CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Bacterial strain

E.coli strain XL-1 blue competent cell

Strain:	XL-1 blue
Genotype:	*rec*A1, *end*A1, *gyr*A96, *thi-1*, *hsd*R17, (r_{k-}, m_{k+}), *sup*E44, *rel*A1,Δ(*lac-pro*AB) [F', *pro*AB,*lac*I^q, *lac*ZΔM15,Tn*10*(*tet*^{r})]

E.coli strain DH5-α competent cell

Strain:	DH5-α
Genotype:	Φ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *rec*A, 1 *end*A1, *hsd*R17 (r_{k-},m_{k+}), *pho*A, *sup*E44, λ– *thi*, *gyr*A96, *rel*A1

4.1.2 Cell line

HEK 293T/17 cell (ATCC: CRL-11268)

Organism:	*Homo sapiens* (Human)
Source:	Kidney
Morphology:	Epithelial
Neomycin susceptibility:	Resistance

HEK 293 cell (ATCC: CRL-1573) kindly provided by Dr. Bruno Vincent

Organism:	*Homo sapiens* (Human)
Source:	Kidney
Morphology:	Epithelial
Neomycin susceptibility:	Sensitive

4.1.3 Virus

Dengue virus serotype 2 (DENV-2; strain 16681)

4.1.4 Reagents for bacterial culture and materials for cloning

Ampicillin sodium salt	Sigma-Aldrich Co., St.Louis, MO, USA
Bacteriological agar	Laboratories CONDA, Madrid, Spain
Deoxyribonuleotide triphosphate (dNTP)	Thermo Fisher Scientific,Waltham MA, USA
Dnase enzyme	Thermo Fisher Scientific,Waltham, MA, USA
DreamTaq DNA polymerase	Thermo Fisher Scientific, Waltham, MA, USA
Gel/PCR DNAfragment extraction kit	Geneaid Biotech, New Taipei city, Taiwan
High speed plasmid mini kit	Geneaid Biotech, New Taipei city, Taiwan
Improm-II™ reverse transcriptase	Promega, Madison, WI, USA
Kanamycin sulfate	Bio Basic Inc., Ontario, Canada
Phusion™ DNA polymerase	Thermo Fisher Scientific, Waltham, MA, USA
Restriction enzyme	Thermo Fisher Scientific, Waltham, MA, USA
T4 ligase	Vivantis, Oceanside, CA, USA
Trizol™ reagent	Ambion®, Waltham, MA, USA
Tryptone powder	Bio Basic Inc., Ontario, Canada
Yeast extract	Laboratories CONDA, Madrid, Spain

4.1.5 Plasmids

Vector used for eukaryotic expression system were pcDNA3.1 hygro+ (**Figure 4.1**) (Invitrogen) and pC2 (**Figure 4.2**) and pC2 were adapted from pEGFP-C2 (**Figure 4.3**) (Clontech, Mountain View, CA, USA) by cutting out the

GFP gene using *Nhe*I and *Xho*I sites, maintained the *Xho*I site after filled in and ligation. Multiple cloning site confirmed by sequencing using CMV-F universal primer. The other plasmid, pcDNA-EGFP (**Figure 4.4**), was used to estimate transfection efficiency.

4.1.6 Primers

All of primers in this study synthesized by Macrogen (Seoul, Korea) are shown in **Table 4.1 and 4.2**. For **Table 4.1,** all of them were overhanged with restriction recognition site at their 5' end as indicated. The forward primer contained a Kozak sequence for the initiation of protein translation at the 5' end. These primers were used for amplification of DENV-2 genes inserted fragments, whereas primers in **Table 4.2** used for pcDNA-D2-opt.prM-E colony PCR screening.

4.1.7 Tissue culture media and accessory reagents

Dulbecco's modified Eagles medium, DMEM	Gibco™ Invitrogen, Waltham, MA, USA
Bovine serum albumin ≥ 96% fatty acid free	A6003; Sigma-Aldrich, Co., St. Louis, MO, USA
Fetal bovine serum, FBS	Gibco™ Invitrogen, Waltham, MA, USA
Trypsin	Gibco™ Invitrogen, Waltham, MA, USA
G418	Gibco™ Invitrogen, Waltham, MA, USA
Oleic acid reagent	O-1383-5G; Sigma-Aldrich, Co., St. Louis, MO, USA

4.1.8 Antibodies

Anti-dengue complex	A4416; Sigma-Aldrich, Co., St. Louis, MO, USA
Goat anti-mouse IgG conjugated horseradish peroxidase (HRP)	A4416; Sigma-Aldrich, Co., St. Louis MO, USA

Pan specific anti-dengue E protein	MA1-27093, Pierce, Rockford, IL, USA
Rabbit anti-mouse IgG conjugated horseradish peroxidase (HRP)	A4416; Sigma-Aldrich, Co., St. Louis MO,USA
Chicken anti-mouse IgG conjugated with Alexa 594	Molecular probes, Eugene, Oregon, USA
Goat polyclonal IgG actin antibody	sc-1616; Santa Cruz, Dallas, Texas, USA
Rabbit anti-goat IgG conjugated with HRP	34102, Pierce, Rockford, IL, USA

4.1.9 Chemicals

1,4-dithio-DL-threitol (DTT)	Bio Basic, Inc., Markham, Ontario, Canada
2-mercaptoethanol	Merck & Co., Inc., Kenilworth, NJ, USA
40% acrylamide/bisacrylamide	National Diagnostics, Atlanta, GA, USA
Acetic acid (glacial)	Merck & Co., Inc., Kenilworth, NJ, USA
Ammonium sulfate	Bio Basic, Inc., Markham, Ontario, Canada
Bovine serum albumin, BSA fraction V	PAA Laboratories GmbH, Pasching, Austria
Bradford reagent	BIO-RAD, Hercules, CA, USA
Broad range protein marker	BIO-RAD, Hercules, CA, USA
Chloroform	BDH, Poole, Dorset, UK
Coomassie Brilliant Blue G250	Research Organics, Cleveland,OH, USA
Diethyl pyrocarbonate, DEPC	Bio Basic, Inc., Ontario, Canada
Disodium hydrogen phosphate, Na_2HPO_4	Merck KGaA, Darmstadt, Germany
Enhanced Chemiluminescense Plus system ECL plus	GE Healthcare, Buckinghamshire, UK

Ethanol	Merck KGaA, Darmstadt, Germany
Ethylenediaminetetraacetate, EDTA	USB Corporation, Cleveland, OH, USA
Gene Ruler DNA ladder Mix	Thermo Scientific Inc., Waltham, MA, USA
Glycerol	Amersham Biosciences, Piscataway, NJ, USA
Glycine	Research Organic, Inc., Cleveland, OH, USA
Hydrogen chloride, HCl	Merck KGaA, Darmstadt, Germany
Isopropanol	Merck KGaA, Darmstadt, Germany
Methanol	Merck KGaA, Darmstadt, Germany
Pierce® ECL Western blotting substrate	Thermo Scientific Inc., Waltham, MA, USA
Protease inhibitor cocktail for mammalian cell	Bio Basic, Inc., Markham, Ontario, Canada
Protein G Sepharose™ 4 Fast Flow	Amersham Pharmacia Biotech AB, Sweden
Seakem LE agarose	Cambrex, Bio Science Rockland Inc., Rockland, ME, USA
Skimmed milk	Fonterra, Selangor, Darul Ehsan, Malaysia
Sodium carbonate, $NaHCO_3$	Merck KGaA, Darmstadt, Germany
Sodium chloride, NaCl	Merck KGaA, Darmstadt, Germany
Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4 \cdot H_2O$	Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulfate, SDS	Bio Basic, Inc., Markham, Ontario, Canada
Sodium hydroxide, NaOH	Merck KGaA, Darmstadt, Germany
Tetramethylethylenediamine, TEMED	Bio Basic, Inc, Markham, Ontario, Canada
Trypan Blue	Research Organic, Inc., Cleveland, OH, USA

Tris base	Research Organic, Inc., Cleveland, OH, USA
Triton X-100	USB Corporation, Cleveland, OH, USA
Tween 20	AMERESCO, Solon, OH, USA

4.1.10 Instruments and laboratory supplied

Biological safety cabinet	NUAIRE, Plymouth, MN, USA
Centrifuge 5702	Eppendorf, Hamburg, Germany
CO_2 incubator	Thermo Scientific Heraeus, Australia
Electrophoresis Power Supply Biorad power PAC300	Biorad, Hercules, CA, USA
Hemacytometer (Blood counting chamber)	BOECO, Hamburg, Germany
Image scanner	Amersham Biosciences
Labnet ProBlot Lab Rocker25	Labnet International, Inc, Edison NJ, USA
Light microscope Nikon ECLIPSE TS100	Nikon Instruments INC, Melville, NY, USA
Magnetic stirrers	JEIO TECH CO., LTD, Seoul, Korea
Microcentrifuge 5418	Eppendorf, Hamburg, Germany
Microcentrifuge 5424	Eppendorf, Hamburg, Germany
Microcentrifuge 5424R	Eppendorf, Hamburg, Germany
Multipor II electrophoresis system	Amersham Biosciences, Corston, Bath, UK
Nikon TIS Inverted fluorescent microscope	Nikon, Tokyo, Japan
Thermomixer model 5436	Eppendorf, Hamburg, Germany
Tomy MX 301 high speed refrigerated micro centrifuge	Prolabmas, Jakarta, Indonesia
VeritiTM Thermal Cyclers and GeneAmp® PCR System 9700	Applied Biosystems®, Waltham, MA, USA

Vortex Genie2 shaker	Genie Scientific industry
Water bath	Memmert GmbH+ Co.KG, Schwabach, Germany
Statspin® Cytofuge2	Iris Sample Processing, Brea, CA, USA

4.1.11 Miscellaneous materials

5 mL serological pipette, sterile	Corning Inc., Corning, NY, USA
10 mL serological pipette, sterile	Corning Inc., Corning, NY, USA
175 cm^3 cell culture flask, sterile	Corning Inc., Corning, NY, USA
500 mL bottle top vacuum filter, 0.22 μm PES, sterile	Corning Inc., Corning, NY, USA
100×20-mm culture plate, sterile	Corning Inc., Corning, NY, USA
6-well culture plate, sterile	Corning Inc., Corning, NY, USA
12-well culture plate, sterile	Corning Inc., Corning, NY, USA
24-well culture plate, sterile	Corning Inc., Corning, NY, USA
Blotting Paper, Whatman® 3MM Chr Paper	Whatman International Ltd., Maidstone, UK
Protein® Nitrocellose Transfer Membrane, 0.2 μm pore size	Whatman International Ltd., Maidstone, UK
50,000 MWCO PES Vivaspin concentrators	Sartorius, Gottingen, Germany
0.22 μm Millex Syringe-driven filter unit	Merck Millipore, Darmstadt, Germany

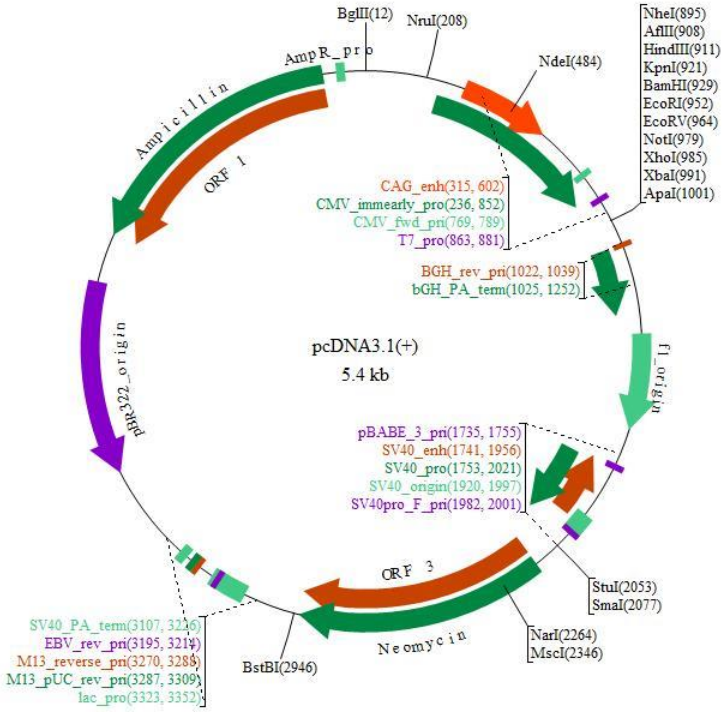

Figure 4.1 Physical map of pcDNA 3.1 hygro+ vector

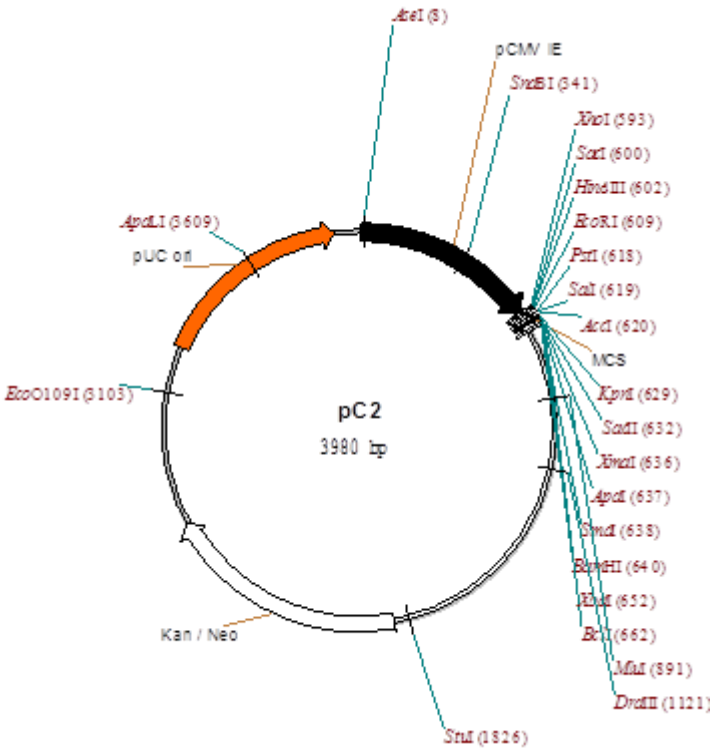

Figure 4.2 Physical map of pC2 vector

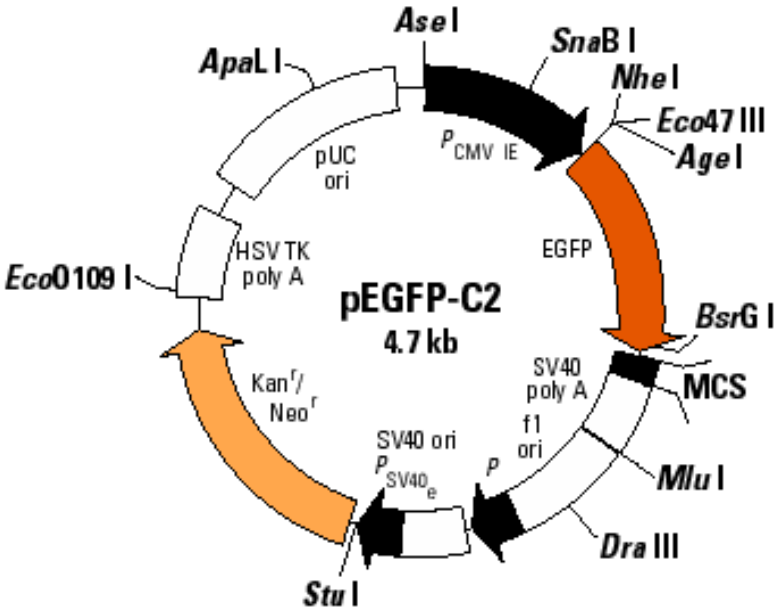

Figure 4.3 Physical map of pEGFP-C2

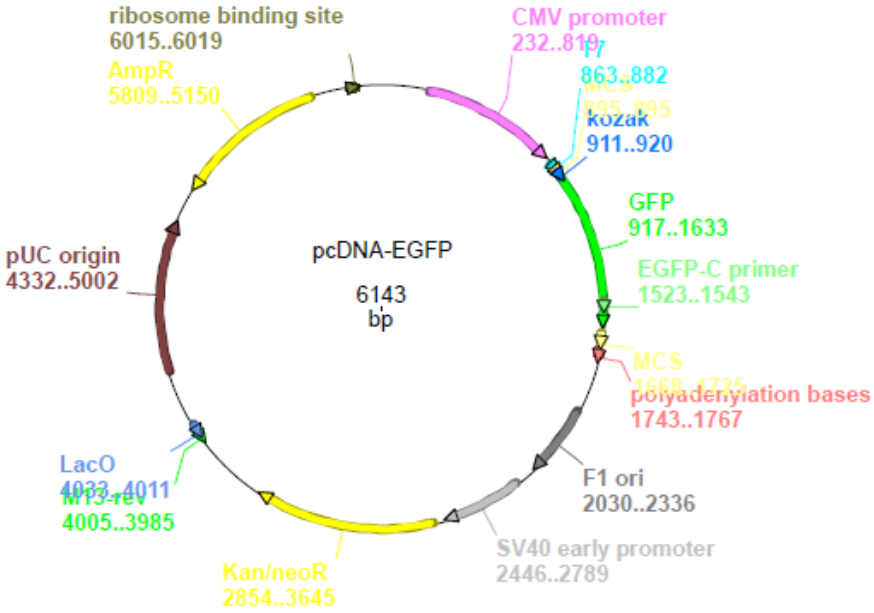

Figure 4.4 Physical map of pcDNA-EGFP

4.2 Methods

4.2.1 Construction of DENV-2 VLPs

4.2.1.1 Construction of DENV-2 VLPs in expression vector

Five types of DENV VLPs were constructed. Four DENV-2 VLPs were constructed in pC2 eukaryotic expression vector including : (1) pC2-D2prM-E containing nucleotide sequence encoding full-length DENV-2 prM and E protein, (2) pC2-D2YPTI-prM-E containing nucleotide sequences encoding YPTI motif, full-length DENV-2 prM and E protein, (3) pC2-D2C-prM-E containing gene fragments encoding signal peptide (19 aa of C-terminal DENV-2 capsid protein, C), full-length prM and E proteins, and (4) pC2-D2YPTI-C-prM-E containing nucleotide sequences encoding YPTI motif, gene fragments encoding signal peptide, full-length prM and E proteins. The other construct is pcDNA-D2opt.prM-E which is the pcDNA 3.1 hygro+ expression vector containing the VSV-G signal sequence followed by the optimized prM and E gene sequences for mammalian codon usage. The detail of each construct are shown in **Figure 4.5.**

4.2.1.2 DENV-2 cDNA synthesis

For DENV-2 cDNA generation, DENV-2 RNA was extracted from partially purified DENV-2 stock using Trizol reagent (Molecular research center, Ohio, USA) and converted to cDNA by reverse transcription (RT). The reaction contained 1 µg of DENV-2 RNA, random hexamer, Improm-II^{TM} reverse transcriptase (Promega, Madison, WI, USA), RT master mix composing of nuclease free water, 1X reverse transcriptase buffer, 25 mM $MgCl_2$, 10 mM deoxynucleotide-triphosphate (dNTP) and 1U/µl ribonuclease inhibitor. DENV-2 cDNA generation was performed in a thermocycler (Applied Biosystems®, Waltham, MA, USA) using the manufacturer's protocol. In brief, reactions were incubated at 25 °C for 5 min for primer binding and continued with 42 °C for 1 hr for cDNA extension. Before PCR amplification mixture was incubated in 72 °C for 15 min to inactivate RNase.

4.2.1.3 PCR amplification

The inserted fragments of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E were amplified from DENV-2 cDNA using specific primers as shown in **Table 4.1.** All of primers were designed based on the full-length nucleotide sequence encoding E protein complete genome sequence from dengue virus serotype 2 (GenBank accession No. NC001474.2) and synthesized by Macrogen (Seoul, Korea). Each primer was overhanged with restriction recognition site for cloning as indicated. At 5' terminus, a Kozak consensus sequence was added in forward primers whereas a stop codon was added in reverse primers. PCR reaction composed of 0.1 µM (each) of primers, 10 mM dNTPs, 1X *phusion* buffer, 3% DMSO, 0.4 U *phusion*^{TM} polymerase enzyme (Thermoscientific, Waltham, MA, USA) and 1 µl of cDNA. The PCR reaction and PCR conditions are shown in **Table 4.2.** PCR reaction was performed in a thermocycler (Applied® Biosystems) and PCR products were analyzed on 1% gel electrophoresis. The expected DNA band of PCR product was 2,000 bp.

4.2.1.4 Preparation of inserted fragments and vector

PCR product size approximately 2,000 bp containing the expected DENV-2 genes were cut from agarose gel then extracted and purified using gel/PCR DNA fragments extraction kit (Geneaid Biotech Ltd., New Taipei city, Taiwan**).** The purified inserted fragments were digested with restriction enzymes that were compatible with restriction recognition sites for cloning in each primers. For vector, pC2 plasmid were digested with the same restriction enzymes as inserted fragment. Digested plasmids were run on 1% agarose gel, cut and purified as mentioned above.

4.2.1.5 Optimized DENV-2 prM-E VLPs construction

DENV-2 prM-E gene was optimized by altering gene sequences into preferable codon set of mammalian to increase expression level of target protein in mammalian cell expression system. Optimized prM-E gene (61) was synthesized by Genscript company (Piscataway, New Jersey, USA) in pUC57 vector. This construct was transformed to *E.coli* DH5-α competent cell and cultured in 50 ml LB broth containing 50 µg/ml ampicillin (Sigma-Aldrich Co., St.Louis, MO, USA). Extracted plasmids were digested with *BamH*I and *Xho*I. Inserted fragment

approximately 2,055 bp was extracted and purified from 1% agarose gel using gel/PCR DNA fragments extraction kit (Geneaid**).** In case of pcDNA 3.1 hygro+ vector, it was digested with the same restriction enzyme as inserted fragment.

4.2.1.6 DNA ligation

The concentration of vectors and inserted fragments was estimated on 1% agarose gel comparing with 200 ng of GeneRuler 1 kb DNA Ladder (Thermoscientific). Vector and inserted fragments were ligated at the molar ratio of 1:5 (vector : inserted fragments) using 200 U of T4 ligase enzyme (Vivantis, Oceanside, CA, USA) with standard ligation buffer (Vivantis) at 16 °C for overnight.

4.2.1.7 Plasmid transformation

Ligation reactions were transformed into *E.coli* XL-1 blue or *E.coli* DH5-α competent cells. The competent cells were prepared using $CaCl_2$ method following standard protocol. In brief, a single colony of *E.coli* was cultured in LB broth. When *E.coli* culture reached OD_{600} of ~0.5, the culture was chilled on ice for 15 min and centrifuged at 3,000 *xg* at 4 ° C for 10 min. Then, cells were resuspended in cold Tfb1 buffer containing $CaCl_2$. After that, cells were chilled on ice and pelleted as indicated above. Pellet was resuspended in cold Tfb2 buffer containing $CaCl_2$. Lastly the competent cells were aliquoted in sterile 1.5 ml tubes and quickly frozen in liquid nitrogen. The transformation efficiency of the competent cells was estimated before use by transformation with control plasmid with a known concentration. Before transformation, ligase enzyme was inactivated by incubation the ligation mixture at 65° C for 20 min. Ligation reaction was transformed into competent cells by the heat shock method as follows, ligation mixture was added to competent cells and incubated on ice for 30 min. Then, transformant were incubated at 42 °C for 90 sec and immediately put on ice for 2 min. Finally, 750 µl of fresh LB broth was added, and cells were incubated at 37 °C for 1 hr with shaking at 220 rpm. Transformed cells were spread on LB agar containing antibiotic then incubated at 37°C, overnight. For D2prM-E, D2YPTI-prM-E, D2C-prM-E and D2YPTI-C-prM-E constructs, transformants were plated on LB agar containing 100 µg/ml kanamycin were whereas pcDNA_D2opt.prM-E was plated on LB agar containing 50 µg/ml ampicillin. The selected colonies were screened by restriction enzyme digestion analysis or colony PCR screening prior to sequencing.

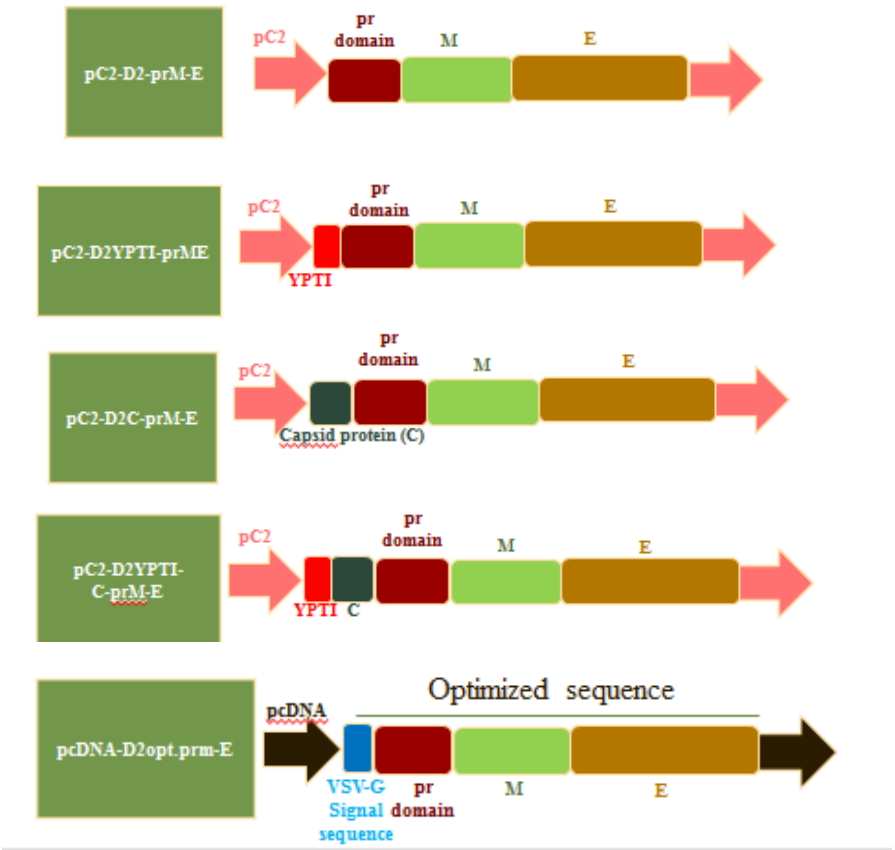

Figure 4.5 Schematic diagram of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2C-prM-E, pC2-D2YPTI-C-prM-E and pcDNA-D2opt.prM-E construct.

Table 4.1 The sequence of primers used for amplification of DENV-2 genes inserted fragments

Serotype/direction	Primers sequence	%GC	Tm (°C)
DENV-2/ Fw*Xho*I-D2prM-E	5'TATA*CTCGAG*CGCCACCATGT TCCATTTAACCACACG3’	41	48
DENV-2/ Fw*Xho*I-D2YPTI-prM-E	5'TATA*CTCGAG*CGCCACCATGTATCCT AC CATCTTCCATTTAACCACACG3’	41	48
DENV-2/ Fw*Xho*I-D2C-prM-E	5'TATA*CTCGAG*CGCCACCATGAATAG GA GACGCAGATCTGC 3'	53	71
DENV-2/ Fw*Xho*I-D2YPTI-C-prM-E	5'TATA*CTCGAG*CGCCACCATGTATCCT AC CATCAATAGGAGACGCAGATCTGC 3'	50	73
DENV-2 / Rv*Apa*I-D2prM-E	5'TATA*GGGCCC*TTAGGCCTGCACCA TGACTCC3’	67	59

** CTCGAG = *Xho*I restriction site
GGGCCC = *Apa*I restriction site
CGCCACCATG = Kozak sequence

Table 4.2 PCR reaction and condition for inserted fragments amplification

Reagent	**Concentration**	**Volume of Small scale PCR (20 µl) (per 1 reaction)**
MilliQ water	-	12.4
phusion buffer	5X	4
dNTP	10 mM	0.4
Forward primer	10 µM	1
Reverse primer	10 µM	1
Phusion enzyme	2U/µl	0.2
cDNA	-	1

Step	**ºC**	**Time**	**cycles**
Denaturation	98	30 sec	-
	98	10 sec	35
Annealing	55	15 sec	
Extension	72	1 min	
	72	7 min	-

4.2.1.8 Restriction enzyme digestion analysis, colony PCR screening and sequencing

In the case of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E construction, the selected colonies on LB agar with 100 µg/ml kanamycin were randomly picked and grown in LB broth containing 50 µg/ml kanamycin at 37˚C 220 rpm for overnight for restriction enzyme digestion analysis. DNA plasmids were extracted using Geneaid kit (Geneaid**)** according to the manufacturer's instructions. For the first 4 constructs, approximately 100 ng of DNA plasmid were digested with *Xho*I and *Apa*I enzymes. The expected DNA band patterns were approximately 4,000 and 2,000 bp of vector and inserted fragments, respectively**.** The digested and undigested plasmid DNAs were analyzed by 1% agarose gel electrophoresis

For pcDNA_D2opt.prM-E, colony PCR technique was used to screen candidate clones. The selected colonies on LB agar containing 50 µg/ml ampicillin were picked and put into PCR reaction containing specific primers (**Table 4.3**) to amplify inserted fragment with size approximately 2,055 bp. All of the candidate clones were submitted to nucleotide sequencing using capillary sequencing by Macrogen. CMV-F and pFastbBact_Reverse primers were used as 5’ terminal and 3’ terminal sequencing primers for pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E constructs whereas CMV-E and BHG-R was used for pcDNA_D2opt.prM-E sequencing. Specific internal primers were used if needed.

4.2.2 Cell culture

HEK 293T (human embryonic kidney 293T) cells and HEK 293 (human embryonic kidney 293) were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM, GibcoTM Invitrogen, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GibcoTM Invitrogen), at 37˚C with 5% CO2 in humidified incubator (Heraeus instrument; Langenselbold, Germany).

4.2.3 DENV-2 VLPs transfection

One day before transfection, the proper amount of HEK293T and HEK293 cells as indicated in **Table 4.4** were pre-seeded in 12 or 6 well plates or 60 mm^2 culture dishes. In the transfection reaction, pEGFP-C2 and pcDNA-EGFP were used as control plasmids to estimate transfection efficiency. The transfection reaction was performed using Lipofectamine^{TM} LTX (Invitrogen) according to manufacturer's protocol. Briefly, plasmids were incubated with Plus^{TM} reagent in the presence of opti-MEM (Gibco^{TM} Invitrogen) for 5 min. Then, the plasmids were mixed with Lipofectamine^{TM} LTX and incubated at room temperature for 25 min. DNA-lipofectamine complex were added drop-wise to the cells. The transfected cells were rocked well and incubated at 37°C with 10% CO_2 for 4-6 hrs and fresh culture media was replaced. Volume of each transfection reagents of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2C-prM-E, pC2-D2YPTI-C-prM-E is shown in **Table 4.5A** and pcDNA-D2opt.prM-E transfection is shown in **Table 4.5B**. Cell lysate, supernatant and DNA pellet were collected for 3 days. Supernatants from transfected cells were spun at 200 *xg* for 5 min to get rid of detached cells and only liquid phase was kept. Some transfected cells were cytocentrifuged onto cover slips using Statspin® Cytofuge2 (Iris Sample Processing, Brea, CA, USA) for immunofluorescent assay. All samples were kept at appropriated temperature for validation.

In order to investigate the effect of cellular lipid content on E protein expression. After 4-6 hrs post transfection, culture media was replaced with fresh media containing 100 µM oleic acid to increase lipid accumulation in the transfected cells. Stock oleic acid with 12.38 mM concentration was prepared. Free fatty acid BSA 0.5 g was dissolved in 1.8 ml Tris-HCl pH 8.0. Next, 6.8 mg of oleic acid was added. The complex of oleic acid and BSA was filtrated with 0.22 µM filter. Culture media with or without 100 µM oleic acid was used to culture pcDNA-D2opt.prM-E transfected cells after 4-6 hrs post transfection. E protein band with or without oleic acid addition was quantitated using Quantity one software and actin expression was determined as internal control.

4.2.4 Validation of DENV-2 E protein expression

4.2.4.1 Western blot analysis

Envelope protein expression was investigated in cell lysate and supernatant by Western blot analysis to detect the expression of E protein. Cell lysate and culture media were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a standard protein marker (All blue prestained Precision Plus Protein standards, Bio-Rad) and electro-blotted onto nitrocellulose membranes using the Mini trans-Blot Electrophoretic transfer Cell (Bio-Rad). After the membranes were blocked with 5% skim milk, E protein expression was detected by pan-specific anti-dengue E protein (MA1-27093, Pierce, Rockford, IL, USA) followed by an anti-mouse IgG conjugated with horseradish peroxidase (A9044, sigma, MO, USA) at dilutions of 1:1000 and 1:10000, respectively. Signal was detected by Amersham ECL Plus western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) and exposed to autoradiography films for an appropriate period of time.

4.2.4.2 Total DNA and episomal DNA extraction

For confirmation of the presence of DENV-2 VLPs in transfected cells, total DNA and episomal DNA were extracted from the transfected cell pellets. For total DNA extraction, 300 µl lysis buffer was added to the cell pellet. Lysed cells were incubated at 65 °C for 2-3 hrs with tube inversion every 15 min. After that, an equal volume of isopropanol was added and tubes inverted a few time prior to pelleting DNA by centrifugation at 18000 *xg* for 5 min. Pellets were carefully washed with 500 µl of 70% ice cold ethanol and incubation at room temperature to dry. Lastly, total DNA was resuspended in 40 µl sterile distilled water. In case of episomal DNA extraction, cell pellet with lysis buffer was centrifuged at 23,000 xg for 40 min. Pellet was treated with RNase A at 37°C for 60 min. Then, 750 µl phenol and 200 µl chloroform were added and stored overnight at -20°C. DNA pellet was spun at 10,000 xg for 30 min, rinsed with ice cold 70% ethanol and resuspended in 40 µl distilled water. The presence of constructed VLPs in the transfected cells was detected by PCR technique using corresponding primer sets and PCR condition is shown in **Table 4.6.**

4.2.4.3 Dot blot analysis

Supernatants from transfected cells were collected at day 2 post transfection and 100 µl of each sample was evaporated until remained only 10 µl. Two nitrocellulose membranes were labelled the region of blotting. Then, 1 µl of supernatants with or without evaporation were slowly dotted into membrane. The spots were left to air dry and dotted again in the same volume of certain samples. When membranes were completely dried, non-specific signal was blocked by 5% skim milk in TBS-T for 1 hr at room temperature followed by incubated with pan-specific anti-dengue E protein at dilution of 1:100. After that, membranes were washed with TBS-T for 5 min, 3 times and incubated with goat anti-mouse IgG conjugated with HRP at dilution of 1:3000 for 1 hr at room temperature. Then, they were washed three times again with TBS-T (15 min x 1, 5 min x 2). DENV-2 E protein was detected by incubation with Amersham ECL Plus western Blotting Detection Reagents (GE Healthcare) and exposure to autoradiography films for an appropriate period of time.

4.2.4.4 Immunofluorescent assay (IFA)

IFA technique was used for estimation of the number of VLP harboring cells. After 1 day post transfection, the transfection efficiency was validated by observation of GFP expression of the control plasmids, pEGFP-C2 for the constructs using pC2 as a vector and pcDNA-EGFP for the construct using pcDNA as a vector, under a Nikon TIS Inverted fluorescent microscope (Nikon, Tokyo, Japan). The number of the GFP expressing cells was determined in each transfection experiment and this can be assumed to be transfection efficiency of the constructed plasmids. For DENV E expressing cells, approximately $1.2x10^5$ transfected cells were cytocentrifuged onto cover slips and fixed with 100% ice-cold methanol for 12 min at room temperature, washed with 1X phosphate buffer saline (PBS) and permeabilized with 0.3% Triton-X 100 in 1X PBS. Then, cells were incubated with pan specific anti-dengue E protein at dilution 1:100 in the dark moist chamber for overnight followed by chicken anti-mouse IgG conjugated with Alexa 594 at dilution 1:200 in the dark moist chamber for 1 hr. Cell nuclei were stained with 4’,6-diamidino-2-phenyllindole (DAPI) (Invitrogen). Finally, cover slips were mounted onto glass slides using Prolong® Gold antifade reagent (Invitrogen).

Table 4.3 The sequence of primers used for pcDNA-D2-opt.prM-E colony PCR screening

Direction	Primers sequence	%GC	Tm (°C)
Forward primer	5'TATAGGATCCGCCACCATGAA3'	48	57
Reverse primer	5'TATACTCGAGTCATCAGGCCTG3'	50	57

Table 4.4 The amount of HEK 293T and HEK 293 cells seeded for transfection

Size	Surface area(cm^2)	Amount of cells	
		HEK 293T	HEK 293
12 well plate	4	4×10^5	5×10^5 (50% confluence)
6 well plate	10	8×10^5	2×10^6
60 mm^2 culture dish	20	1.5×10^6	4×10^6

Table 4.5 Volume of each transfection reagents and plasmids that were used to transfect HEK 293 cell and HEK 293T cell per 1 reaction (**A**) and volume of transfection reagents for pcDNA-D2-opt.prME transfection into HEK 293 cells per 1 reaction (**B**). Negative control was performed by incubation the cells with transfection components without plasmid.

A

Components	**pEGFP-C2**	**pC2-D2 prM-E**	**pC2-D2 YPTI prM-E**	**pC2-D2 CprM-E**	**pC2-D2 YPTI-CprM-E**
Cell	HEK 293T			HEK 293T and HEK 293 cells	
Amount of plasmid	1 µg or 8 µg				
Optimem (µl)	1 µg			8 µg	
	200			500	
Plus reagent (µl)	2			5	
Lipofectamine LTX (µl)	3			15	

B

Components	**pcDNA-EGFP**	**pcDNA-D2opt.prME**
Cell	HEK 293	
Amount of plasmid	2.5 µg	
	8.0 µg	
	16.0 µg	
Optimem (µl)	500	
Plus reagent (µl)	16	
Lipofectamine LTX (µl)	20	

Table 4.6 PCR reaction and condition for detection of DENV-2 VLPs in the transfected cells in both of genomic DNA and episomal DNA

Reagent	Concentration	Volume of Small scale PCR (per 1 reaction)
MilliQ water	-	6.2
DreamTaq buffer	10X	2
dNTP	10 mM	0.7
Forward primer	10 µM	0.5
Reverse primer	10 µM	0.5
DreamTaq enzyme	2U/µl	0.2
DMSO	100%	0.3 (only CprM-E and YPTI-C-prM-E amplification)
DNA sample	-	4

Step	ºC	Time	cycles
Denaturation	95	30 sec	-
	95	10 sec	35
Annealing	55	15 sec	
Extension	68	2 min	
	68	5 min	-

The presence of DENV-2 E protein expressing cells were observed under Nikon TIS Inverted fluorescent microscope (Nikon).

4.2.5 Stable transfection

The transfected cells which stably express DENV E protein were selected by G418 (Gibco^{TM} Invitrogen). This antibiotic is an aminoglycoside antibiotic which is structurally similar to Gentamycin, so it is used for selection and maintenance of eukaryotic cells expressing *Neo* gene. In this experiment, pC2 vector in the DENV-2 VLPs constructs contain Kanamycin/Neomycin resistance gene. Two constructs including pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E, which could effectively express E protein, were transfected to HEK 293 cell line which is sensitive to kanamycin. First, the optimal concentration of G418 was optimized. HEK 293 cells were treated with different concentration of G418: 1,000, 1,200, 1,400, 1,500, 1,700, 1,900, 2,000 µg/ml and observed for 10 days to find the least concentration that showed 100% HEK 293 cells death. Then, the transfected cells were maintained in culture media containing optimal concentration of G418 and the selective media was replaced once a week. DENV-2 VLPs positive cells in stable transfection were observed once a month using IFA technique and Western blot to detect DENV-2 E protein expression in cell lysate and supernatant.

4.2.6 Partial protein purification

To purify DENV-2 VLPs, supernatant from stable pC2-D2C-prM-E transfected cells were collected. Supernatant was concentrated by filtration and FBS was eliminated using 50,000 MWCO PES Vivaspin concentrators (Sartorius, Gottingen, Germany). Supernatant from DENV-2 infected cells was used as a positive control. First, supernatant was filtered through 0.22 µm Millex Syringe-driven filter unit (Merck Millipore, Darmstadt, Germany). Then, 10 ml filtered supernatant was added in Vivaspin column and resupended with 10 ml ice-cold PBS. The first flow-through was collected from column after PBS resuspension. After that, samples were centrifuged at 6000 *xg* for 30 min using Tomy MX 301 high speed refrigerated micro centrifuge (Prolabmas, Jakarta, Indonesia) prior to second flow-through collection. Ice-cold PBS was added on top of column for resuspension again

and centrifuged at the same speed. This step was repeated for 3 times and flow-through was collected from column at each step after centrifugation. Concentrated supernatant in column was centrifuged until it remained approximately 500 µl. then supernatant was transferred to 1.5 ml tube. All flow-through and concentrated supernatants were analyzed by Western blot.

4.2.7 Protein precipitation by ammonium sulfate and acetone

Beside of column purification, proteins in supernatant were precipitated using ammonium sulfate ($(NH_4)_2SO_4$) or acetone. Ammonium sulfate precipitation is a simple method to fractionate proteins from supernatant. Charged proteins will form large complexes and precipitate out by centrifugation. First, 50 ml of supernatant was stirred on stirring plate. While it was stirring, ammonium sulfate powder was slowly added to sample until saturation and continued stirring overnight in cold room. After that, samples were centrifuged at 15,000 *xg* for 15 min and supernatant was discarded and pellet was resuspensed with PBS.

In case of acetone precipitation, required volume of acetone was cooled at -20°C. Supernatant was added in acetone-compatible tube then four times the sample volume of cold acetone was added to the tube. Sample was vortexed and incubated at -20°C for 60 min. After that, sample was centrifuged 10 minutes at 13,000-15,000 *xg* and supernatant was discarded. Protein pellet were left in the uncapped tube at room temperature for 30 min but not over-dried. Lastly, 40 µl of non-reducing loading buffer was added and vortexed thoroughly for dissolved protein pellet prior to analysis by Western blot. Precipitated supernatants from these 2 methods were analyzed by Western blot. In this experiment, supernatant from DENV-2 infected cells were used as a positive control.

4.2.8 Immunoprecipitation (IP)

Immunoprecipitation was performed to pull down the expressed DENV-2 E protein in supernatant by using specific antibody for concentrated DENV-2 protein in supernatant**.** Supernatants from pC2-D2C-prM-E, pC2-D2YPTI-C-prM-E and pcDNA_D2opt.prM-Etransfection were harvested at day 2 post transfection. Five hundred of each supernatant was eliminated non-specific binding by incubation with

50% slurry of protein G sepharose beads (Amersham Pharmacia Biotech AB, Sweden) and gentle inverted the tubes at 4°C for 2 hrs. After pre-clearing, supernatant was centrifuged at 6,000 *xg* for 3 min at 4°C and separated supernatant into 2 tubes equally. The first tube was not added antibody and used as negative control. The second tube was added 2 µg of pan specific anti-dengue E protein. These 2 tubes were inverted at 4°C for overnight. After that, the mixture were added protein G sepharose beads, inverted at 4°C for 4 hrs and centrifuged at 6,000 *xg* for 3 min again. Supernatant was discarded after centrifugation and washed with 500 µl of PBS for 3 times (with no vortexing). Thirty µl of 3X loading dye was added and boiled at 100°C for 5 min. The mixture was centrifuged at 14,000 *xg* for 2 min and collected only supernatant without protein G sepharose beads contamination. This supernatant was analyzed by Western blot.

4.2.9 Oil Red O staining

To estimate the amount of lipid component, Oil Red O dye was used to stain neutral lipid or triglyceride in the transfected cells. The transfected cells with and without 100 mM oleic acid treatment were washed with 1 ml of PBS. After complete PBS removal, cells were fixed with 1 ml of 10% formalin and incubated for 10 min at room temperature. Formalin was replaced with 1 ml of fresh 10% formalin and incubated at least 2 hrs at room temperature. Then, formalin was removed and 1 ml of 60% isopropanol was added for 10 min. After remove isopropanol, the transfected cells were stained with Oil Red O working solution for 10 min. In washing step, the transfected cells were washed with distilled water immediately for 3-4 times. Absolute isopropanol was used as an elution reagent to elute Oil Red O dye from lipid component in the transfected cells. Finally, an optimal density at 490 nm (OD490) of the eluted samples was measured by spectrophotometer using 100% isopropanol as blank.