peroxidase antibody complex (DAKO) diluted in PBS containing 0.05% Tween-20 and 2.0% FBS at the dilution of 1:2000. The plate was incubated at 37ºC for 1 hour. Finally, the cells were washed with PBS five times, and then the substrate-chromogen solution [0.05 mg/ml of 3, 3´ diaminobenzidine (Sigma) and 0.06% of H_2O_2 in PBS] was added into each well. The reaction was allowed to proceed at room temperature in a dark chamber for 15-30 minutes. Cells were washed

several times with water, and 40 isolated foci, which were randomly selected, were photographed under a light microscope at a magnification of 100. All cells with dark brown precipitates in the cytoplasm were counted in each focus. The arithmetic means and standard deviation were calculated. Statistic analysis of difference of averages was tested by T-test (SPSS program package, SPSS 13.0 for Windows).

9.5. Determination of prM cleavage efficiency

The cleavage efficiency of prM protein was studied using C6/36 cells. Two-day- old cell monolayer at the confluency of 90-100% in T-25 cm^2 flask was infected with dengue virus at the MOI of 0.2 FFU/cell for 48 hours. Infected cells were starved by replacing the culture medium with the methionine- and cysteine-free Dulbecco's modified Eagle medium containing 1.5% dialyzed FBS. Cells were incubated at 37ºC in a 5% CO_2 incubator for 2 hours. After the starvation, 50-75 μCi of L-[^{35}S]methionine and L-[^{35}S] cysteine in an *in vitro* cell labeling mixture (Redivue pro-mix; Amersham, Buckinghamshire, England) were added into the medium. Then cells were continued incubating at 37ºC for 18 hours.

The culture medium was harvested, and cell debris was removed by centrifugation at 5,000 rpm at 4ºC for 5 minutes. For concentrating the viral particles, the culture supernatant was added with one-half volume of 21 gm% polyethylene glycol 8000 and 6.9 gm% NaCl. The solution was mixed with a gently agitating motion with a rotator overnight at 4ºC. Then, the solution was centrifuged 30 minutes at 14,000 rpm at 4ºC. Pellets were resuspended with TES buffer and left overnight at 4ºC for 1-2 days. Suspension of concentrated virus was overlaid on a 5-55% (w/v) sucrose gradient in TES buffer, and then the tube was centrifuged at 35,000 rpm at 4ºC in a SW40 rotor (Beckman) for 24 hours. Fifteen fractions of about 0.7 ml each were collected by an upward displacement method from the bottom of the centrifugation tube. Two μl of each fraction were dotted onto a glass fiber filter paper. The paper was allowed to dry at room temperature, and the radioactive signal were counted in a scintillation counter.

Twenty five μl of each fraction were analyzed by electrophoresis in a 2% SDS-15% polyacrylamide gel. The gel was soaked in a solution containing 25%

isopropanol, 15% acetic acid for 15 minutes and the gel was dried at 70ºC under vacuum for 4 hours. The dry gel was exposed to X-Ray film (Kodak) for 1-2 months at -70ºC. The radioactive signals generated from radiolabeled viral protein bands were visualized by developing the films. Film developing was performed as follows. A film was soaked in a developer solution (Kodak) for 3 minutes, and then the film was washed with tap water for 1 minute. Next step, the film was fixed in a fixer solution (Kodak) and then washed in tap water.

Fractions showed a high level of radioactive signal were pooled and the pooled fraction was subjected to electrophoresis in a 2% SDS-15% polyacrylamide gel. For recording the signal on the X-ray film, the gel was first soaked in an enhancer solution (Amplify fluorographic reagent, Amersham), and then it was dried by heating at 70ºC under vacuum. Otherwise, the gel was dried without soaking in an enhancer solution and then exposed to a phosphoimager screen (Amersham) for 2 months. For quantitating the signals of a radiolabeled protein band, the signal was detected by using a phosphoimager (Typhoon 9210, Amersham). These digital images were analyzed by the ImageQuant software (Amersham). Area with radioactive signals of E, prM and M proteins were marked, and the background signal was subtracted out. The signal intensities of each protein band were divided by a number of methionine-plus-cysteine residues in E, prM, and M proteins, which are 33, 16 and 5 residues (Keelapang et al., 2004), respectively.

The percentage of the prM protein cleavage was calculated as follows. An amount of a signal of M protein was divided by total amount of prM signal-plus-M signal, and the result was then multiplied by 100. For control, total signal of E protein was divided by total signal of prM and M proteins, which should be approximately 1.0.

$$\text{Signal ratio} = \frac{\text{Adjusted M signals}}{\text{Adjusted prM-plus-M signals}}$$

$$\text{Percentage of prM cleavage} = \text{Signal ratio of the M protein} \times 100$$

9.6 Study of viral particle export by single-step kinetics experiment

The export of infectious virus particles was studied using PS cells in the single-step kinetics experiment. PS cells were plated into a 35-mm^2 culture dish, and were incubated at 37ºC for two days. Cells were counted and about at 1.0×10^6 cells/dish were infected with 1 ml of viral suspension. The amount of virus used for infection was 1.0×10^6 FFU so that the MOI of about 1.0 was achieved. Cells were incubated with viruses for 2 hours at 37ºC with gentle tapping of the dish every 15 minutes. Then, cells were washed for 5 times with 1X L-15 medium, and 1 ml of L-15 containing 3% FBS was added. Then, the dishes were further incubated at 37ºC in a moist chamber.

At specified time points, i.e. 0, 4, 12, 14, 16, 20, 24, 36 and 48 hours after adsorption, the culture media were collected and centrifuged at 5,000 rpm for 5 minutes to remove detached c ells and cellular debris. The clarified culture supernatant was collected and adjusted with FBS to the final concentration of 20%. The supernatant was stored in small aliquots at -70ºC until use.

After the collection of culture media, infected PS cell monolayers were washed with 2 ml of 1X L-15 medium for five times to remove extracellular viruses. Then, 1 ml of L-15 medium containing 20% FBS was added into the dish. The dish was brought to -70ºC and was left frozen for 1 hour. Then it was taken to thaw on ice. After three cycles of freezing and thawing, cell lysates were harvested and subjected to centrifugation at 5,000 rpm for 5 minutes in a microcentrifuge. The clarified cell lysates were collected and kept in small aliquots at -70ºC until the virus titration was performed.