CHAPTER V
RESULTS

5.1 Construction of DENV-2 VLPs

Five types of DENV VLPs were constructed. For four constructs including pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E and pC2-D2-YPTI-C-prM-E, four pairs of DENV-2 prM-E gene specific primers were used to generate each inserted fragment from DENV-2 cDNA. The specificity and purity of the PCR products were analyzed by electrophoresis on 1% agarose gels. **Figure 5.1** shows that a single band with an expected size approximately 2,000 bp was observed for all RT-PCR reactions. PCR reactions without cDNA template or using negative RT as template (the RT reaction without DENV-2 RNA) were used as negative controls whereas the pcDNA Hygro(+)/D2prM-E plasmids provided by Assoc. Prof. Nopporn Sittisombut was used as a template in positive control. An absence of PCR product in both of negative controls confirmed the specificity of PCR product. The PCR products were used for cloning into pC2 vector as mentioned above. Both of vector and inserted fragments concentration were estimated prior to ligation in 1% agarose gel **(Figure 5.2)**. Vector and inserted fragments were ligated at the molar ratio of 1:5. Ligation mixture was transformed to *E.coli* XL-1 blue. Randomly selected colonies from LB agar containing 100 µg/ml kanamycin were screened by restriction enzyme digestion analysis. The double digested plasmids with *Xho*I and *Apa*I suggested that these 4 DENV-2 VLPs could be constructed after generating the expected bands of 4,000 bp and 2,000 bp **(Figure 5.3 - Figure 5.6)**. Candidate clones were submitted to DNA sequencing by Macrogen**.** All sequencing results were blasted against the NCBI database and they showed 95% similarity with DENV-2 strain 16681 polyprotein mRNA and they showed that all of the desired plasmids, pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E and pC2-D2-YPTI-C-prM-E were successfully constructed. **(Figure 5.7 - Figure 5.10).**

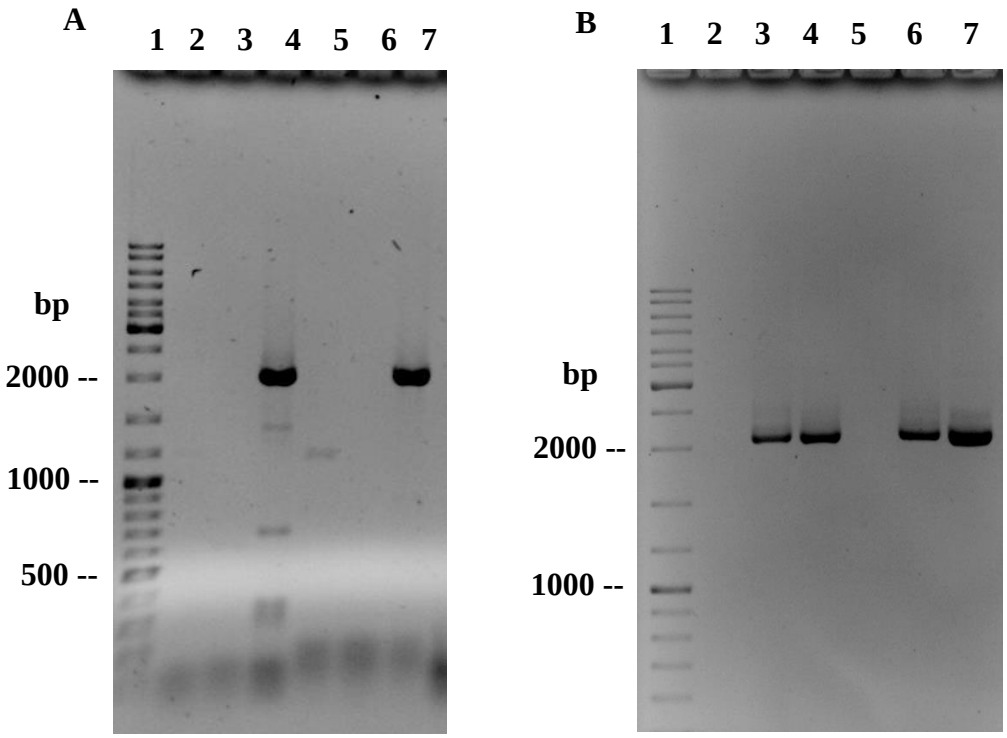

Figure 5.1 Gel electrophoresis of PCR products with specific primers for amplification of first strand cDNA of DENV-2 with prM-E, YPTI-prM-E primers (A) and C-prM-E, YPTI-C-prM-E primers (B)

(A)
Lane 1 marker
Lane 2 negative RT for prM-E primer
Lane 3 negative PCR for prM-E primer
Lane 4 PCR product for prM-E primer
Lane 5 negative RT for YPTI- prM-E primer
Lane 6 negative PCR for YPTI- prM-E primer
Lane 7 PCR product for YPTI-prM-E primer

(B)
Lane 1 marker
Lane 2 negative PCR for C-prM-E primer
Lane 3 PCR product for C-prM-E primer
Lane 4 positive control for C-prM-E primer
Lane 5 negative PCR for YPTI-C-prM-E primer
Lane 6 PCR product for YPTI-C-prM-E primer
Lane 7 positive control for YPTI-C-prM-E primer

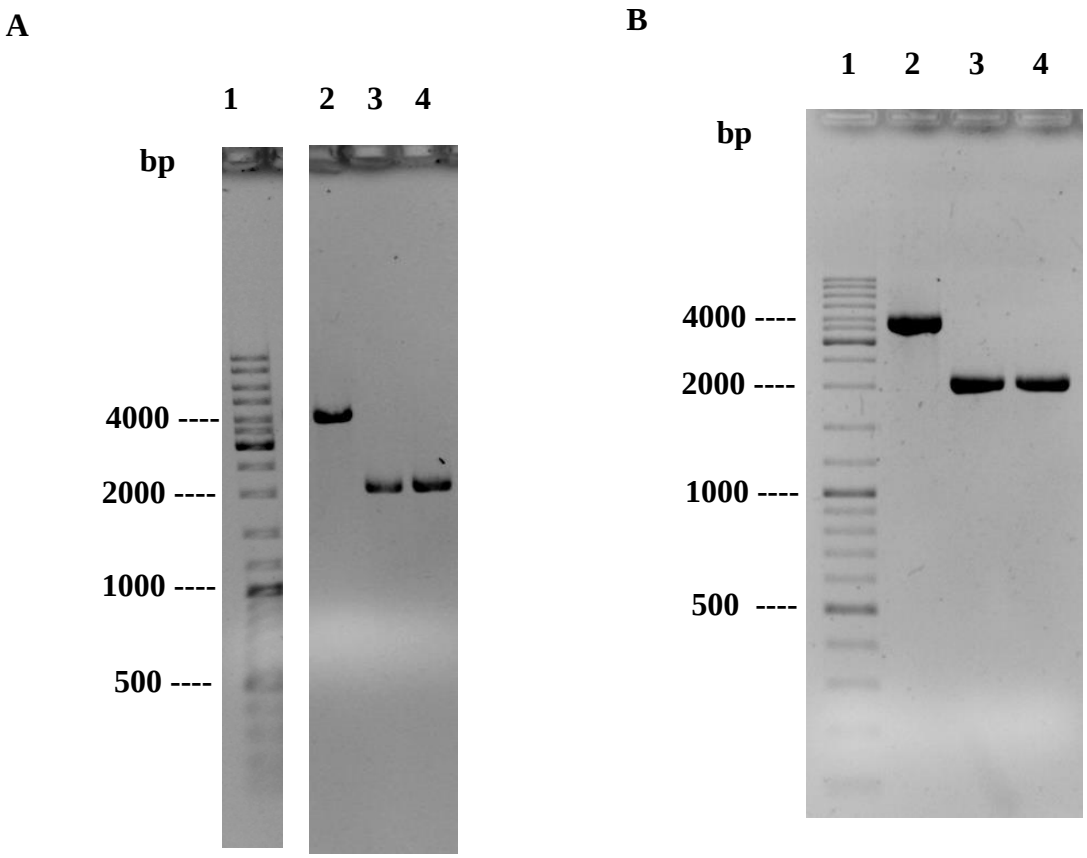

Figure 5.2 Concentration estimation of pC2 vector and 4 types of inserted fragments.

One µl of the *Xho*I and *Apa*I digested pC2, prM-E fragment, YPTI-prM-E fragment (A), C-prM-E fragment and YPTI-C-prM-E fragment (B) were loaded on 1% agarose gels to compare their concentration with 200 ng of GeneRuler 1kb DNA Ladder.

(A)
Lane 1 marker
Lane 2 digested pC2 vector with *Xho*I and *Apa*I
Lane 3 prM-E inserted fragment
Lane 4 YPTI- prM-E inserted fragment

(B)
Lane 1 marker
Lane 2 digested pC2 vector
Lane 3 C-prM-E inserted fragment
Lane 4 YPTI-C-prM-E inserted fragment

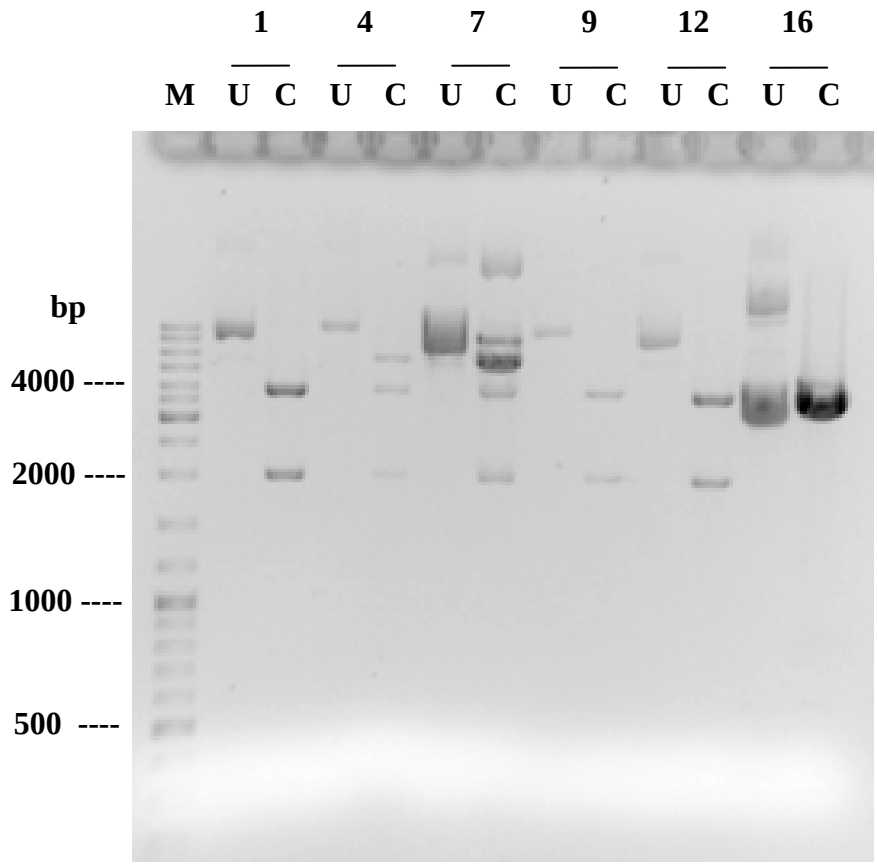

Figure 5.3 Restriction enzyme digestion analysis for screening of pC2-D2-prM-E construct.

Clones No.1, 9 and 12 generated the expected bands with size approximately 4,000 and 2,000 bp. M stands for marker. U and C are uncut and *Xho*I+*Apa*I cut plasmids, respectively.

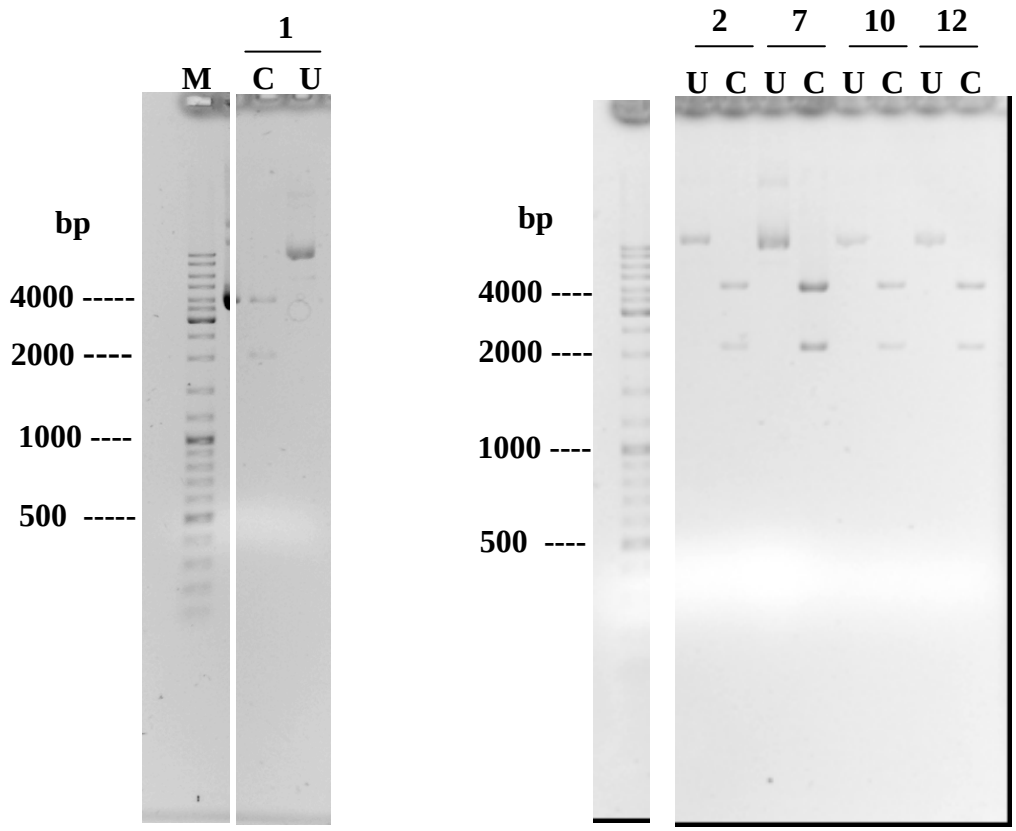

Figure 5.4 Restriction enzyme digestion analysis for screening of pC2-D2YPTI-prM-E construct.

Clones No.1, 2, 7, 10 and 12 generated expected bands with size approximately 4000 and 2000 bp DNA bands. M stands for marker. U and C are uncut and *Xho*I+*Apa*I cut plasmids, respectively.

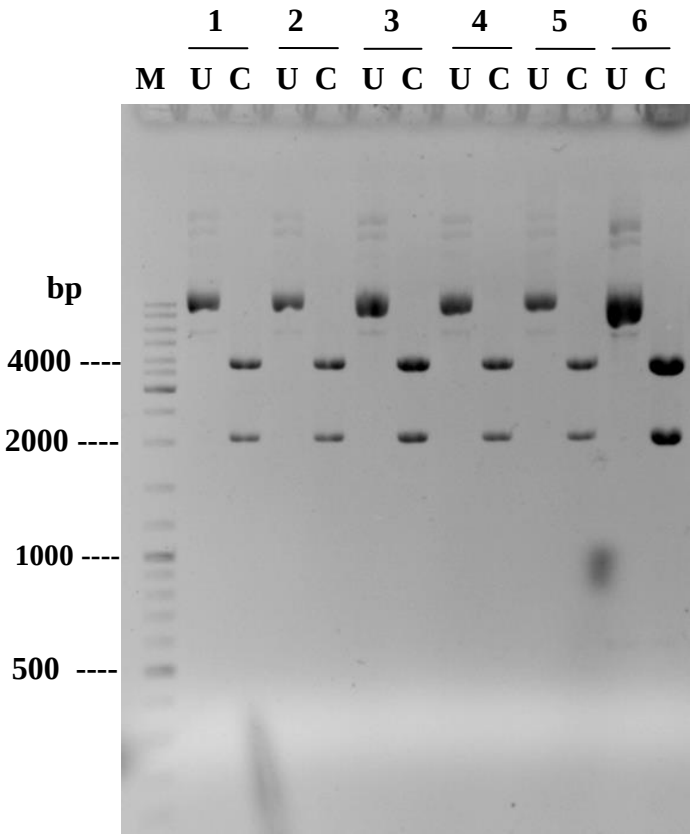

Figure 5.5 Restriction enzyme digestion analysis for screening of pC2-D2-C-prM-E construct.

Clones No.1- 6 generated expected bands with size approximately 4,000 and 2,000 bp DNA bands. M stands for marker. U and C are uncut and *Xho*I+*Apa*I cut plasmids, respectively.

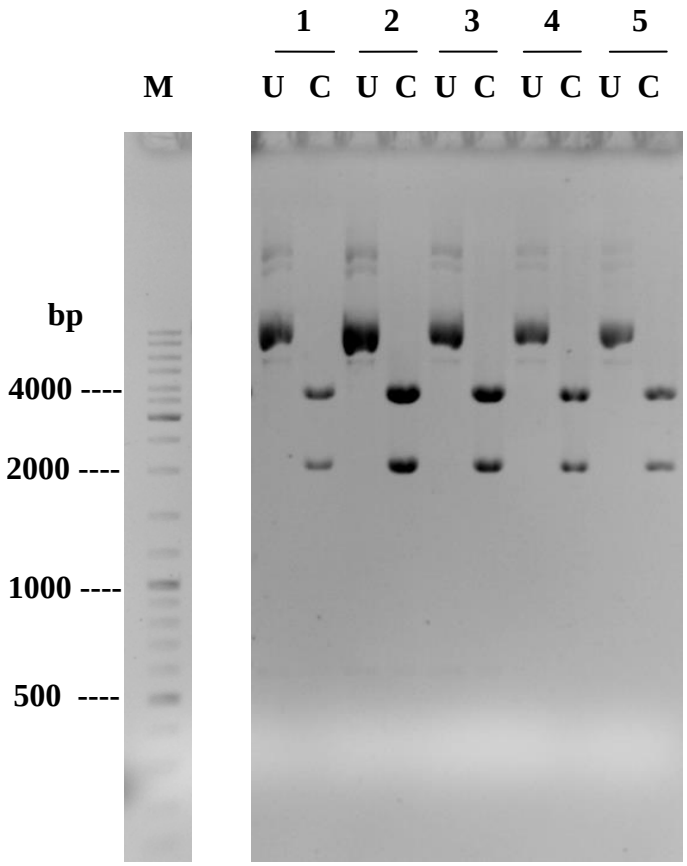

Figure 5.6 Restriction enzyme digestion analysis for screening of pC2-D2-YPTI-C-prM-E construct.

Clones No.1- 5 generated expected bands with approximately 4,000 and 2,000 bp DNA bands. M stand for marker. U and C are uncut and *Xho*I+*Apa*I cut plasmids, respectively.

Figure 5.7 Nucleotide sequences alignment of pC2-D2prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2).

Nucleotide sequences alignment of pC2-D2prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2) shows 1 silent mismatched nucleotide at position 596 from Guanine (G) to Thymine (T) nucleotide. Both corresponding codons translate to threonine.

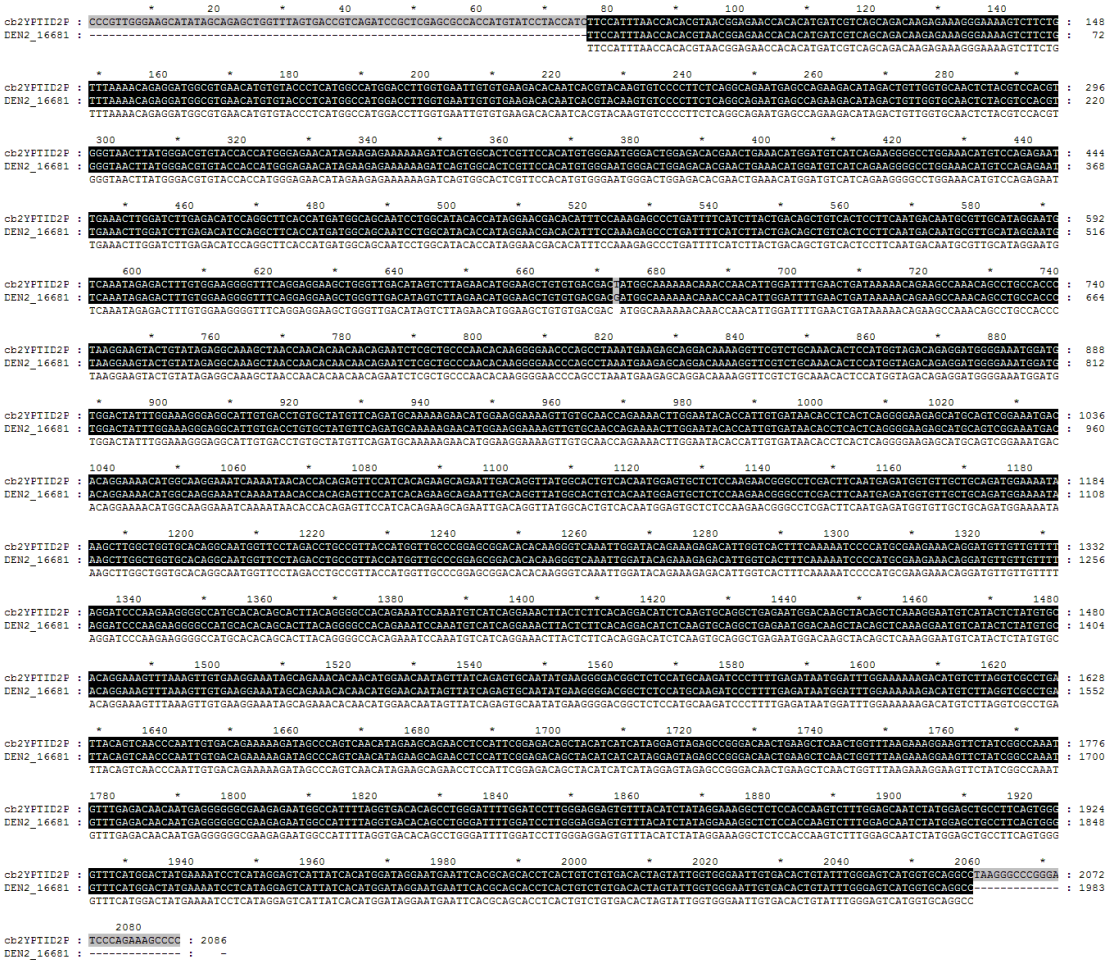

Figure 5.8 Nucleotide sequences alignment of pC2-D2YPTI-prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2).

Nucleotide sequences alignment of pC2-D2YPTI-prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2) shows 1 silent mismatched nucleotide at position 596 from Guanine (G) to Thymine (T) nucleotide. Both corresponding codons translate to threonine.

Figure 5.9 Nucleotide sequences alignment of pC2-D2-C-prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2).

Nucleotide sequences alignment of pC2-D2-C-prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2) shows 1 silent mismatched nucleotide at position 596 from Guanine (G) to Thymine (T) nucleotide. Both corresponding codons translate to threonine.

Figure 5.10 Nucleotide sequences alignment of pC2-D2-YPTI-C-prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2).

Nucleotide sequences alignment of pC2-D2-YPTI-C-prM-E with the corresponding genes of DENV-2 strain 16681 polyprotein mRNA sequence (Accession No. NC001474.2). shows 1 silent mismatched nucleotide at position 596 from Guanine (G) to Thymine (T) nucleotide. Both corresponding codons translate to threonine.

For pcDNA-D2opt.prM-E construct, a codon optimized prM-E gene was synthesized by Genscript company (Piscataway, New Jersey, USA) and cloned into the pUC57 vector at an *EcoR*V recognition site **(Figure 5.11)**. Nucleotide sequencing result of this construct was aligned with the targeted insert sequence and it showed 100% identity. This construct was transformed to *E.coli* DH5-α competent cells and cultured in 50 ml LB broth containing 50 µg/ml ampicillin. DNA plasmids were extracted and digested from pUC57 using *BamH*I and *Xho*I to subclone into pcDNA 3.1 hygro+ vector. The digested plasmid was run on a 1% agarose gel and the correct bands pattern were composed of 2710 and 2055 bp. The result of restriction enzyme digestion analysis is shown in **Figure 5.12**. Large scale digestion of vector and inserted fragment was performed and generated the corrected bands **(Figure 5.13).** Inserted fragment and digested pcDNA 3.1 hygro+ vector were extracted and purified from 1% agarose gel and their concentrations were also estimated prior to ligation **(Figure 5.14)**. Vector and inserted fragment with concentration of 7.2 ng/µl and 6.4 ng/µl respectively, were ligated at the molar ratio of 1:5. Ligation mixture was transformed to *E.coli* DH5-α. Randomly selected colonies from LB agar containing 50 µg/ml ampicillin were screened for the correct construct by colony PCR technique. Clones no.1, 2, 5, 14, 16, 17 showed the correct band (**Figure 5.15)**. Therefore, clone numbers 14 and 17 were submitted to sequencing by Macrogen. Sequence alignment of clone numbers 14 and 17 with referenced DENV-2 optimized prM-E are shown in **Figure 5.16 - Figure 5.17**, respectively. The candidate clone no.17 presented C nucleotide insertion at position 717 whereas clone no.14 showed 100% identical with DENV-2 opt.prM-E gene.

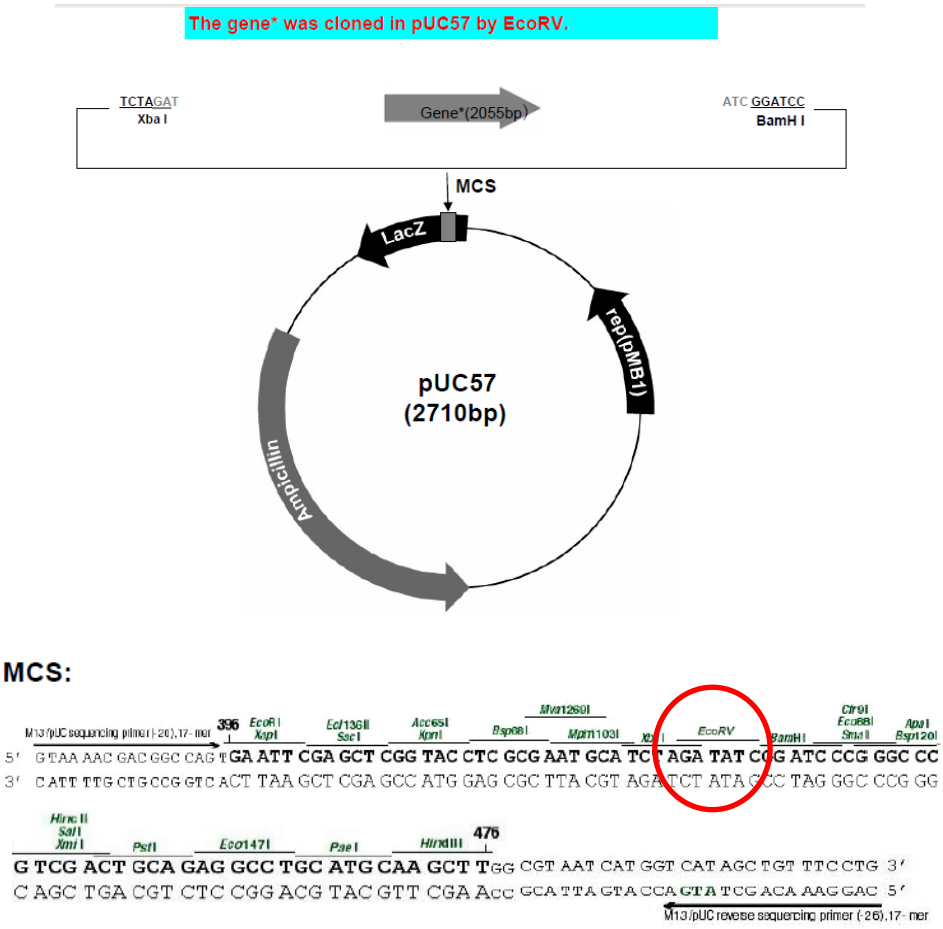

Figure 5.11 pUC57 plasmid diagram showing multiple cloning sites.

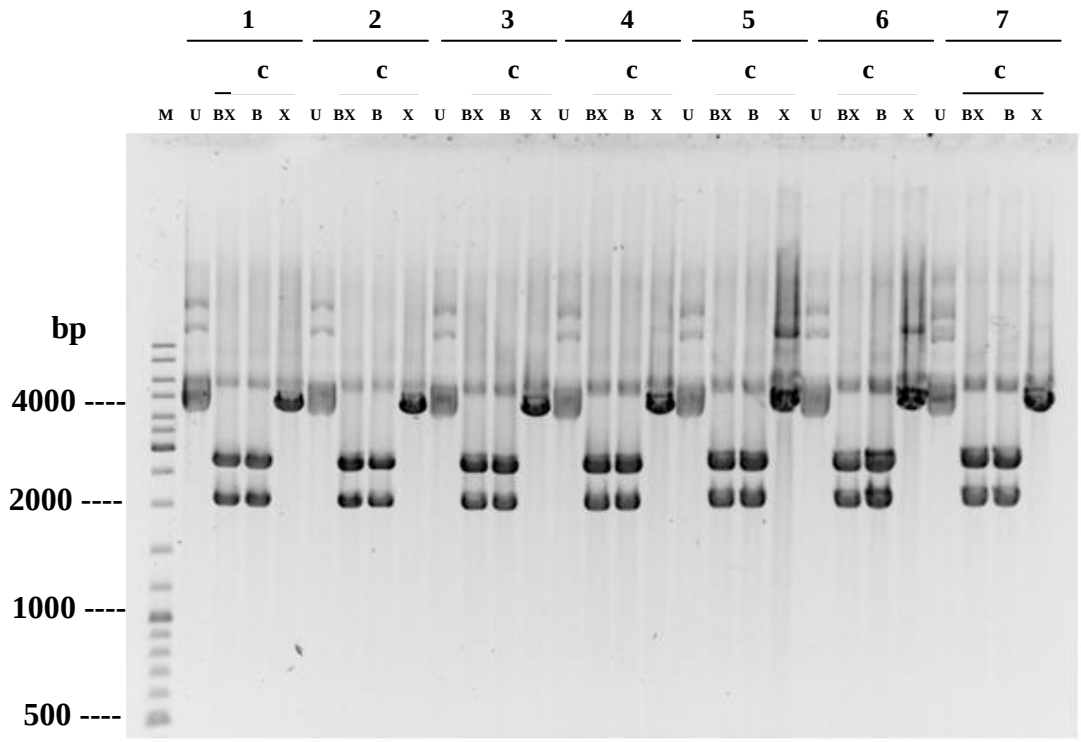

Figure 5.12 Restriction enzyme digestion analysis for screening of pUC57-opt.prM-E construct.

Clones No.1-7 generated expected bands with size 2,710 and 2,055 bp. M stands for marker. U and C are uncut and cut plasmid. B or X are *BamH*I or *Xho*I cut plasmids whereas BX is double digestion with these 2 restriction enzymes.

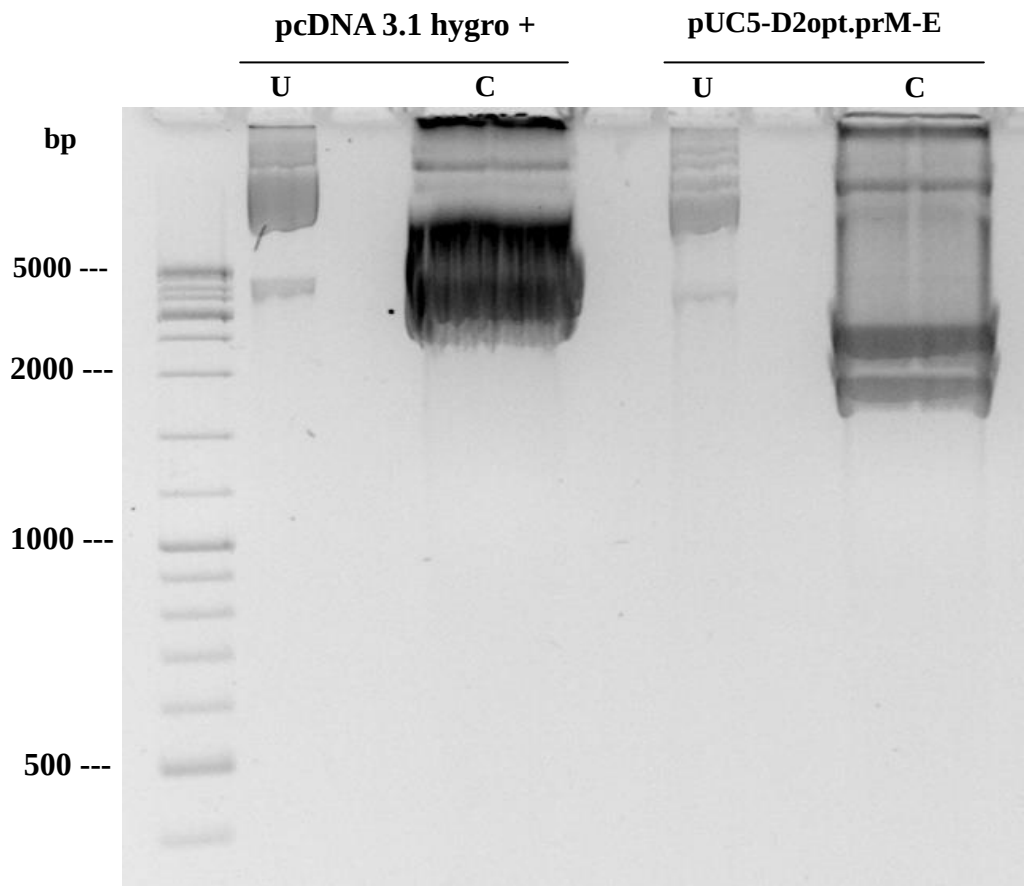

Figure 5.13 Large scale digestion of pcDNA 3.1 hygro + vector and pUC57-D2opt.prM-E was performed.

Both of vector and inserted fragment were transformed in *E.coli* and cultured in 50 ml of LB broth containing 25 µg/ml ampicillin. Plasmid were extracted and digested with *BamH*I and *Xho*I prior to ligation. U and C are uncut and cut plasmid.

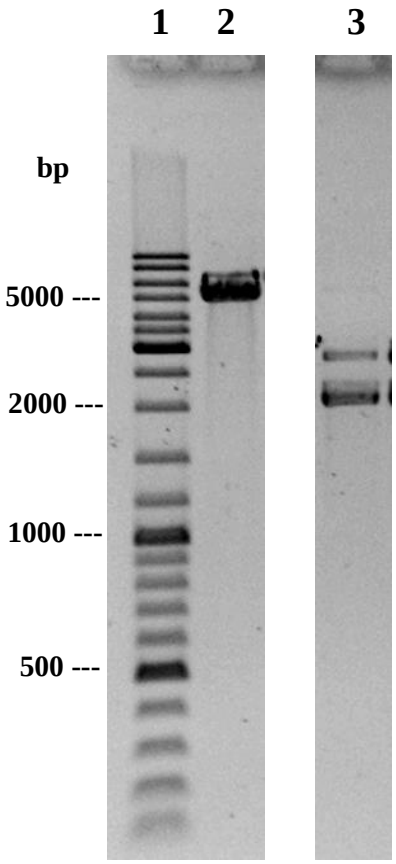

Figure 5.14 Concentration of the digested inserted fragment and digested pcDNA 3.1 hygro+ vector were estimated on 1% agarose gel prior to ligation.

Vector and inserted fragment were ligated at the molar ratio of 1:5.

Lane 1 Marker

Lane 2 Digested pcDNA3.1 hygro+

Lane 3 Digested D2opt.prME

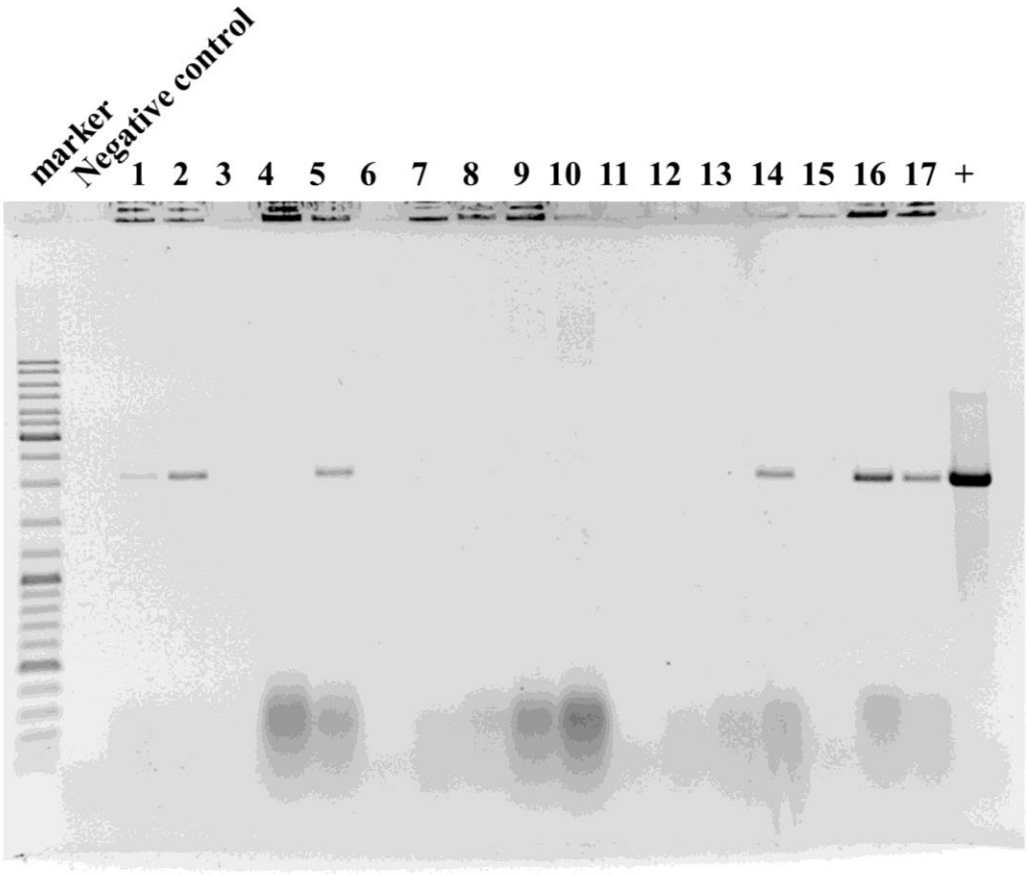

Figure 5.15 Gel electrophoresis of colony PCR screening for candidate clones of pcDNA-D2opt.prM-E.

Clones No.1, 2, 5, 14, 16, 17 generated expected bands with size approximately 2,055 bp. Negative control was a negative PCR without DNA template and + stands for positive control which pUC57-D2opt.prM-E.

Figure 5.16 Nucleotide sequence alignment of pcDNA-D2opt.prM-E clone No. 14 with the referenced DENV2-optimized prM-E sequence.

Nucleotide sequence alignment of pcDNA-D2opt.prM-E clone No. 14 with the referenced DENV 2-optimized prM-E sequence shows 100% identical with the reference sequence.

Figure 5.17 Nucleotide sequence alignment of pcDNA-D2opt.prM-E clone No. 17 with reference DENV2-optimized prM-E sequence.

Nucleotide sequence alignment of pcDNA-D2opt.prM-E clone No. 17 with reference DENV 2-optimized prM-E sequence shows C insertion at position 717.

5.2 Validation of DENV-2 E protein expression

5.2.1 Western blot analysis

Envelope protein expression in the cells and supernatant of the cells transfected with DENV-2 VLPs constructs by Western blot analysis. Firstly, the transfection efficiency was estimated at 1 day post transfection using 1 µg of pEGFPC-2 per $4x10^5$ HEK 293T cells and 8 µg of pcDNA-EGFP per $2x10^6$ HEK 293 cells as the transfection control. The transfection efficiency of both constructs were observed under an inverted fluorescent microscope as shown in **Figure 5.18.** An abundance of green fluorescent expressing cells suggested that the plasmids were highly transfected into the cells. After that, 1 µg of pC2-D2prM-E and pC2-D2YPTI-prM-E were transfected to HEK293T. Cell lysates and supernatants were collected for 3 day post transfection to investigate E protein expression by Western blot analysis. Pan specific anti-dengue E protein and rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) were used to detect E protein expression. Results show that DENV-2 E protein size approximately 50 kDa could not be clearly detected in cell lysate. In addition, a faint 50 kDa band of protein was also detected in mock (transfection reaction without any plasmid). For supernatant of pC2-D2prM-E and pC2-D2YPTI-prM-E, they showed a non-specific band at the same size of DENV-2 E protein in all 3 post transfection including mock **(Figure 5.19).** It might be cross-reaction between some non-specific proteins and antibodies in E protein detection. The non-specific cross reaction was confirmed by detection of a strong signal in DMEM+10% FBS using a pan specific anti-dengue E protein and rabbit anti-mouse IgG conjugated with HRP. This complex generated signal covering the same size as E protein causing the difficulty in detection of VLPs releasing in the supernatant **(Figure 5.20)**. In order to solve the problem, goat anti-mouse IgG conjugated with HRP was used instead and no cross reaction with DMEM+10% FBS complex was detected (**Figure 5.21**). On the others hand, opti-MEM was used to culture transfected cell instead of DMEM+10% FBS in all 3 days post transfection.

Then, 1 µg of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E and pC2-D2-YPTI-C-prM-E were transfected to HEK 293T again and cultured in opti-MEM after transfection. Western blot analysis showed that DENV-2 E protein

size approximately 50 KDa could be detected in the pC2-D2-C-prM-E cell lysate since day 1 post transfection and substantially decreased in day 2 and 3 post transfection whereas pC2-D2-C-YPTI-prM-E transfected cells could express low amounts of E protein at day 2 and could not be detected at day 3 post transfection. There was no detectable E protein expression in either pC2-D2prM-E or pC2-D2YPTI-prM-E including supernatant of all constructs for all 3 days post transfection as shown in **Figure 5.22-5.24**. Interestingly that only the constructs containing a signal sequence (19 amino acid sequence at 3' terminus of capsid protein (C)) including pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E could express the E protein in cell lysate. This signal sequence might facilitate the expression of E protein inside the transfected cell but it was not sufficient to enhance the secretion of VLPs out of cells.

5.2.2 Detection of the DENV-2 constructs in the transfected cells

Regarding deficient E protein expression in the DENV-2 constructs transfected cell, the presence of DENV-2 VLPs plasmids in the cell at day 1 and day 3 post transfection was confirmed by PCR. Four DENV-2 VLPs, pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E and pC2-D2-YPTI-C-prM-E, were transfected to HEK 293T. Total DNA and episomal DNA were extracted from the transfected cells and DENV-2 prM-E gene was amplified by 4 pairs of specific primers as mentioned above. PCR product was ran on 1% agarose gel. Surprisingly, result showed that the VLPs constructs including C-prM-E and YPTI-C-prM-E which could express E protein could not be detected by PCR amplification. On the other hand, prM-E and YPTI-prM-E plasmid DNA which did not express DENV E protein detected by Western blot analysis but could be detected in the cells (**Figure 5.25**).

5.2.3 Dot blot analysis

Dot blot analysis was performed to detect DENV-2 E protein in concentrated supernatant. One µl of non-evaporated and evaporated supernatant from pC2-D2-C-prM-E and pC2-D2YPTI-C-prM-E transfected cell were dotted onto 2 nitrocellulose membranes. First membrane was probed with mouse monoclonal pan-specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP whereas the second membrane was probed with only goat anti-mouse IgG

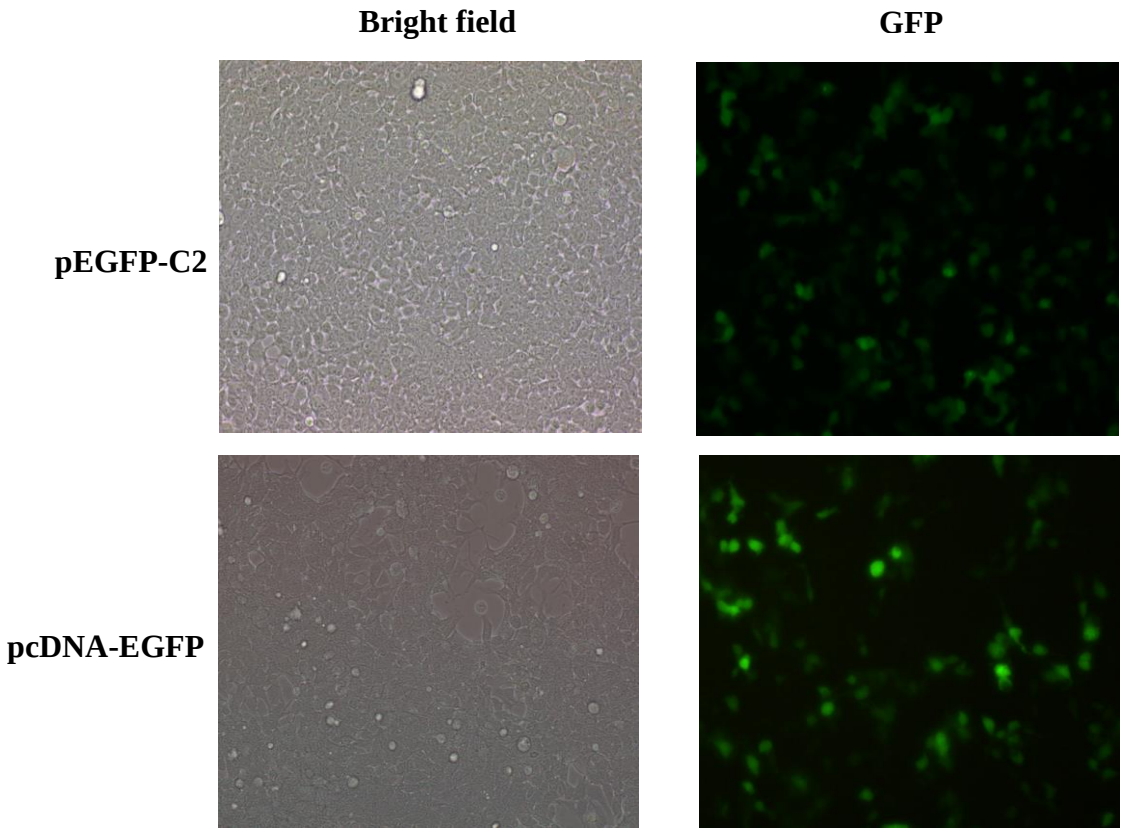

Figure 5.18 Transfection efficiency of pEGFP-C2 and pCDNA-EGFP in HEK 293T and HEK 293 cell respectively.

Transfection efficiency of pEGFP-C2 and pCDNA-EGFP in HEK 293T and HEK 293 cell respectively were estimated . Images were assessed under an inverted fluorescent microscope using FITC filter.

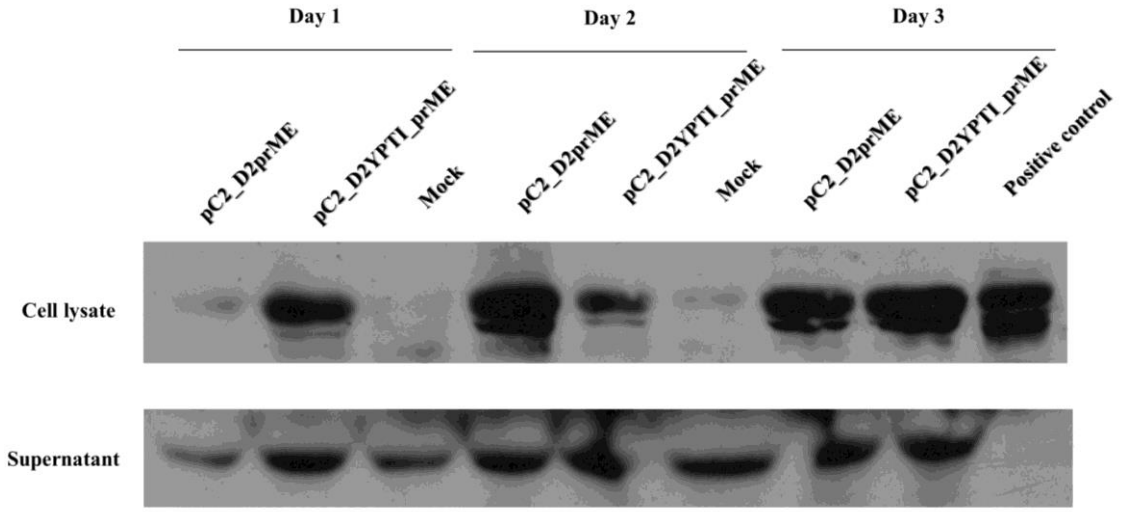

Figure 5.19 The Western blot analysis to detect the E protein expression in HEK 293T transfected with 1 µg pC2-D2prM-E and pC2-D2YPTI-prM-E.

Cell lysates and supernatants of pC2-D2prM-E and pC2-D2YPTI-prM-E were separated on 10% polyacrylamide gel and blotted on nitrocellulose membrane. Membrane was probed with pan specific anti-dengue E protein followed by rabbit anti-mouse IgG conjugated with HRP.

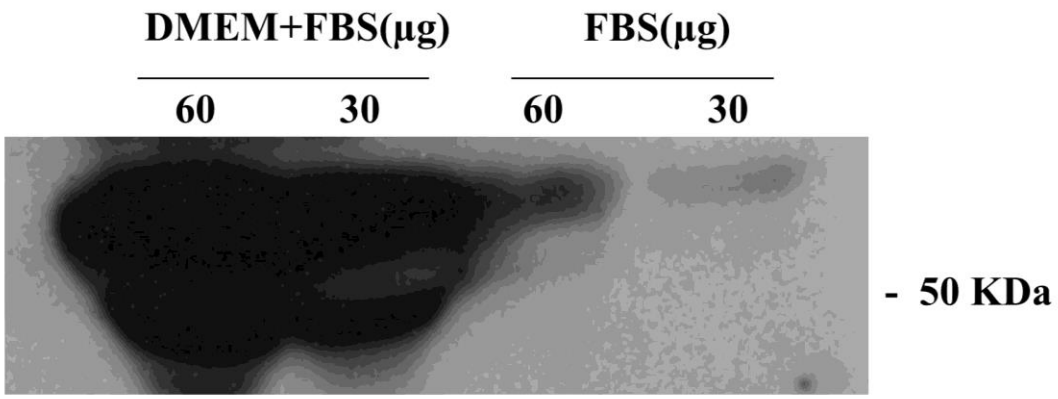

Figure 5.20 Western blot analysis using the mouse monoclonal pan specific anti-dengue E protein followed by rabbit anti-mouse IgG conjugated with HRP.

Various amounts of FBS or with DMEM media as indicated were applied on 10% SDS-PAGE prior to Western blot analysis. It demonstrated a cross reaction of antibody with DMEM+10%FBS complex causing the difficulty in detection of DENV-2 E protein releasing in the supernatant.

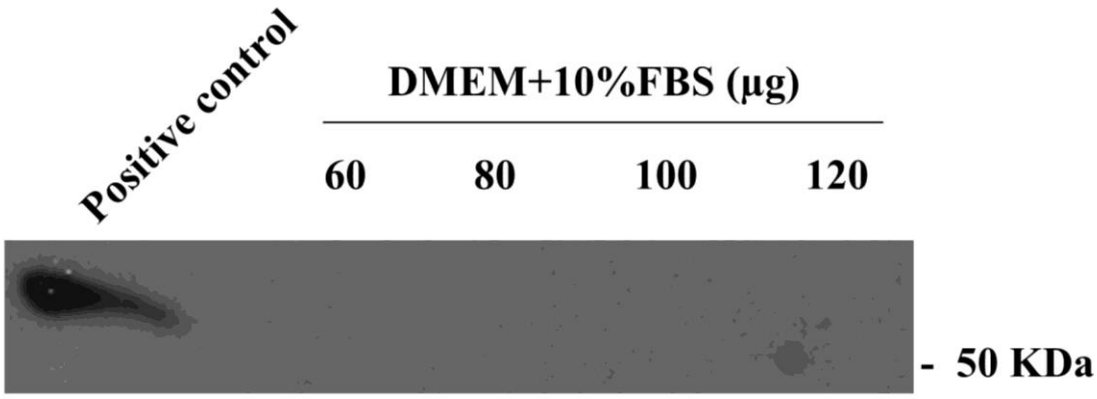

Figure 5.21 Western blot analysis using the mouse monoclonal pan specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP.

Various amounts of DMEM+10%FBS media as indicated were applied on 10% SDS-PAGE prior to Western blot analysis. Cell lysate of HEK293T infected with DENV-2 was used as positive control.

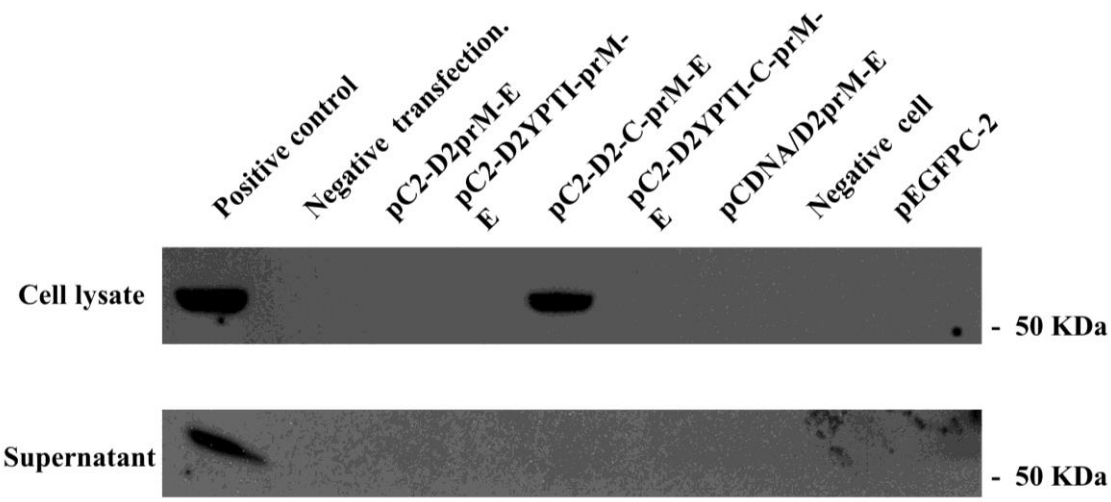

Figure 5.22 Western blot analysis to detect E protein in the cell lysate and supernatant of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E, pcDNA hygro+/D2prM-E and pEGFPC-2 plasmid transfected cells at day 1 post transfection.

Cell lysate and supernatant of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E, pcDNA hygro+/D2prM-E and pEGFPC-2 were collected at day 1 post transfection. Negative controls included negative transfection and negative cells which were HEK293T transfected with no plasmid and HEK293T without transfection, respectively. Expected band size of E protein is approximately 50 KDa.

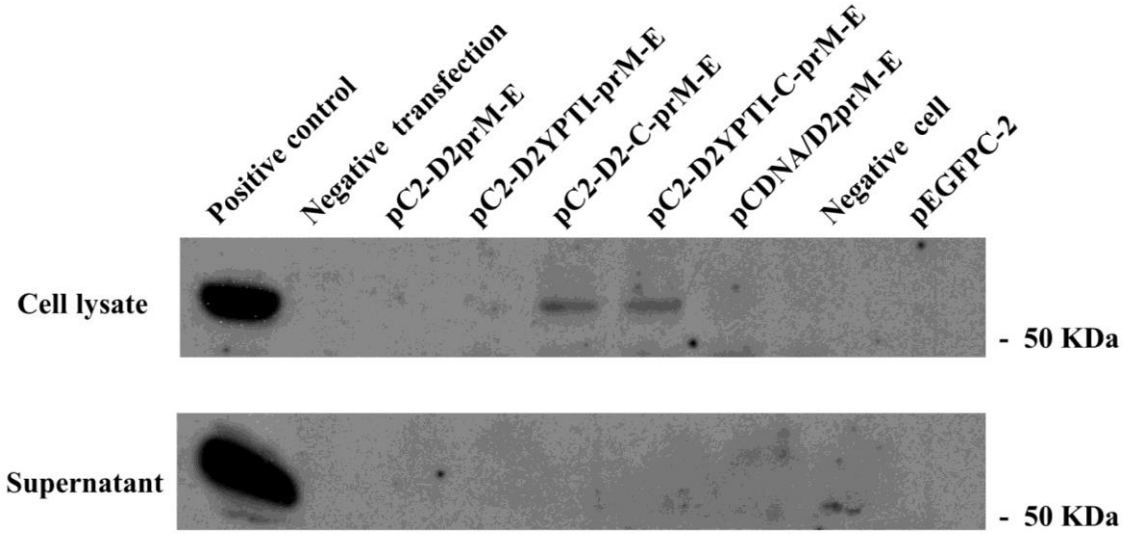

Figure 5.23 Western blot analysis to detect E protein in the cell lysate and supernatant of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E, pcDNA hygro+/D2prM-E and pEGFPC-2 plasmid transfected cells at day 2 post transfection.

Cell lysate and supernatant of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E, pcDNA hygro+/D2prM-E and pEGFPC-2 plasmid transfected cells at day 2 post transfection. Negative controls included negative transfection and negative cells which were HEK293T transfected with no plasmid and HEK293T without transfection, respectively. Expected band size of E protein is approximately 50 KDa.

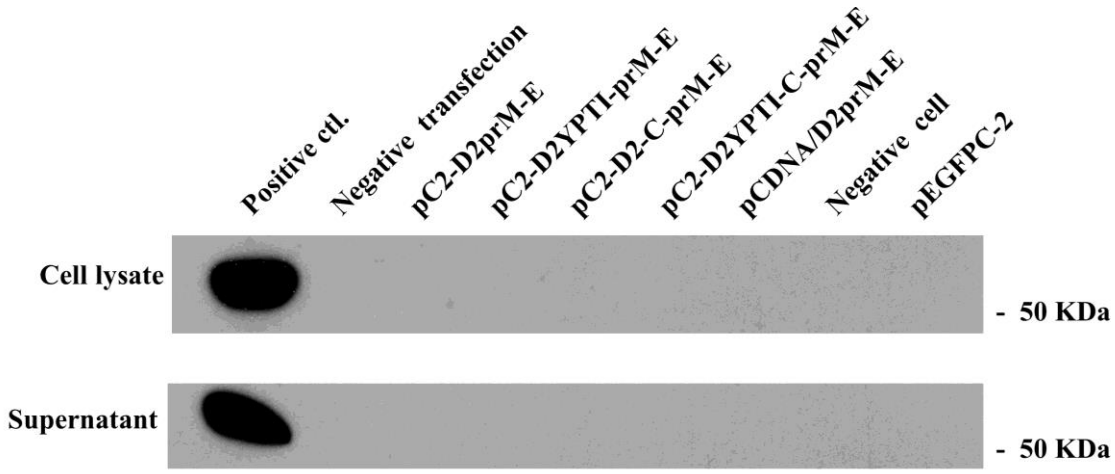

Figure 5.24 Western blot analysis to detect E protein in the cell lysate and supernatant of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E,pcDNA hygro+/D2prM-E and pEGFPC-2 plasmid transfected cells at day 3 post transfection.

Cell lysate and supernatant of pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E, pcDNA hygro+/D2prM-E and pEGFPC-2 plasmid transfected cells at day 3 post transfection. Negative controls included negative transfection and negative cells which were HEK293T transfected with no plasmid and HEK293T without transfection, respectively. Expected band size of E protein is approximately 50 KDa.

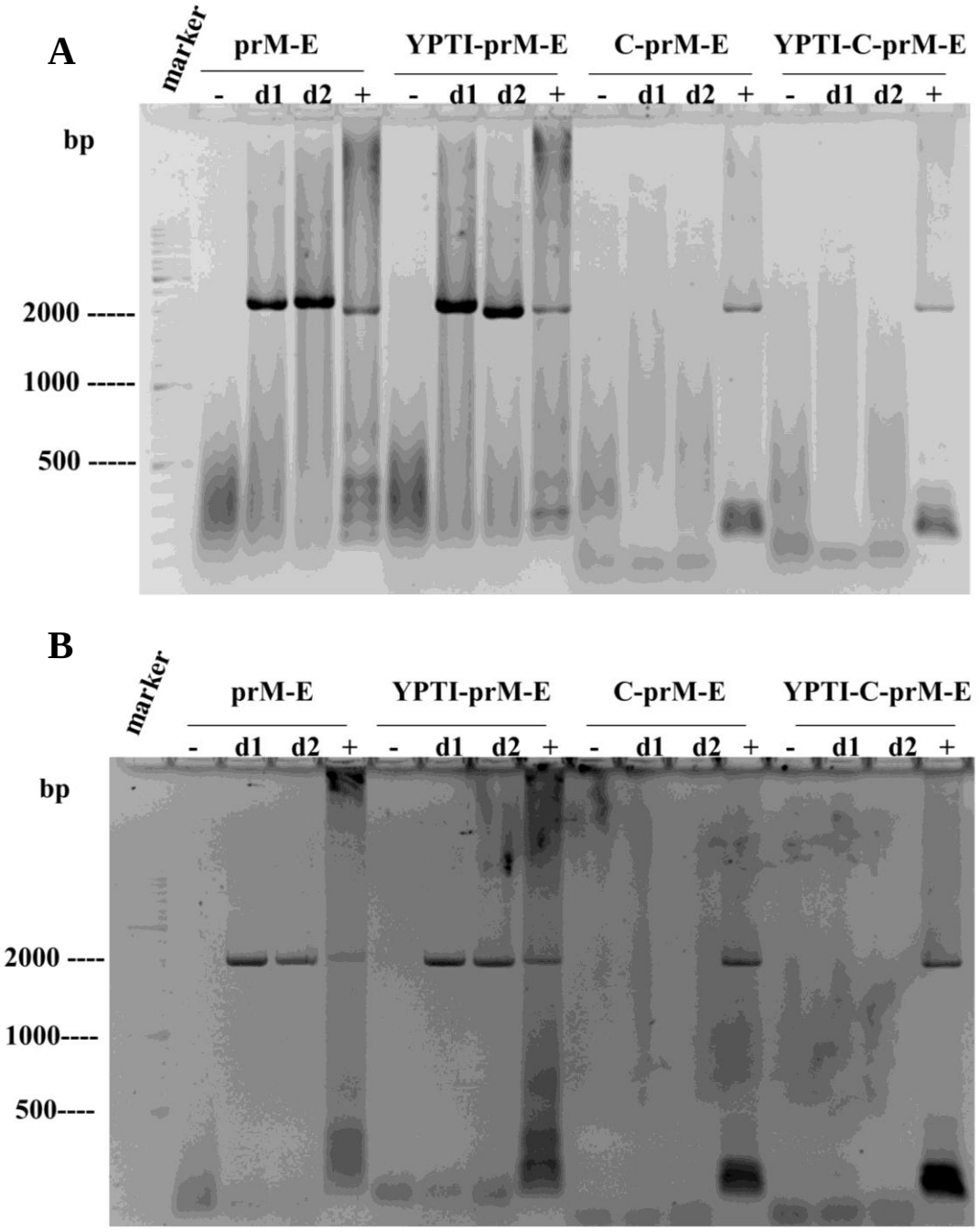

Figure 5.25 PCR detection of DENV-2 VLPs plasmids from total DNA (A) and episomal DNA (B) of the transfected cells.

Episomal and total DNA were extracted from VLPs transfected cell. PCR reactions were carried out using the corresponding primer sets. Lane 1 represents to negative control. Lanes 2 and 3 represent to DNA pellet which were collected in day 1 and day 3, respectively and lane 4 represents to positive control which DENV-2 gene fragment was used as a template.

conjugated with HRP to observe non-specific signal. In this experiment, supernatant from negative transfection and supernatant from DENV-2 infected cells were used as negative and positive control, respectively. The first membrane showed higher background and higher intensity of signals than the control membrane in all of pC2-D2-C-prM-E, pC2-D2YPTI-C-prM-E and positive samples. In case of supernatant from pC2-D2-C- prM-E and pC2-D2YPTI-C-prM-E transfection, they showed the similar level intensity of E protein dot when observed by eyes **(Figure 5.26)**.

5.3 Stable transfection

The concentration of G418 in stable transfection were optimized. HEK 293 cells were preseeded in 24 well plate at 50% confluence. After that, HEK 293 cells were treated with G418 in range of 500 – 2000 µg/ml in triplicate. Morphological changes and cell death were observed for 10 days. Fresh selective media were replaced every 3 days for each concentration. All treated cells in each well were observed daily under microscope. The result showed that 1500 µg/ml of G418 was the least concentration that made 100% untransfected cell die at day 4 post treatment (**Figure 5.27**). So this concentration was used in DENV-2 VLPs transfected cell selection in stable transfection. After that, pC2-D2-C-prM-E and pC2-D2YPTI-C-prM-E were transfected to HEK 293 cells and cultured in DMEM + 10% FBS containing 1500 µg/ml G418 for 1 month. DENV-2 E protein expression was validated by Western blot analysis and they showed band of E protein expression in cell lysate of both constructs whereas no detectable band of E protein expression in supernatants **(Figure 5.28)**.

5.3.1 Immunofluorescent assay (IFA)

The number of DENV E protein expressing cells in pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E stable transfection was estimated by IFA. The cells expressing E protein were detected with anti-dengue complex followed by chicken anti-mouse IgG conjugated with Alexa 594 and observed under Nikon TIS inverted fluorescent microscope. DENV-2 VLPs harboring cell could be stained with the

anti-dengue antibody and show red color. IFA results of pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E stable transfection are shown in **Figure 5.29**. Results showed that the pC2-D2-C-prM-E harboring cells remained approximately 20-30% in 1 (**Figure 5.29A)** and 2 months **(Figure 5.29B)** stable transfection and pC2-D2-C-YPTI-prM-E stable transfected cells gave similar result.

5.3.2 Partial protein purification

In order to detect dengue E protein in supernatants, supernatant from DENV-2 infected cells and pC2-D2C-prM-E stable transfection were concentrated by filtration and FBS was eliminated using 50,000 MWCO PES Vivaspin column prior to analyze by Western blot. SDS-PAGE from this fraction showed a huge band of FBS in concentrated sample (**Figure 5.30)** so by this method FBS could not be eliminated and it may interfere the detection the band of E protein expression by Western blot analysis as shown in **Figure 5.31**.

5.3.3 Protein precipitation by Ammonium sulfate

An alternative method to concentrate supernatant is protein precipitation. Fifty ml of supernatant from pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E were precipitated using ammonium sulfate ($(NH_4)_2SO_4$) and analyzed by Western blot as well. Western blot result showed no band of E protein expression in supernatant of both constructs but it showed E protein band in the supernatant of DENV-2 infected cells precipitated with ammonium sulfate and DENV-2 virus stock (**Figure 5.32**). Because FBS contamination might interfere the E protein detection in Western blot analysis, some of the stable transfected cells were subculture in opti-MEM containing 1500 µg/ml G418 and precipitated with ammonium sulfate again. The result showed that the band of E protein expression could not be detected in neither pC2-D2C-prM-E nor pC2-D2YPTI-C-prM-E stable transfection but it showed E protein band from opti-MEM of DENV-2 infected cells both with or without protein precipitation **(Figure 5.33).**

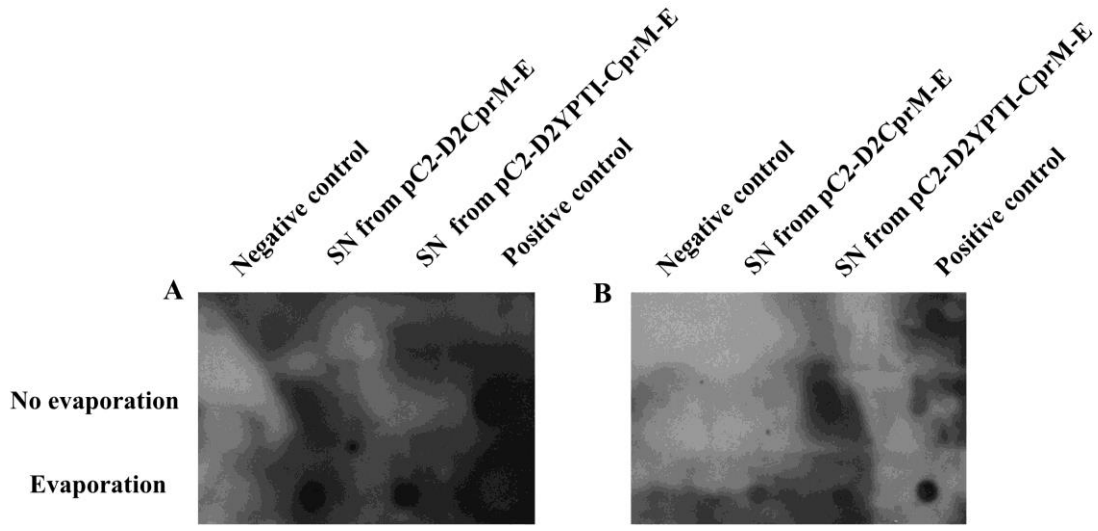

Figure 5.26 Dot blot analysis to detect DENV-2 E protein in the concentrated supernatant from pC2-D2-C-prM-E and pC2-D2YPTI-C-prM-E transfection.

Supernatants with or without evaporation were dotted onto 2 nitrocellulose membrane and probed with pan specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP (A) whereas second membrane was probed with only goat anti-mouse IgG conjugated with HRP (B). Fresh culture media and supernatant from DENV-2 infected cells were used as negative and positive controls, respectively.

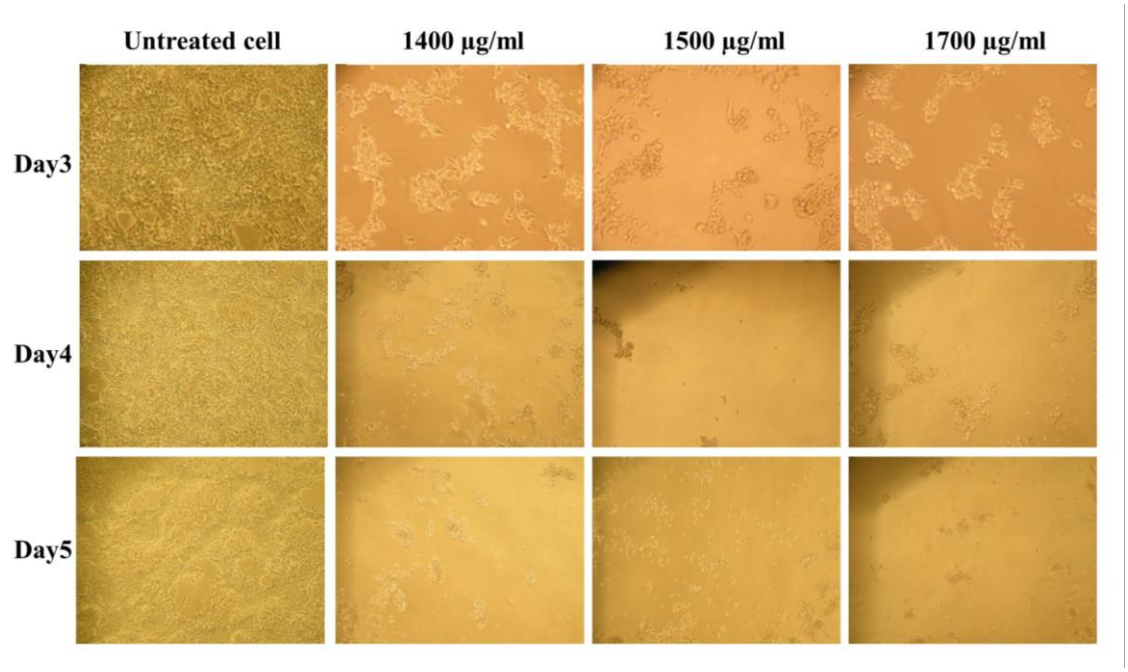

Figure 5.27 Cell morphology and survival of HEK 293 cells treated with G418 observed under light microscope.

HEK 293 cells were treated with G418 in range of 500 – 2000 mg/ml and observed for 10 days. This figure shows only the selected concentration of G418 and day of treatment.

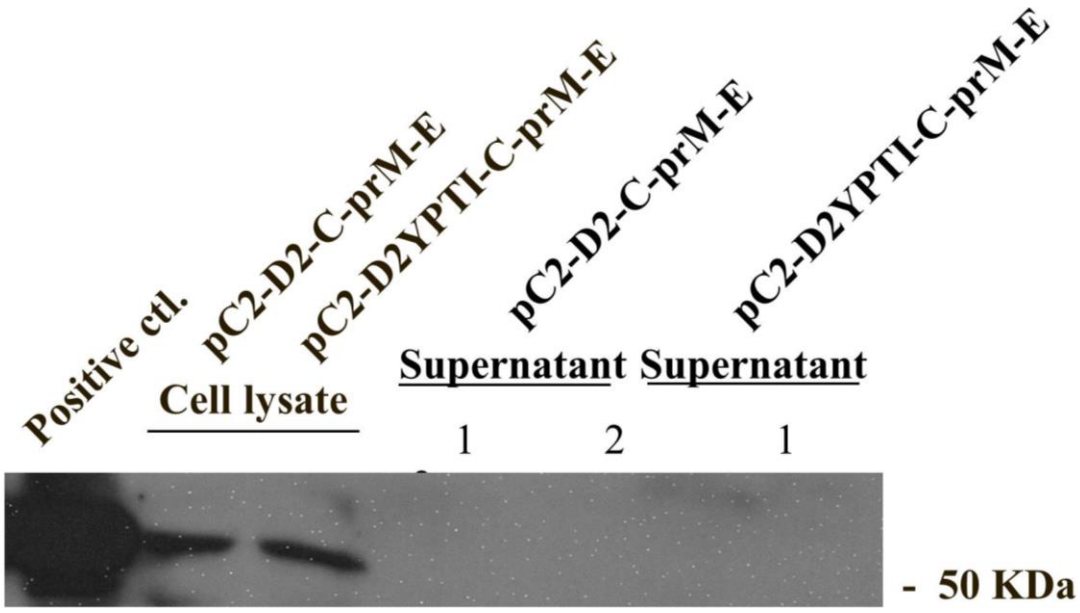

Figure 5.28 Western blot analysis to detect E protein expression of 1 month stably transfected of pC2-D2-C-prM-E and pC2-D2YPTI-C-prM-E in HEK 293 cells.

pC2-D2-C-prM-E and pC2-D2YPTI-C-prM-E constructs were transfected to HEK 293 cells and cultured in DMEM + 10% FBS containing 1500 µg/ml G418 for 1 month. DENV-2 E protein expression was validated by Western blot analysis. DENV E protein was detected by pan-specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Cell lysate of HEK29T3 infected with DENV-2 was used as positive control. Expected band size of E protein is approximately 50 KDa.

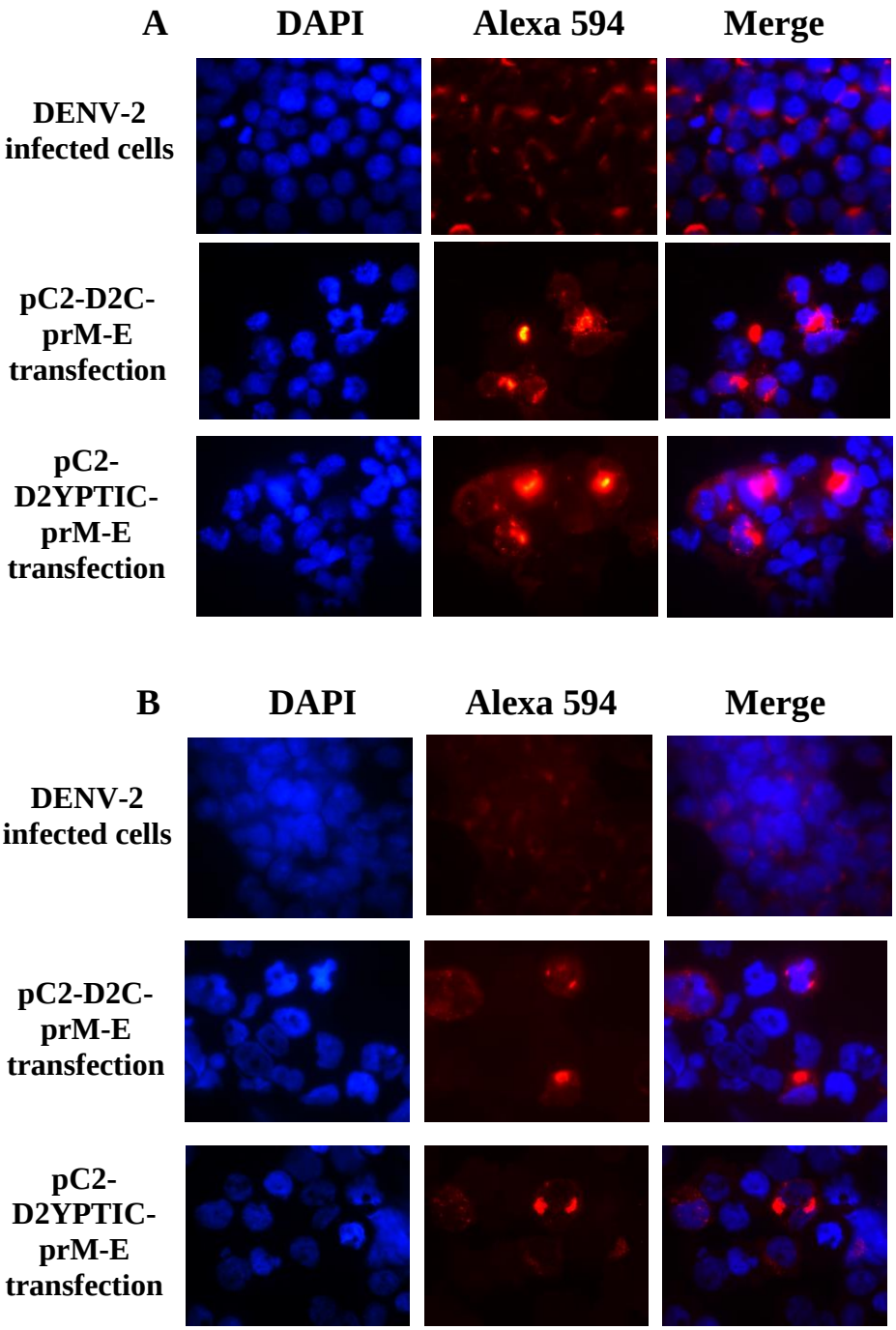

Figure 5.29 The number of cells harboring pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E for stable transfection was estimated by IFA at 1 month (A) and 2 month stable transfection (B).

Cells were incubated with anti-dengue complex followed by chicken anti-mouse IgG conjugated with Alexa 594 to stain E protein and showed in red color. Cell nuclei were stained by DAPI and showed in blue color.

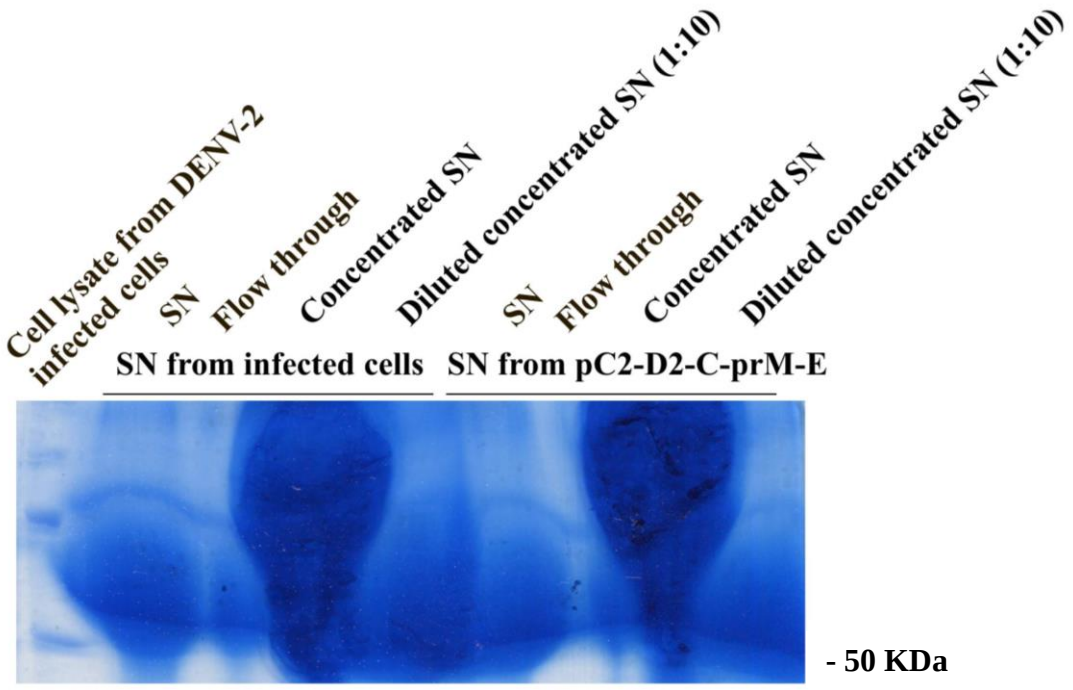

Figure 5.30 SDS-PAGE of supernatant from pC2-D2C-prM-E stable transfection after FBS elimination using 50,000 MWCO PES Vivaspin column.

Supernatant from DENV-2 infected cells was used as a control. Flow-through and concentrated supernatant were collected after spin through 50,000 MWCO PES Vivaspin column.

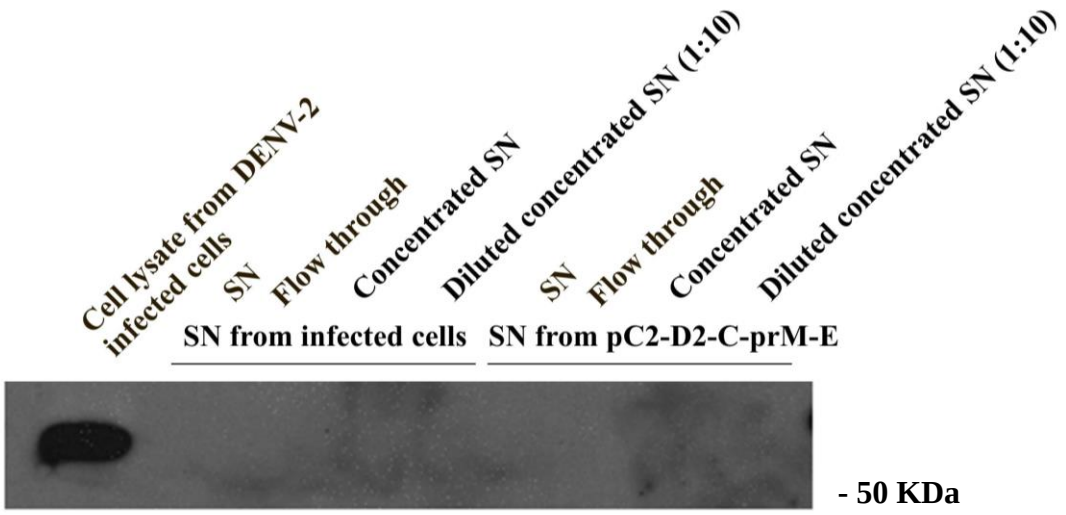

Figure 5.31 Western blot analysis to detect E protein expression in supernatant from DENV-2 infected cells and pC2-D2C-prM-E stable transfection.

Supernatant from DENV-2 infected cells was used as a control. Flow-through was collected after spin through 50,000 MWCO PES Vivaspin column to eliminate FBS. DENV E protein was detected by pan-specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Expected band size of E protein is approximately 50 KDa.

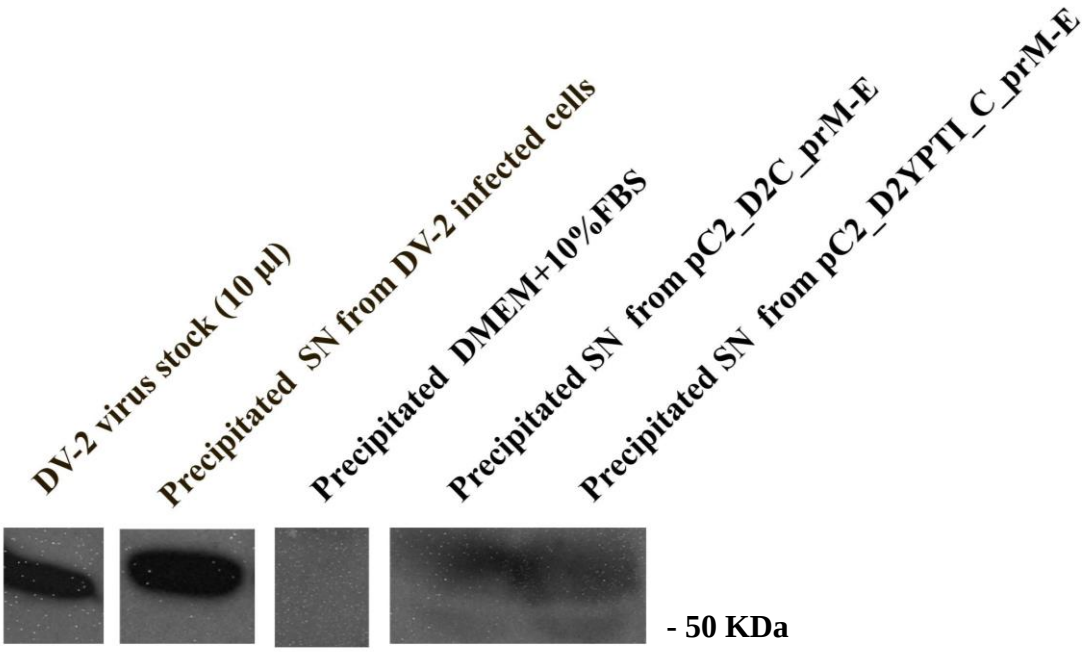

Figure 5.32 Western blot analysis to detect E protein after partial protein precipitation.

Supernatant 50 ml from pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E were precipitated using ammonium sulfate. Supernatant from DENV-2 infected cells precipitated with ammonium sulfate and DENV-2 virus stock were used as positive controls and precipitated DMEM+10%FBS was used as negative control. DENV E protein was detected by pan-specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Expected band size of E protein is approximately 50 KDa.

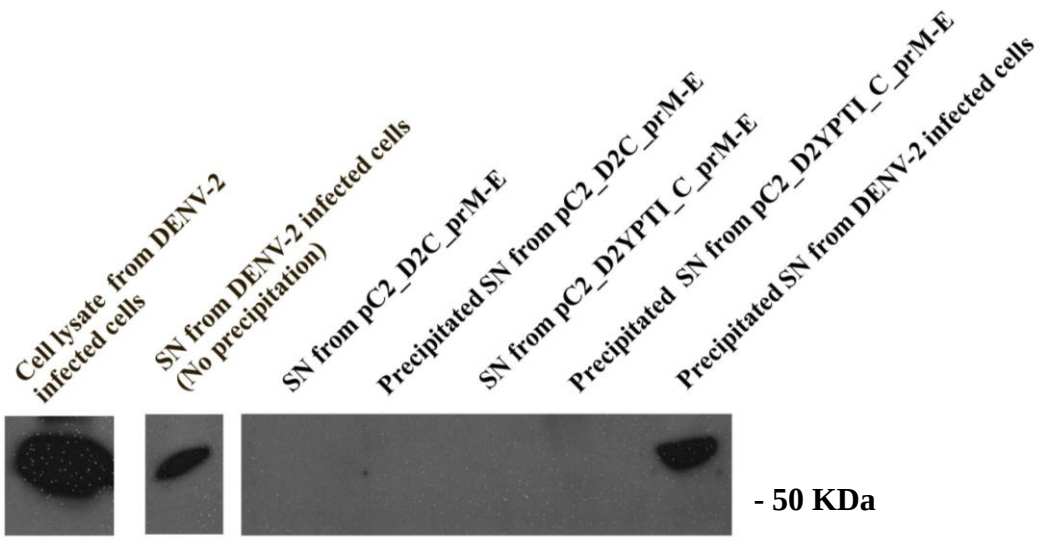

Figure 5.33 Western blot analysis to detect E protein in supernatant after protein precipitation.

Stable transfection of pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E were subcultured in opti-MEM to get rid of FBS and collected to precipitate protein using ammonium sulfate. Supernatant from DENV-2 infected cells with and without precipitation with ammonium sulfate were used as positive control. DENV E protein was detected by pan-specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Expected band size of E protein is approximately 50 KDa.

5.4 DENV-2 prM-E codon optimization

5.4.1 Immunofluorescent assay (IFA)

The number of DENV E protein expressing cells in pcDNA-D2opt.prM-E was estimated by IFA. Cells were incubated with anti- dengue complex followed by chicken anti- mouse IgG conjugated with Alexa 594 and observed under Nikon TIS Inverted fluorescent microscope. DENV-2 VLPs harboring cell could be stained with the anti-dengue antibody and show red color of E protein staining. IFA results of pcDNA-D2opt.prM-E transfection are shown in Figure 5.34. Percentage of pcDNA-D2opt.prM-E harboring cells were approximately 20-30% which was approximately the same as pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E and pcDNA-D2opt.prM-E (Figure 5.35).

5.4.2 Western blot analysis and protein precipitation by ammonium sulfate

Optimized construct 5 µg were transfected to 5 x 10^5 of HEK 293 cells in 12 well plate and harvested at day 1, 2 and 3 post transfection. From this experiment, opti-MEM was used to culture transfected cells instead of DMEM + 10% FBS to eliminate FBS contamination. The Western blot results showed that no detectable band of E protein expression in both of cell lysate and supernatant from pcDNA-D2opt.prM-E transfected cells in all 3 day post transfection as shown in **Figure 5.36**. To concentrate protein in supernatant, 8 µg of pcDNA-D2opt.prM-E were transfected to 4x10^6 HEK293 cells in 60 mm^2 culture dish. Supernatant was precipitated using ammonium sulfate ($(NH_4)_2SO_4$) and analyzed by Western blot as well. The result showed no band of E protein expression in supernatant but it showed E protein band in ammonium sulfate precipitation from supernatant of DENV-2 infected cells (**Figure 5.37**).

5.4.3 Immunoprecipitation (IP)

Immunoprecipitation was performed to concentrate and precipitate DENV-2 E protein from supernatant using specific antibody against DENV E protein. Five µg of pcDNA-D2opt.prM-E was transfected to HEK 293 cells. Opti-MEM from

transfected cells were collected for 3 days after transfection. Protein G sepharose beads were used to preclear the collected supernatants to get rid of non-specific binding between specific antibody and others proteins. Then, 500 µl of supernatant from day 1- 3 post transfection was pulled down by pan-specific anti-dengue E protein prior to forming the complex with protein G. Supernatants from transfected cells were incubated with or without this antibody. After washing step, protein in supernatant which formed complex with protein G and specific antibody was eluted and separated on 10% polyacrylamide gel. Proteins in the gel were transferred to nitrocellulose membrane. E protein detection in this experiment was detected by the same specific antibody that was used in pull-down step. The result showed that only E protein in supernatant from DENV-2 infected cells could be pulled down whereas no detectable band of E protein in opti-MEM from pcDNA-D2opt.prM-E transfected cells in all 3 days post transfection **(Figure 5.38)**. This IP was used to detect DENV-2 E protein from supernatants of pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E transfected cells. The result showed no detectable band of E protein in supernatants after precipitation. The big band was the heavy chain of antibody used in pulled down step **(Figure 5.39).**

5.4.4 Detection of protein with non-reducing loading dye and acetone precipitation

Since there was a big problem in detection of DENV E protein by Western blot analysis, protein samples were tried to analyze in their native form by using non-reducing buffer instead of reducing loading buffer. Because non-reducing buffer is the loading buffer without reducing agents so it preserves their native structure of protein. C400 Visible Fluorescent Western Imaging System (Azure biosystems, Dublin, CA, USA) was used in detection step of this experiment. In the same time, saturated amount of ammonium sulfate caused a bent protein band affecting protein migration, acetone was used instead to precipitate E protein from the supernatant. After precipitation, remaining acetone was allowed to completely evaporate by uncapping the tube so acetone would not interrupt protein migration during gel electrophoresis. Five ml of supernatant from infected cells was precipitated with acetone. From the result, an obvious band of protein showed in precipitated supernatant loaded with

non-reducing loading dye. The intensity of E protein was substantially decreased when more concentration of DTT was added in supernatant from infected cells **(Figure 5.40)**.

5.4.5 Protein precipitation by acetone

To confirm the E protein production in the supernatant was not from detached floating cells, supernatant from the pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E transfected cells were centrifuged to excluded all detach cells prior to precipitated with acetone. The result in **Figure 5.41A** showed band of E protein in both of cell lysate and supernatant at day 2 after 16 µg of pcDNA-D2opt.prM-E transfection and it substantially increased in 3 day post transfection **(Figure 5.41B)**. The same method was used with pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E which contained DENV-2 native signal sequence but no band of E protein expression was detectable in precipitated supernatant from the transfected cells as shown **in Figure 5.42.**

5.4.6 Optimization of the VLPs concentration for transfection

The amount of transfected VLPs plasmids per cells was optimized to find the optimal amount of plasmid per cells which could increase the expression of E protein in both of cell lysate and supernatant. Various concentration of pcDNA-D2opt.prM-E which were 2.5, 8 and 16 µg, were transfected to 1.8 x 10^6 HEK 293 cells. Cell lysate and supernatant were collected at day 3 post transfection. From **Figure 5.43**, E protein could be detected in both cell lysate and supernatant of all transfection condition. However, 8 ug of plasmid transfection showed the highest yield whereas increasing amount of plasmid to 16 µg did not improve E protein expression. So the optimal transfection condition is 8 ug plasmids / 1.8 x 10^6 HEK 293 cells at day 3 post transfection.

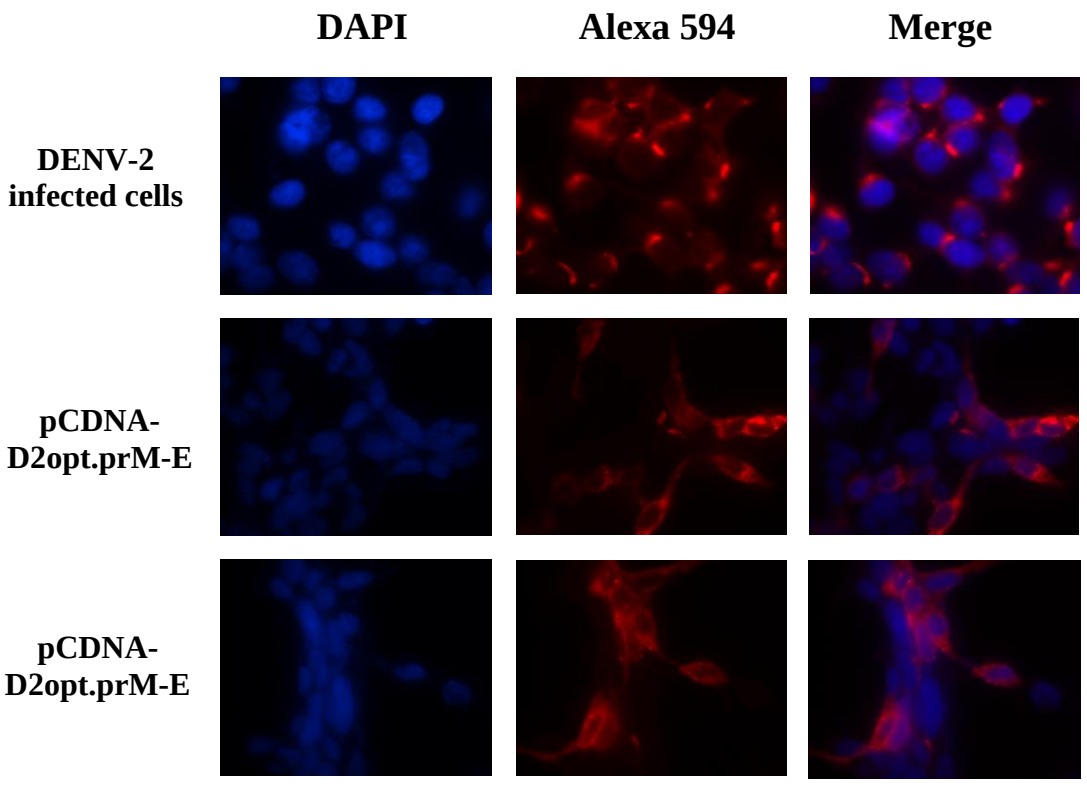

Figure 5.34 IFA results of pcDNA-D2opt.prM-E were observed to estimate the number of cells harboring DENV-2 VLPs.

Cells were incubated with anti-dengue complex followed by chicken anti-mouse IgG conjugated with Alexa 594 to stain E protein and showed in red color. Cell nuclei were stained by DAPI and showed in blue color.

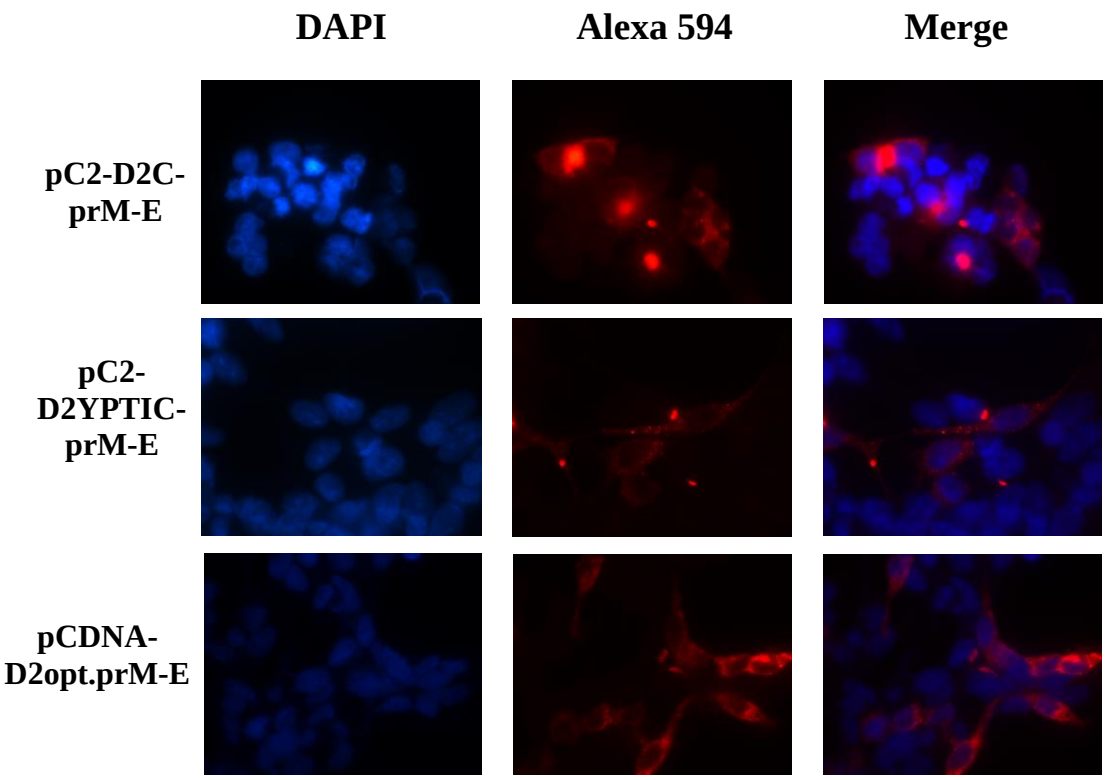

Figure 5.35 IFA results of pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E and pcDNA-D2opt.prM-E

IFA results of pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E and pcDNA-D2opt.prM-E were observed to estimate the numbers of cells harboring DENV-2 VLPs. Cells were incubated with anti-dengue complex followed by chicken anti-mouse IgG conjugated with Alexa 594 to stain E protein and showed in red color. Cell nuclei were stained by DAPI and showed in blue color.

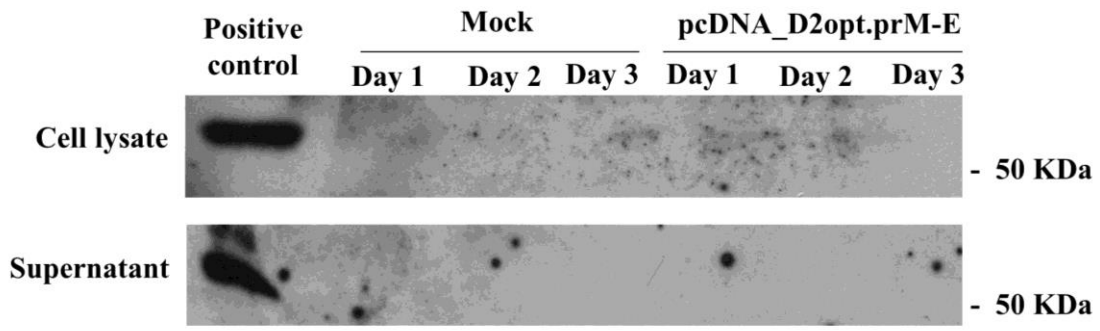

Figure 5.36 Western blot result of 5 µg pcDNA-D2opt.prM-E transfection. The construct was transfected to 5 x 10^5 of HEK 293 cells in 12 well plate and harvested at day 1, 2 and 3 post transfection.

E protein in cell lysate and supernatant from pcDNA-D2opt.prM-E transfection were detected by the pan-specific anti- dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Cell lysate and supernatant of HEK293 infected with DENV-2 were used as positive control. Expected band size of E protein is approximately 50 KDa.

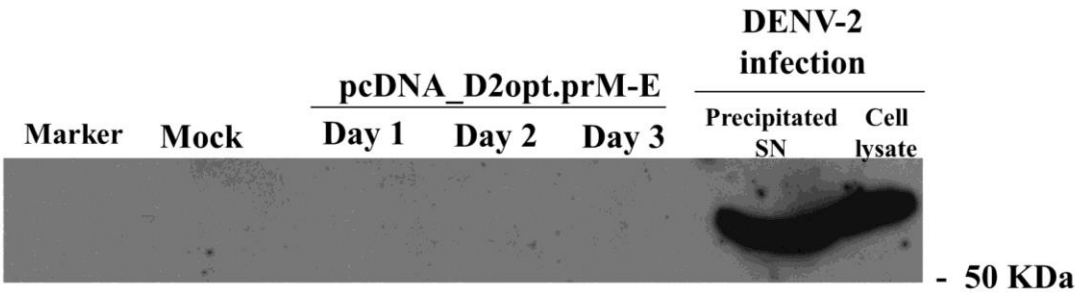

Figure 5.37 Western blot analysis of 8 µg pcDNA_D2opt. prM-E transfection. The construct was transfected to 4 x 10^6 of HEK 293 cells in 60 mm^2 plate and harvested at day 1, 2 and 3 post transfection.

E protein in cell lysate and supernatant from pcDNA-D2opt.prM-E transfection were detected by the pan-specific anti- dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Precipitated supernatant and cell lysate from DENV-2 infected cells were used as positive control. Expected band size of E protein is approximately 50 KDa.

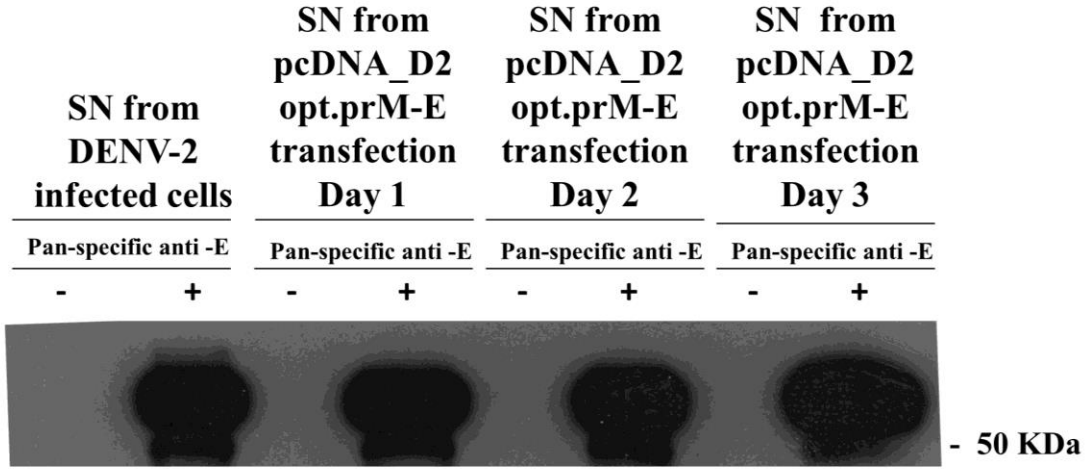

Figure 5.38 Immunoprecipitation of supernatant from pcDNA-D2opt.prM-E transfection.

Five hundred µl of supernatant from pcDNA-D2opt.prM-E transfected cells were collected at 3 days after transfection and pulled down with or without pan-specific anti-dengue E protein (Negative control). E protein detection in this experiment was detected by the same specific antibody that was used in pull-down step followed by goat anti-mouse IgG conjugated with HRP. Supernatant from DENV-2 infected cell was used as positive control.

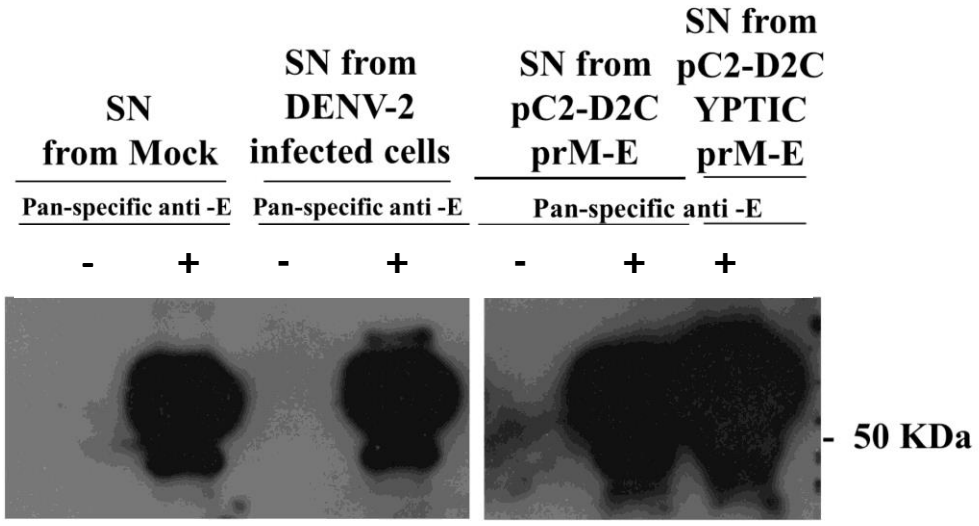

Figure 5.39 Immunoprecipitation of supernatant from pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E transfection.

Five hundred µl of supernatant from pcDNA-D2opt.prM-E transfected cells was pulled down with or without pan-specific anti-dengue E protein (negative control). E protein detection in this experiment was detected by the same specific antibody that was used in pull-down step followed by goat anti-mouse IgG conjugated with HRP. Supernatant from DENV-2 infected cells was used as positive control.

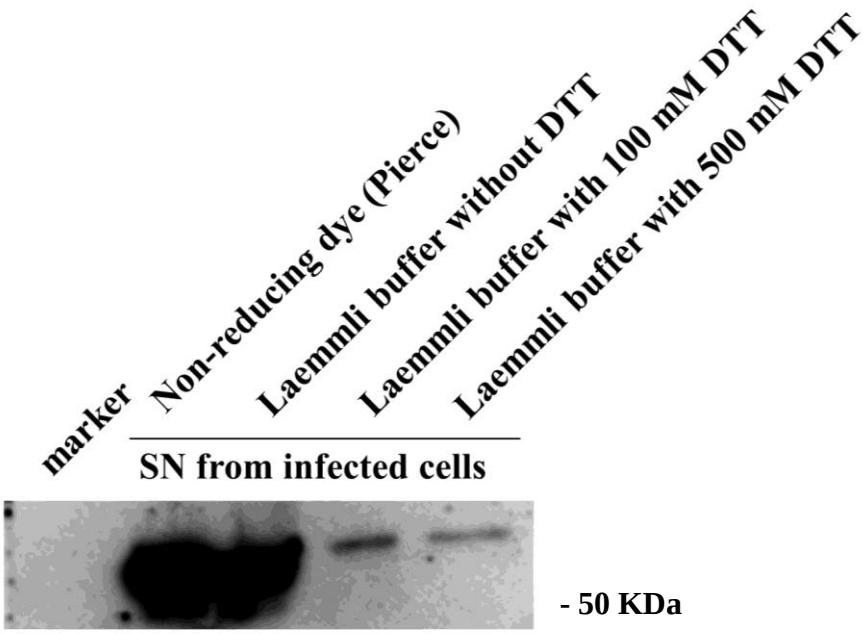

Figure 5.40 Western blot analysis of the acetone precipitated sample using non-reducing loading dye.

Five ml of supernatant from infected cells was precipitated with acetone and they were separated on 10% polyacrylamide gel using non-reducing or reducing loading buffer as indicated. E protein was detected by the pan-specific anti- dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Expected band size of E protein is approximately 50 KDa.

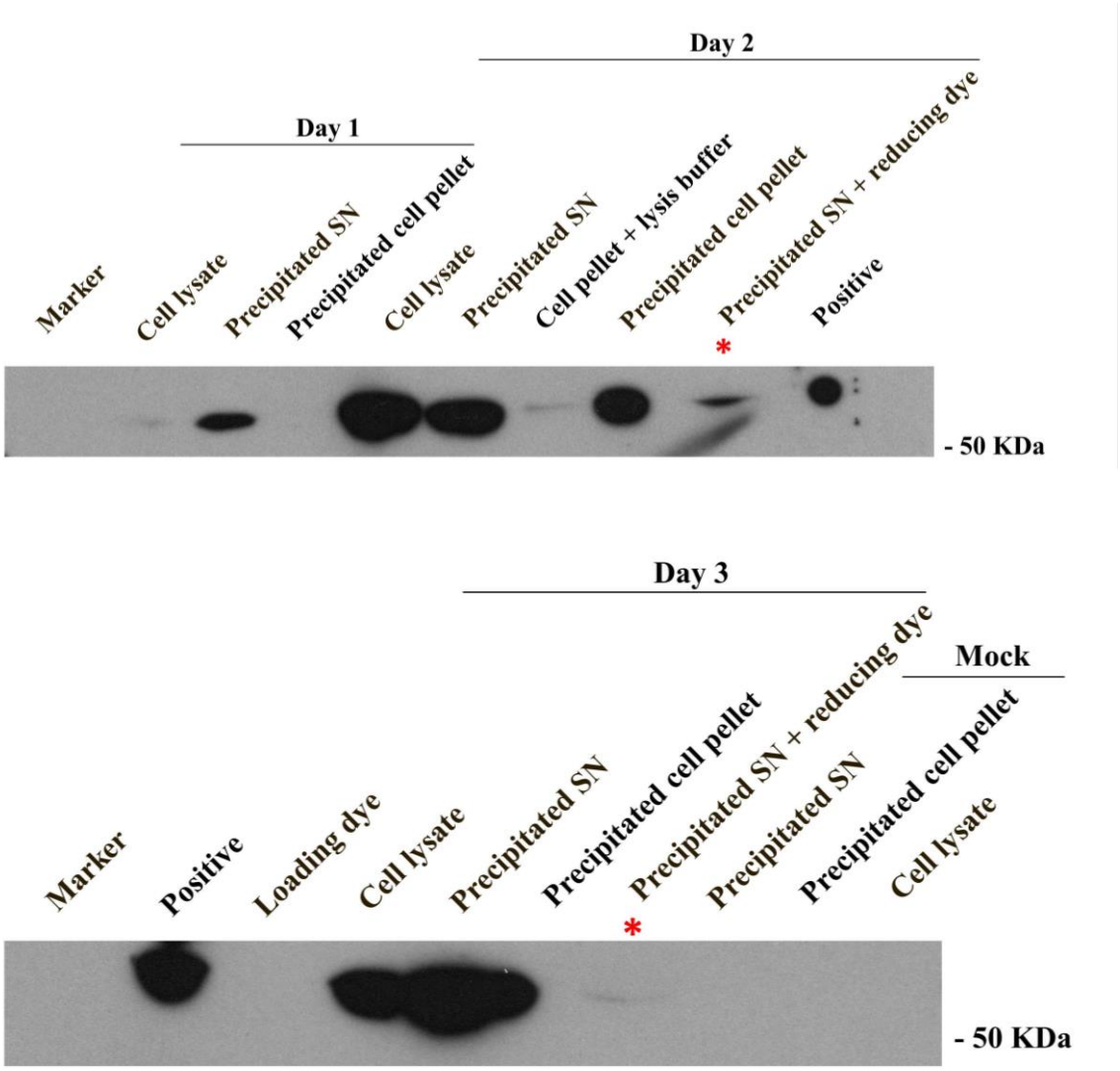

Figure 5.41 Western blot analysis to detect E protein from supernatant of pcDNA-D2opt.prM-E transfection.

Supernatant and cell pellet after centrifugation from pcDNA-D2opt.prM-E transfected cell at day 1, 2 **(A)** and 3 **(B)** post transfection were precipitated with acetone. Sample loading buffer was non-reducing loading buffer except for * which 5x SDS loading buffer containing 0.5M DTT was used. Precipitated supernatant from DENV-2 infected cells was used as control. DENV E protein was detected by pan specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Expected band size of E protein is approximately 50 KDa.

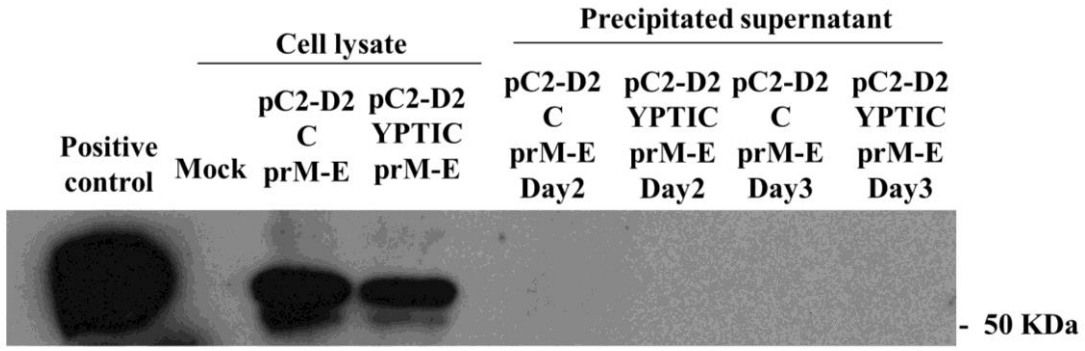

Figure 5.42 Western blot analysis to detect E protein from supernatant of 16 µg of pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E transfection in both of cell lysate and precipitated supernatant.

Cell lysate and precipitated supernatant of 16 µg of pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E transfection were run on 10% SDS-PAGE. Precipitated supernatant from DENV-2 infected cells by acetone used as control. DENV E protein was detected by pan specific anti-dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Expected band size of E protein is approximately 50 KDa.

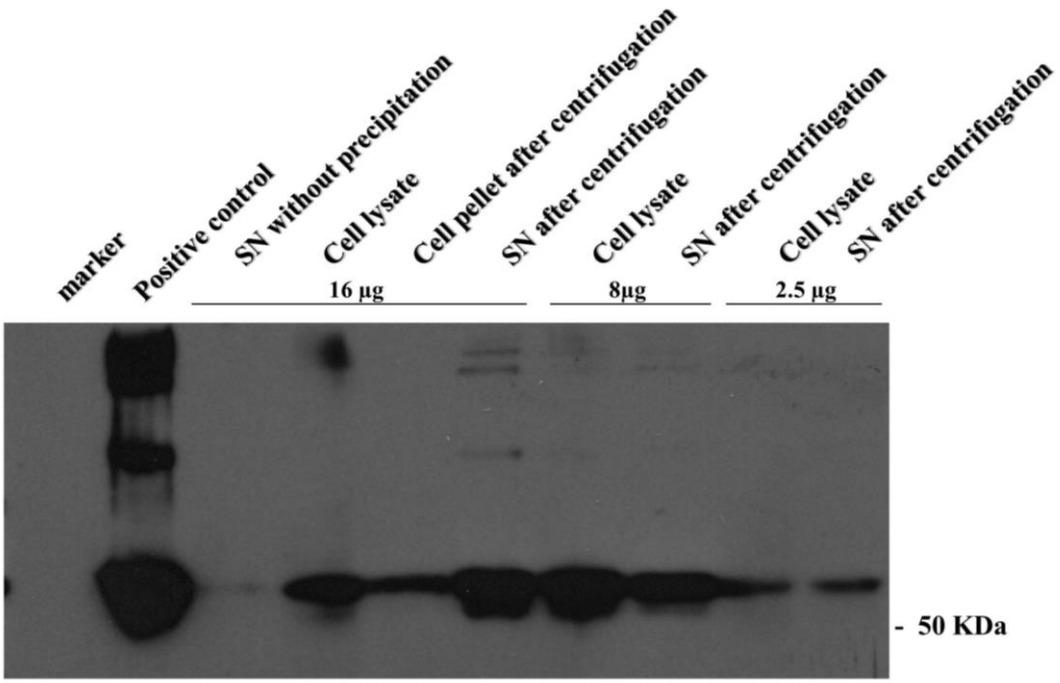

Figure 5.43 Optimization of the amount of transfected VLPs plasmids per cells. Various amounts of pcDNA-D2opt.prM-E was transfected to 1.8 x10^6 HEK 293 cells.

Cell lysate and supernatant of 2.5, 8,16 µg of transfected VLPs were collected at day 3 post transfection. DENV E protein was detected by pan specific anti dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Expected band size of E protein is approximately 50 KDa. Cell lysate from DENV-2 infected cells was used as control.

5.5 Effect of lipid droplet formation in DENV-2 VLPs transfected cells

5.5.1 Determination of lipid droplet accumulation in transfected cells by Oil Red O staining

Because oleic acid could stimulate lipid droplet formation in cell culture, the amount of lipid droplet formation in the transfected cells with and without 100 µM oleic acid treatment was estimated using Oil Red O staining by measuring an OD490 of the Oil Red O dye elution from transfected cells. From **Table 5.1,** the OD490 values from elutions with or without 100 µM oleic acid treatment are shown for both of triplicate samples and mean values. For 100 µM oleic treatment, it showed 0.956 ± 0.02 OD value and it was almost 2 times increasing when compared with no oleic acid treatment that showed only 0.585± 0.05 OD value. Therefore, oleic acid treatment could increase the lipid accumulation in the transfected cells.

5.5.2 E protein expression of the transfected cells with or without oleic acid treatment

Cell lysates from 8 µg pcDNA-D2opt.prM-E transfection in 1.8 x 10^6 HEK 293 cells with or without 100 µM oleic acid treatment were collected at day 3 post transfection. Western blot analysis was used to detect E protein expression as the protocol mentioned above. The result showed higher intensity of E protein band in the transfection with oleic acid treatment than that of the transfection without oleic acid treatment **(Figure 5.44)**. After that, the intensity of E protein band was quantitated using Quantity One software. E protein expression was normalized with actin expression. From **Figure 5.45,** E protein expression in pcDNA-D2opt.prM-E transfected cells with 100 µM oleic acid treatment was significantly increased when compared with the transfected cells without oleic acid treatment. Data is shown as a bar graph of mean ± SD value. Student's T-test (independent sample) was used to compare and to assess statistical analysis. Significant value was set at $p < 0.05$ (*).

Table 5.1 Optical density value at 490 nm (OD490) of eluted Oil Red O dye from the transfected cells with or without 100 µM oleic acid treatment.

Samples	OD490 values	Mean value ± SD
No oleic acid	0.638	0.585 ± 0.05
	0.518	
	0.598	
100 µM oleic acid	0.978	0.956 ± 0.02
	0.946	
	0.944	

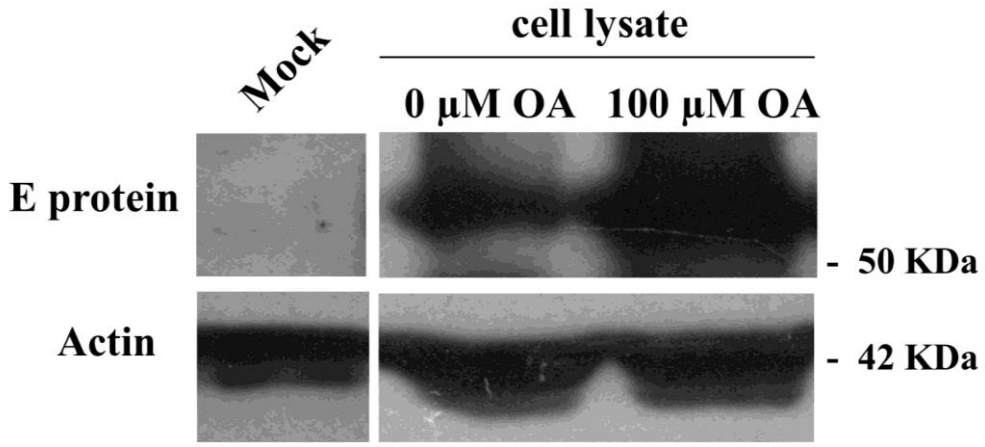

Figure 5.44 E protein expression of the pcDNA-D2opt.prM-E transfection between with or without 100 µM oleic acid treatment.

Cell lysates from 8 µg pcDNA-D2opt.prM-E transfection in 1.8 x 10^6 HEK 293 cells with or without 100 µM oleic acid treatment were collected at day 3 post transfection. DENV E protein was detected by pan specific anti dengue E protein followed by goat anti-mouse IgG conjugated with HRP. Actin was detected by goat polyclonal IgG anti actin followed by rabbit anti goat IgG conjugated with HRP. Expected band size of E protein and actin are approximately 50 and 42 KDa, respectively. OA stands for oleic acid.

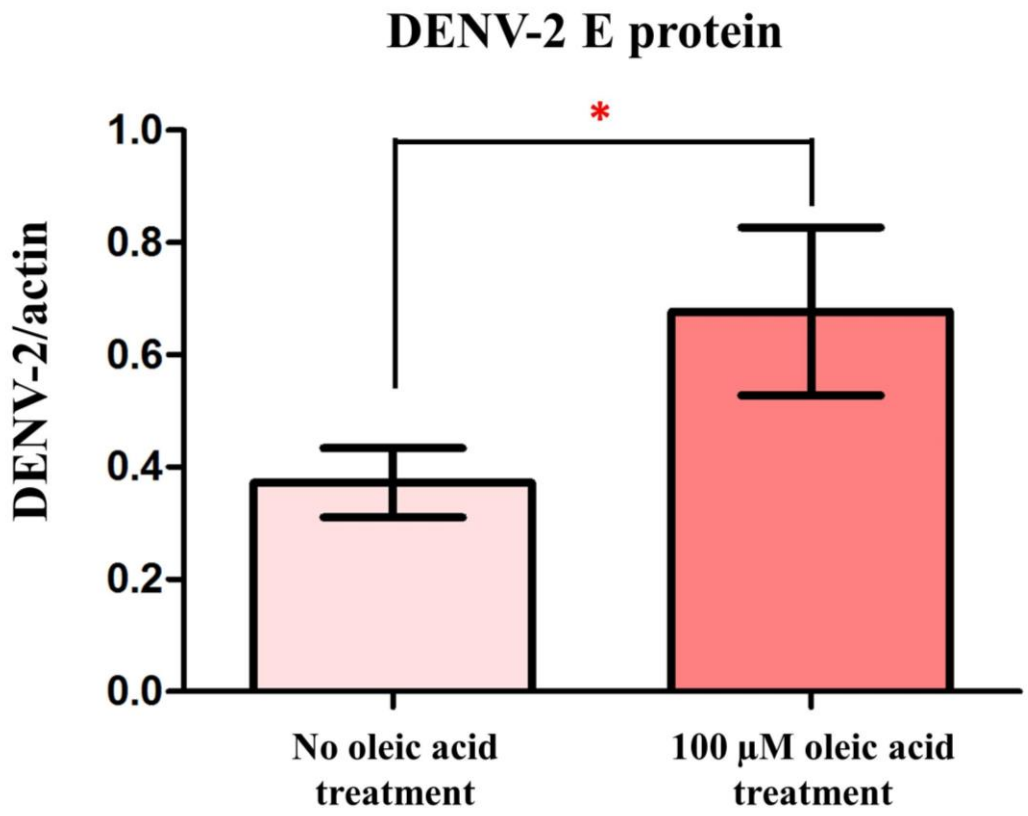

Figure 5.45 Bar graph of relative E protein expression in the pcDNA-D2opt.prM-E transfected cell lysates with or without 100 µM oleic acid treatment.

E protein expression of pcDNA-D2opt.prM-E transfected cells was shown as bar graph of the mean ± SD value. Student's T-test (independent sample) was used for statistical analysis. Asterisk represents significant difference ($p < 0.05$).