

Codon optimization of the prM-E coding region generates stable genome-length cDNA clone for a chimeric dengue 2/3 virus that can be propagated in *Escherichia coli*

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Abstract In an effort to generate a chimeric dengue virus containing the prM-E sequence from DENV-3 and with the rest of the viral genome derived from a DENV-2 strain 16681-3pm by *in vitro* ligation of subgenomic fragments, it was found that the ligated chimeric genome could not be stably propagated as genome-length cDNA clone in *Escherichia coli*. Native DENV-3 prM-E coding region may contain certain nucleotide sequence that results in plasmid instability when amplified in *E. coli*. In this study, we modified codons in the DENV-3 viral sequence into those that are most frequently used in human genome. Employing the Gibson assembly method, the modified prM-E fragment was introduced into the full-length cDNA clone of strain 16681-3pm to replace the corresponding sequence in a DENV-2 genome. The full-length plasmid cDNA clone intended for the generation of a chimeric virus, D3 prMEopt/16661-3pm, was successfully propagated in *E. coli*. *In vitro* transcription of this clone produced full-length RNA transcripts that generated infectious viruses when introduced into cultured mammalian cells. Based on kinetics experiments, the replication of D3 prMEopt/16661-3pm with modified codons in the prM-E gene was comparable to the one generated by the *in vitro* ligation approach. By combining the use of modified codons and the Gibson assembly method, a full-length cDNA clone of a chimeric virus containing the DENV-3 prM-E sequence can be stably propagated in *E. coli*, facilitating further manipulation of the viral sequences. Availability of such an infectious cDNA clone would provide a valuable tool for investigating the role of nucleotide/amino acid variations in the DENV-3 prM-E gene in viral replication, immunogenicity, and pathogenicity as well as accelerating dengue vaccine development. **Chiang Mai Medical Journal 2017;56(3): 125-36.**

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Introduction

Dengue virus (DENV) is a mosquito-borne pathogen that is widespread in tropical and

subtropical regions, with approximately 390 million dengue infection cases each year (1).

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At least four antigenically distinct serotypes, DENV-1 to DENV-4, have been found (2). Infection by any of the dengue serotypes can cause similar consequences in humans. The majority of infected individuals have either no symptoms or a mild self-limiting illness (undifferentiated fever or classical dengue fever); however, a few cases may develop a severe life-threatening disease (dengue hemorrhagic fever, either with or without organ impairment) that may require intensive medical care (3). Currently, there is no efficient specific anti-viral drug available for treatment. Along with vector control, dengue vaccine is one of the two most promising means of prevention of that disease. There are several dengue vaccine candidates in the development pipeline. Those entering clinical trials phases include recombinant live-attenuated chimeric vaccine, protein subunit vaccine, and DNA vaccine (4). Dengvaxia, a tetravalent dengue/yellow fever chimeric vaccine, is the only dengue vaccine currently on the market. That vaccine is recommended for children, adolescents, and adults aged 9-45 years living in endemic areas. The efficacy of this vaccine against symptomatic dengue for this group was only 65.6% (5). Further development of dengue vaccine for younger age groups is still an urgent need. Better understanding and basic knowledge of the virus and its pathogenicity may aid the design of new and improved vaccine candidates.

Reverse genetics is a molecular based technology that provides an alternative means of generating a new strain of RNA virus. Resembling flaviviruses, the dengue genome comprises a single, plus-stranded RNA of about 11 kilobases in length. Once it enters a cell, the genome acts as mRNA to generate a large polyprotein that is subsequently cleaved into three structural proteins, capsid (C), premembrane/membrane (prM/M) and envelope (E), as well as into seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) (6). Similar to poliovirus, a complementary DNA (cDNA) clone representing the entire genome of flaviviruses can be cloned on a plasmid and propagated in *Escherichia coli*. This molecular cDNA clone can be used to generate infectious

virus in cultured mammalian cells (7). The availability of infectious clones allows the manipulation of the nucleotide sequences of the virus in order to gain more understanding and basic knowledge and also benefits vaccine design.

A common obstacle to this technology is often related to the instability of a plasmid carrying certain viral genome sequences in *Escherichia coli* (8-12). Several efforts have been made to minimize the problem during construction of full-length genome of flaviviruses, including the use of low copy number plasmids (7, 13, 14) and special bacterial strains (7). Some investigators have assembled full-length cDNA fragments of flavivirus by homologous recombination in yeast (12,15) or in bacterial artificial chromosome (BAC) vectors (16). Alternatively, full-length clones have been constructed using an assembly of viral cDNA fragments via *in vitro* ligation (8,9, 11,17,18).

Another approach involves the manipulation of the genetic sequence of the virus, as it is assumed that a certain natural viral sequence may contain an element deleterious to the bacteria. It has been reported that the insertion of synthetic DNA linker sequences to create redundant translational termination codons in each of the forward and reverse open reading frames at either the C-terminus of the core gene or at the C-terminus of the E gene could stabilize the full-length genome of DENV-3 in *E.coli* (19). Prior to being used as a template for *in vitro* transcription, this linker has to be removed. An alternative approach to minimize the leaky expression of viral genes by prokaryotic promoter was successfully accomplished by the introduction of the silent mutations to putative *E. coli* promoters residing in the 5' end of the genome (20).

In our laboratory, full-length infectious clones of dengue 2 16681 virus have been successfully created in a high-copy number plasmid, pBluescript II KS, with some modifications of the growth conditions of bacterial strains commonly used for cloning (21). By decreasing the growth temperature to 20-25°C and lowering the level of antibiotics, plasmid containing

dengue type 2 full-length cDNA sequence can be stably maintained. This plasmid was successfully used as a backbone for constructing D1 prME/16681-3pm and D4 prME/16681-3pm chimeric viruses (22,23). However, this strategy failed in the case of D3 prME/16681-3pm chimeric viruses. According to previous reports, the construction of a full-length cDNA clone for dengue 3 virus seems to be the most difficult among the four dengue serotypes (10, 18, 24-26). As plasmids containing DENV-3 sequences were unstable when propagated in *E. coli*, molecular cloned infectious viruses were recovered from a full-length template which was constructed by joining cDNA fragments by *in vitro* ligation (10, 18, 24, 26). As the D3 prME/16681-3pm chimeric viruses differed from other chimeric viruses only at the prM and E genes, we thus suspected that the viral-derived prM and E genes of the dengue 3 virus have a strong prokaryotic promoter. In this study, we modified the codons of these genes by switching to codons that are frequently used in humans, thus diminishing putative prokaryotic promoter(s) in this region. A molecular clone of this modified virus can be stably propagated in *E. coli*.

Methods

Cell lines and viruses

An African monkey kidney cell line, Vero (ATCC CCL-81), was maintained at 37°C in 5% CO₂ in humidified air in minimal essential medium (MEM) supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. The DENV-2 strain 03-0347 was isolated in Thailand in 2003 from a patient with dengue hemorrhagic fever. This strain and the prototype DENV-3 strain H-87 were provided by Dr. Prida Malasit, Mahidol University, Bangkok, Thailand. A DENV-2, strain 16681Nde(+), was generated from a full-length cDNA plasmid clone as previously described (27) and was used as a titration control. A plasmid containing synthetic prM and E genes of dengue type 3 virus with codons frequently used in humans was provided by Prof. Dr. Kiat Ruxrungtham, Chulalongkorn University, Bangkok, Thailand.

Prediction of *E. coli* promoter regions in the prM and E-encoding regions in DENV-3

The search for potential prokaryotic promoters was conducted using the Neural Network Promoter Prediction

program version 2.2 from the Berkeley Drosophila Genome Project website (<http://www.fruitfly.org/seqtools/promoter.html>).

Construction of a cDNA clone of dengue 2/3 chimeric viruses

Two approaches were employed to generate an infectious dengue 2/3 chimeric virus. The first approach was performed with native sequence from the DENV-3 strain 03-0347. The nucleotide sequence of contiguous prM and E coding regions was amplified from viral genomic RNA using reverse transcription-polymerase chain reaction (RT-PCR), cloned into a plasmid pBlue-script II KS, and sequenced. The prM and E genes were reamplified using D3 Bg III S (5'-GGGAGATCTGCCG CATGATCATTATGCTGATTCCAACAGCTATGGCG TTCCACTTGACTTCACGCGAT-3') and D3 Kpn2IR (5'-CCCTCCGGAATCAGCTTGTACCACGGCTCCC-3') and digested with restriction endonucleases Bgl II (nt 394) and Kpn 2I (nt 2426) before ligation into a plasmid subclone pBK 16681(165-2678) containing nucleotide sequences 165-2678 of DENV-2 16681 and a silent mutation to create a Kpn 2I recognition site at nucleotide 2426. The natural Kpn 2I recognition site at nucleotide 5557 in a full-length cDNA clone of DENV-2 3 pm backbone was removed by PCR-based site-directed mutagenesis. DENV-3 sequences were then excised by digestion with Pst I (nt 212) and Kpn 2I (nt 2426), purified, and ligated with the Pst I-Kpn 2I large fragment from the 16681-3pmΔKpn2I (5557) backbone. As this plasmid did not propagate in *E. coli*, the full-length sequence of dengue 2/3 chimeric virus was then amplified *in vitro* by long PCR using a high fidelity DNA polymerase (Phusion, Thermo Fisher Scientific) using primer T7 (5'-TAATACGACTCACTATAGGG-3') and Xba Mlu Age C (5'-CTA GAACGCGTACCGGTT-3'). In the second approach, the viral prM and E sequences of DENV-3 were substituted with codons that are frequently used in humans (28) and synthesized by a commercial supplier. The optimized prM/E sequences were amplified using primers D2-D3opt S (5'-TGATTC CAACAGTGATGGCGTTCCACCTGACCAGCAGA GA-3') and D3opt-D2 R (5'-CTCACAACGCAACAAC TATCGGCCTGCACCACGGCGC-3') while the 12 kb backbone was amplified from pBK S1SP6-10723 PDK (22) using primer 438R (5'-GGCCATCACTGTTGGA ATCA-3') and D2 244S (5'-GATAGTGGTTGCGTTGT GAG-3'). The fragments were combined at 50°C for 1 hour using a mixture of T5 exonuclease, Phusion DNA polymerase, and Taq ligase (29). The ligation mixture was transformed into DH5α and the full-length clone was selected using LB agar containing 50 µg/mL ampicillin. The bacteria containing the infectious cDNA clone were cultured at 22.5°C, as previously described (21).

***In vitro* transcription of full-length cDNA clone**

The PCR product and the infectious cDNA clone were digested at the 3' end of the genome by either Xba I or Mlu I prior to transcription. The *in vitro* transcription reaction was set up using SP6 Ribomax kit (Promega). The RNA transcript was collected after incubation at 30°C for 4 hours and stored at -70°C.

Generation of infectious virus from *in vitro* transcribed product

To generate the viruses, confluent monolayers of Vero cells in 35-mm dishes were transfected with *in vitro* transcribed RNA using Lipofectamine 2000 (Thermo Fisher Scientific), following the manufacturer's protocol. After a 4-hour incubation period, the Lipofectamine-RNA mixture was removed and the MEM maintenance medium containing 2% fetal bovine serum (FBS) was added. Culture media of transfected cells were harvested and assayed for the presence of infectious viruses by focus immunoassay titration using Vero cells (27). Virus-containing culture media were stored at -70°C in the presence of 20% FBS.

Indirect immunofluorescence assay (IFA) to detect viral antigens

Transfected cells were washed briefly with PBS, fixed with 3.7% formaldehyde in PBS for 15 minutes at room temperature, and then permeabilized with 2% Triton X-100 in PBS for 10 min at room temperature. The viral antigens were stained with an anti-DENV-3 monoclonal antibody, clone 8A1 (30). The cells were then reacted with a Cy3-conjugated goat anti-mouse IgG antibody (Invitrogen). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI).

Sequence analysis

Viral RNA was extracted from the culture supernatant of DENV-3 infected cells using a viral nucleic acid extraction kit (GeneAid) as described in the manufacturer's protocol. Viral RNAs were employed as a template for cDNA synthesis by reverse transcriptase (Revert-Aid, Thermo Fisher Scientific) and the entire prM+E coding regions were sequenced using Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Single-step replication kinetics

Eighteen hours prior to the experiment, Vero cells were seeded at 5×10^5 cells in a 35-mm tissue culture dish and incubated at 37°C in an incubator with 5% CO₂. Cells were infected with the chimeric virus at a multiplicity of infection (MOI) of 1. After two hours of viral adsorption, the inoculum was removed. The cells were washed five times with incomplete MEM medium

before 2 mL of the maintenance MEM medium with 2% FBS were added. At 0, 8, 12, 16, 20, 24, 36 and 48 hours post infection, the culture supernatants were collected and centrifuged to pellet cell debris. The clarified culture supernatant was stored at -70°C in the presence of 20% FBS prior to the quantitation of extracellular viruses. For cell-associated viruses, cells were washed five times with incomplete MEM medium and then lysed in the presence of 1 mL of MEM medium with 20% FBS by three freeze-thaw cycles. Cell lysate was clarified by centrifugation and stored at -70°C. Infectious virus titers were determined by focus immunoassay titration in Vero cells using an anti-flavivirus monoclonal antibody, clone 4G2, for the detection (27).

Multi-step replication kinetics

Confluent monolayers of Vero cells in T25 flasks were infected with viruses at an MOI of 0.01. After two hours, the inoculum was removed. Cells were then washed 5 times with incomplete MEM medium before 5 mL of maintenance MEM medium with 2% FBS were added. Culture supernatants were collected daily and assayed for infectious virus titers by using the focus immunoassay titration.

Determination of focus size

For each virus, 50 randomly-selected foci from the same titration plate were photographed. Virus-infected cells present in each focus were counted by using the Image Frame Work software (Tarosoft, Bangkok, Thailand).

Statistical analysis

The Mann-Whitney U test was employed in the comparison of infectious virus titer and focus size between D3 prME/16681-3pm and D3 prMEopt/16681-3pm. *P* values < 0.05 were considered as statistically significant.

Results

Putative bacterial promoters in DENV-3 prM- and E-coding sequences were minimized by codon optimization.

We initially failed to clone the prM-E gene of the DENV-3 strain 03-0347 into a plasmid containing nucleotide 165-3490 of a dengue serotype 2 virus, strain 16681, as only a few colonies were obtained from the selection plates. Sequencing of ampicillin-resistant clones revealed various mutations that caused frame shifts in the reading frame of the E gene. As

the same recipient plasmid was successfully employed in the generation of chimeric dengue cDNA fragments with the prM-E genes from DENV-1, DENV-2 and DENV-4, we suspected that the instability of this plasmid was likely a result of the inserted prM-E sequences of DENV-3. That portion of the gene was then analyzed for putative bacterial promoters using the Neural Network Promoter program. Thirteen putative bacterial promoters with scores higher than 0.8 were identified on the sense strand and nine were found on the reverse strand (Table 1). As the expression of these promoters may lead to the generation of RNA or protein products that could restrict bacte-

rial growth, modification of the prM+E protein-coding sequence was accomplished by replacing viral codons with the codons that are frequently used in humans (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606>). Analysis of the codon-optimized gene sequence revealed the presence of only one putative bacterial promoter on the non-coding strand (Table 1).

Propagation of full-length D3 prMEopt/16681-3pm chimeric cDNA sequence in *E. coli*

In the initial attempt, full-length D3 prME/16681-3pm chimeric cDNAs sequence was

Table 1. Predicted prokaryotic promoters within the prME-coding regions of DENV-3 strain 03-0347 and DENV-3opt DENV-3 strain 03-0347

Forward strand			
Start	End	Score ^a	Promoter Sequence ^b
4	49	0.96	CACTTGACTTCACGCGATGGAGAGCCGCGCATGATTGTGGGGAAGAATGA
834	879	0.82	CTTGGTGACATGCGCGAAATTTCAATGTCTAGAACCAATAGAGGGAAAAA
850	895	0.97	AAATTTCAATGTCTAGAACCAATAGAGGGAAAAATGGTGCAATATGAGAA
1011	1056	0.92	TGCCTTGCCTGAGTATGGAACCCCTTGGTCTAGAATGCTCACCACGGACAG
1060	1105	0.85	GGCTTGGATTCAATGAAATGATTTTATTGACAATGAAGAACAAAGCCTG
1176	1221	0.98	ACCAACTTGGAACAGGAAAGAGCTTCTTGTAACATTCAAAAATGCACATG
1324	1369	0.95	ATTTTTCGCGGGGCACTTGAAATGTAGACTTAAGATGGACAAATTGGAAGT
1333	1378	0.93	GGGCACTTGAAATGTAGACTTAAGATGGACAAATTGGAAGTCAAGGGGAT
1360	1405	0.89	GACAAATTGGAAGTCAAGGGGATGAGCTATGCAATGTGCTTGAATACCTT
1510	1555	0.92	GAGGATGGACAAGGGAAAGCCACAAATGGTAGACTGATCACAGCCAACCC
1624	1669	1.00	ATAGTGATTGGAATTGGAGACAAAGCCTTGAAAATTAAGTGTACAAGAA
1860	1905	0.96	AATTGGAATAGGTGTCTCTTAACTGGATAGGGTTGAATTCAAAAaACA
1918	1963	0.87	TCATTTTCATGCATTGTGATAGGAATTATTACACTCTATCTGGGAGCCGT
Reverse strand			
Start	End	Score ^a	
1910	1865	0.91	TGGAAGTGTTTTTTGAATTCAACCCTATCCAGGTTAAGAGGACACCTATT
1788	1743	0.81	TTCCCTAATGAATTCAAGAGCGCCACCCACTGAACCAAAGTCCCAGGCTGT
1664	1619	0.88	TGTACCAGTTAATTTTCAAGGCTTTGTCTCCAATTCCAATCACTATGTTG
1235	1190	0.98	CTTCTTGCTTTTTTGCATGTGCATTTTTGAATGTTACAAGAAGCTCTTTC
1101	1056	0.90	GCTTTGTTCTTCATTGTCAATAAAATCATTTCATTGAAATCCAAGCCTGT
924	879	0.94	TGCACTGTGATGATGACAGTGTATTTGAGGTTCTCATATTGCACCATTTT
704	659	0.98	CAGTTGTTATGTTGGTAATTTTCCCTCAATGCATAACTTCCTTAGGGTC
497	452	0.85	TCATTGTCATGGATGGAGTGACCAGCATTAGTAGTATAAAAAATAACCACT
84	39	0.85	CCAGAAGCTGTCTTAAAAAGTAGGGATTTCCTCTTTCATTCTTCCCCAC
DENV-3opt			
None			
Reverse stand			
Start	End	Score ^a	
68	23	0.93	ACAGCAGGCTCTTGCCTCTCTCGTTCTTGCCACGATCATTCTGGGCTGG

^a The score for the predicted prokaryotic promoter was calculated by a Neural Network promoter prediction search program (http://www.fruitfly.org/seq_tools/promoter.html).

^b Transcription start shown in large font

generated by successive *in vitro* ligation of subgenomic fragments. The native DENV-3 prM-E sequence could be cloned successfully into a recombinant pBluescript II KS plasmid containing nucleotides 165-2678 of DENV-2 strain 16681, but not in a larger plasmid containing nucleotides 165-3490. Sequence analysis of pBluescript II KS 16681 (165-2678) containing the native DENV-3 prM and E genes confirmed the accuracy of the sequence. To generate a full-length cDNA clone, the Pst I-Kpn 2I fragment containing the native DENV-3 prM-E gene was ligated to the Pst I-Kpn 2I large fragment from the 16681-3pmΔKpn 2I (5557) backbone (Figure 1A, 1B). A 10.7 kb full-length cDNA of D3 prME/16681-3pm was obtained by long PCR using a high fidelity DNA polymerase (Phusion, Fermentas) (Figure 1C). Multiple PCR reactions were performed in order to obtain a sufficient quantity for *in vitro* translation.

A full-length cDNA clone of dengue 2/3 chimeric virus with modified codons was constructed using the Gibson assembly approach (Figure 1D). The prM and E genes with optimized codons were amplified by PCR to generate fragments containing 20 nucleotide sequences of the capsid gene and the nonstructural protein 1 gene of DENV-2 strain 16681 at the 5' end and 3' ends, respectively. This PCR fragment and a fragment of pBluescript II KS 16681-3pmΔKpn2I (5557) which lacks the prM- and E-coding regions (Figure 1E) were combined by the action of T5 exonuclease, Phusion DNA polymerase, and Taq ligase. The mixture was transformed into *E. coli* strain DH5α. The growth of bacteria harboring the D3 prMEopt/16681-3pm chimeric cDNA clone was comparable to those with the parental 16681-3pmΔKpn2I cDNA. Digestions of a full-length plasmid clone of D3 prMEopt/16681-3pm with five restriction endonucleases confirmed the expected restriction patterns (Figure 1F).

The replicated D3 prMEopt/16681-3pm chimeric virus with modified codons was comparable to those assembled via the *in vitro* ligation method

In vitro transcripts of the full-length chimeric cDNA plasmid clones with either native DENV-3 prM and E codons or with modified codons generated viable viruses when transfected into Vero cells. Viral E antigens could be detected in both types of transfected cells with 8A1, a mouse monoclonal antibody that is specific for DENV-3 (Figure 1G). For chimeric virus with codon optimization, the virus titer on day 4 post transfection was 1.01×10^4 FFU/mL and the virus reached peak titers (4×10^6 FFU/mL) on days 10–14 while the titer of dengue 2/3 chimeric virus with the native prM-E sequence reached 6×10^4 FFU/mL on day 14 post transfection. DNA sequence analysis of the prM-E gene revealed only one silent mutation (T2247C) at residue 437 of the E protein in the chimeric virus with codon optimization. There were two mutations in the chimeric virus generated by *in vitro* ligation, one silent mutation (A744G) at residue 11 of the M protein and one amino acid substitution from arginine to lysine (G1628A) at residue 513 in the E protein.

To compare the focus size and replication of the two viruses, experiments were conducted using Vero cells. For focus size comparison, the average number of cells infected was counted on the same titration plate to minimize variation related to the number passages of the host cells and the duration of cultivation before fixing. Infected foci appeared as clusters of cells with dark purple cytoplasm and clear nuclei. Representatives of infected foci from each virus are shown in Figure 2A and the numbers of cells in 50 foci of each virus are plotted in Figure 2B. The mean number of infected cells per focus was 43 ± 20 cells for the chimeric virus with native prM-E sequence and 66 ± 32 cells for the chimeric virus with the modified prM-E sequence. The difference in focus size of these two viruses was statistically significant ($p < 0.0001$).

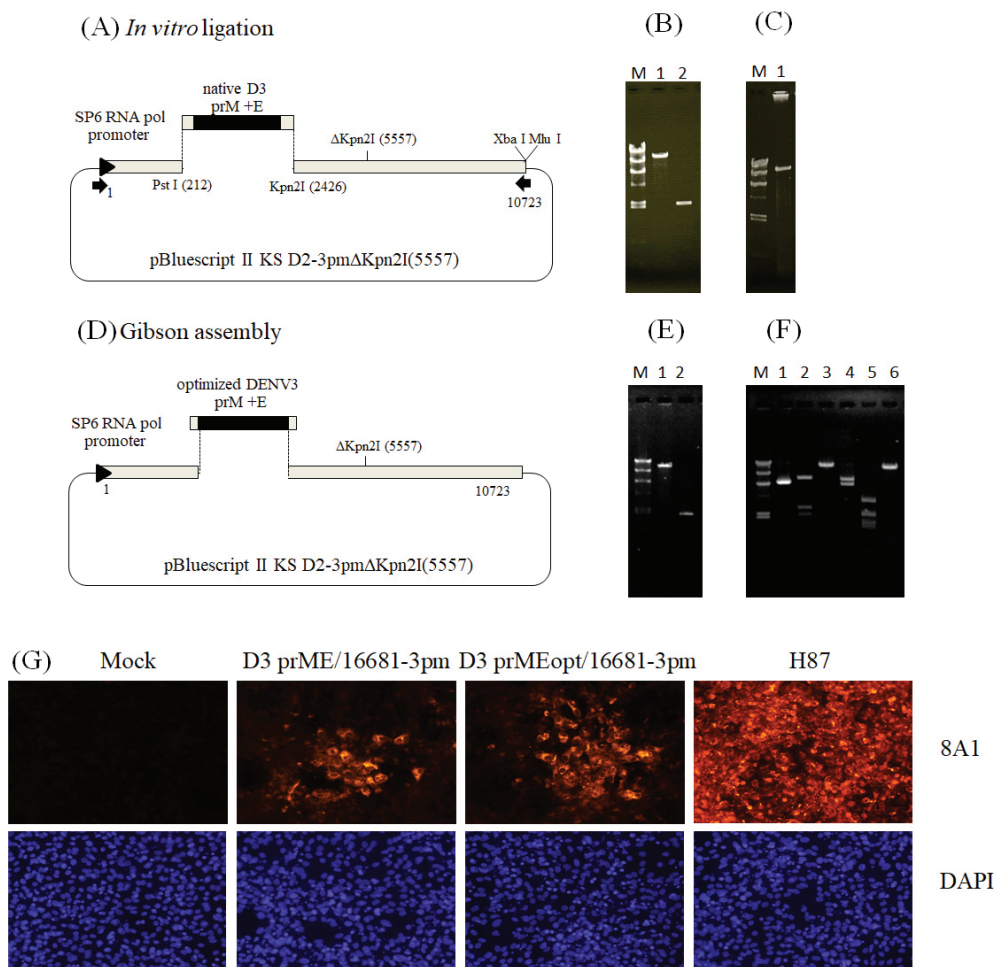


Figure 1. Construction of full-length cDNAs for dengue 2/3 chimeric viruses. **(A)** Diagram of the construction of the complete full-length cDNA clone by *in vitro* ligation. **(B)** Analysis of cDNA fragments used to construct dengue 2/3 chimeric virus with native sequence; large cDNA fragment of 16681-3pmΔKpn2I (lane 1) and prM-E gene form DENV-3 03-0347 (lane 2). **(C)** Analysis of full-length cDNA of D3 prME/16681-3pmΔKpn2I obtained via PCR (lane 1). **(D)** Diagram of the construction of the complete full-length cDNA clone with modified codons in the prM-E coding region by Gibson's assembly. **(E)** Analysis of PCR fragments used to construct dengue 2/3 chimeric virus with modified sequence; large cDNA fragment of 16681-3pmΔKpn2I (lane 1), synthetic prM-E gene of DENV-3 (lane 2). **(F)** Analysis of D3 prMEopt/16681-3pmΔKpn2I plasmid; uncut DNA (lane 1); DNA fragments after digestion with Eco RI, Hind III, Kpn I, Nco I and Pst I, respectively (lanes 2-6). DNA fragments were analyzed on a 0.7% agarose using HindIII-digested λ DNA fragments (lane M) as MW size control. Restriction enzyme cleavage sites in the cloning diagrams are numbered based on the nucleotide position in the viral genome. Position of SP6 promoter is indicated by a triangle. Binding positions of PCR primers used for long PCR are marked with black arrows. Numbering is based on nucleotide sequence of DENV-2 strain 16681. **(G)** Detection of viral protein expression in transfected cells by indirect fluorescent antibody assay. At 7 days post transfection, cells grown on coverslips were fixed and stained for viral E protein using a mouse monoclonal antibody specific for DENV-3 (8A1). Uninfected cells and cells infected with DENV-3 strain H-87 were included as negative control and positive control, respectively. Nuclei were stained with DAPI.

In addition, the viral growths of the two viruses were compared by single-step kinetics and multi-step kinetics experiments. For single-

step replication kinetics, Vero cells were infected with an MOI of 1. Following infection, low levels of infections of both viruses were detected in

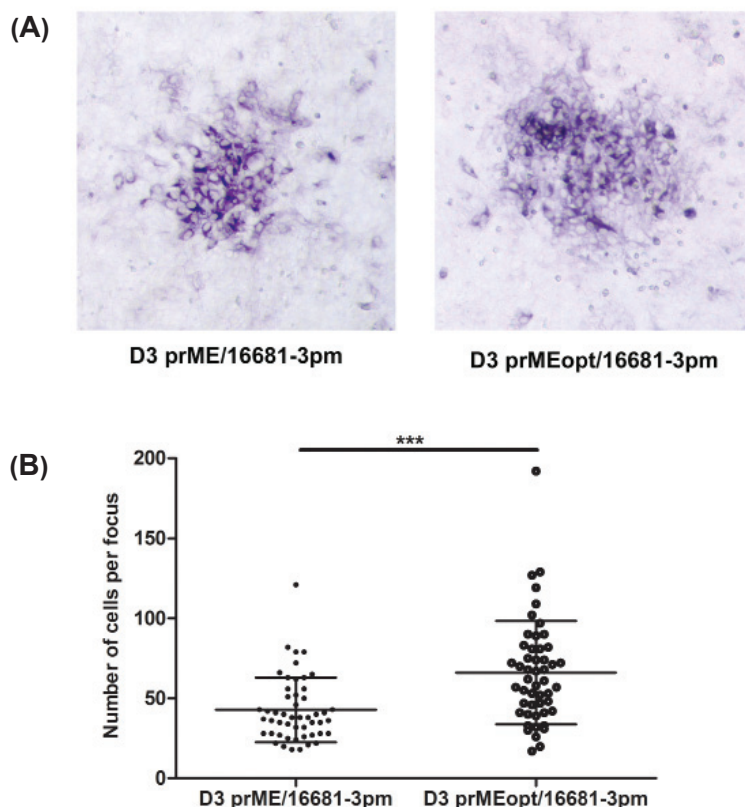


Figure 2. Focus size of dengue 2/3 chimeric viruses. (A) Representative foci of D3 prME/16681-3pm and D3 prMEopt/16681-3pm. The chimeric dengue viruses were titrated using Vero cells. Cells were fixed, permeabilized, and stained for viral antigens using anti-flavivirus monoclonal antibody, clone 4G2. (B) Scatter plot of the number of infected cells per focus of D3 prME/16681-3pm (●) and D3 prMEopt/16681-3pm (○). Fifty separate foci from the same titration plate were photographed and counted. Dots represent data obtained from individual foci. Error bars indicate the standard deviation of the measurement. Asterisks (***) indicate a statistically significant difference between groups ($p < 0.0001$).

either cell-associated samples or extracellular samples during the first 20 hours (Figure 3A and 3B). The viral titers slowly increased over the first 24 hours and reached a plateau level at 36–48 hours post infection. The rates of replication of the two viruses were similar at all time points. For multi-step replication kinetics, Vero cells were infected with a lower MOI (0.01) to allow multiple cycles of replication to occur. As shown in Figure 3C, the titers of chimeric virus with the modification of the prM-E gene were higher than those with native prM and E genes at all time points. However, statistical analysis revealed that the difference of infectious titers between these two viruses was not significant. These results suggest that the modification of the codon usage in the prM

and E gene did not affect viral multiplication process.

Discussion

Reverse genetics is now important for virology because viruses with specific modifications can be generated. However, this methodology may not be readily applied to certain viruses due to the instability of full-length cDNA clones when propagated in *E. coli*. For flaviviruses, the construction of an infectious clone of dengue 3 virus seems to be very tricky. There have been no reports of successful construction of a full-length cDNA clone of dengue 3 virus using authentic viral sequences in *E. coli*. *In vitro* ligation of subgenomic fragments could gen-

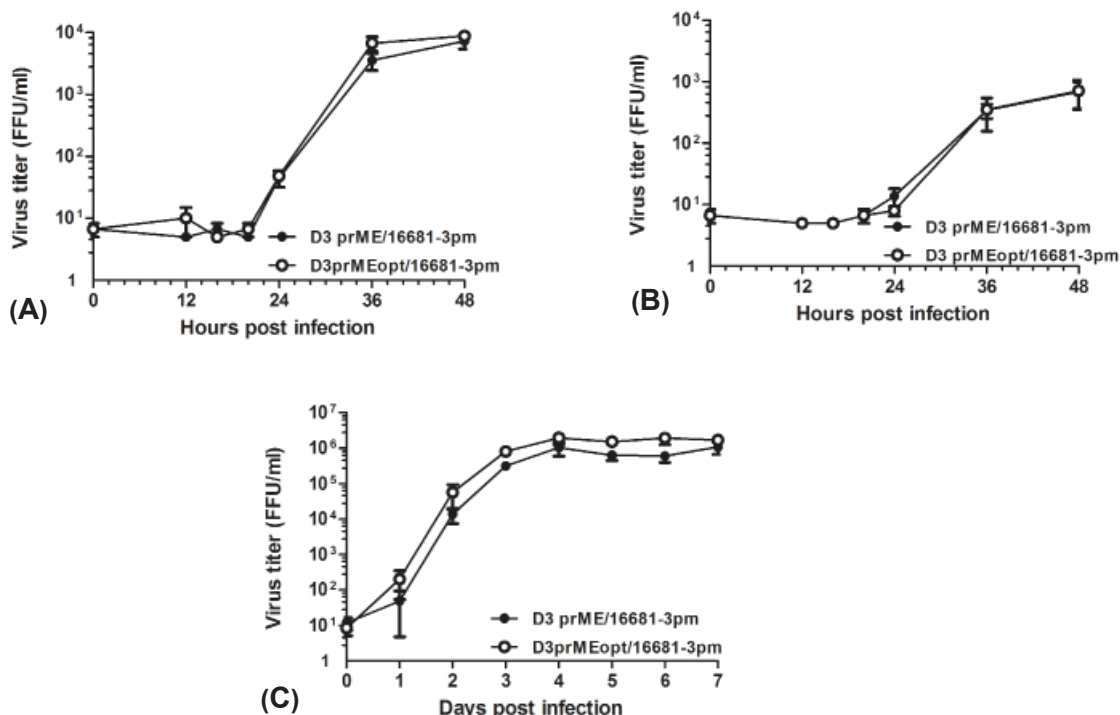


Figure 3. Growth kinetics of dengue 2/3 chimeric viruses. **(A and B)** Single-step replication kinetics experiments of D3 prME/16681-3pm and D3 prMEopt/16681-3pm. Vero cells were infected with chimeric viruses at an MOI of 1. At indicated time points, samples were collected and subjected to focus immunoassay titration. Extracellular virus titers and cell-associated virus titers are plotted on (A) and (B), respectively. **(C)** Multi-step replication kinetics of prME/16681-3pm and D3 prMEopt/16681-3pm. Vero cells were infected with the chimeric viruses at an MOI of 0.01. The infectious virus titers in culture media were determined by focus immunoassay titration using an anti-flavivirus monoclonal antibody, clone 4G2. Data points represent the means and standard errors from 3 independent experiments. The limit of detection was 10 FFU/mL. Titers below the detection limit were plotted as 5 FFU/mL.

erate the full-length cDNA to be used as template for *in vitro* transcription (10, 18, 24, 26); however, there is no infectious clone to serve further manipulation. To aid in cloning, synthetic DNA linker sequences can be inserted to create a redundant translational termination codon which would be removed prior to transcription (19). Alternatively, the putative prokaryotic promoter sequences can be mutated to minimize the possibility of these promoters directly expressing toxic proteins (20). Experiments may be required to identify which putative prokaryotic promoters truly function.

The system described here represents a rapid method of generating stable cDNA clones for dengue 3 viruses, as demonstrated by the construction of a chimeric dengue 2/3

virus. As prM and E genes from non-DENV-3 serotypes have been successfully cloned into DENV-2 backbone in bacteria, it was postulated that the native coding regions for DENV-3 prM and E proteins may contain prokaryotic promoters which would drive the expression of certain proteins that are lethal to *E. coli*. Analysis of the native coding regions for protein prM and E of dengue 3 virus revealed several putative prokaryotic promoters in both strands. In this study, we modified the infectious clone of dengue using codons that are frequently used in humans. Possibly because nearly all potential prokaryotic promoters within dengue 3 sequences are mutated, a stable full-length cDNA clone of dengue 3 virus can be constructed successfully in *E. coli*. However, it

remains uncertain if the instability of the plasmid containing native DENV-3 sequence in *E. coli* is related to the expression of toxic gene products, the metabolic burden of during the maintenance of plasmid, the defective segregation at cell division and at population level, or the bacterial growth conditions employed in this study.

In an effort to simplify the construction process, the modified prM and E gene had been combined with the rest of the DENV-2 viral genome using Gibson assembly technology (29). Construction of a full-length cDNA clone can be accomplished in a few days. As reported by other investigators, the cDNA fragments of flaviviruses and other positive-sense RNA viruses can be combined into a genome-length cDNA construct. Together with a cyto-megalo-virus promoter at the 5' end of the gene and a ribozyme sequence at the 3' end, the complete assembled viral cDNA efficiently generates infectious virus when introduced into cultured cells (31, 32). This method is very useful for studying the diversity of the viruses. However, as mutations may arise during the preparation of cDNA fragments by PCR, sequencing is essential to confirm the viral sequence. Depending on the viral templates, the viruses produced by this method may represent a pool of closely related viruses. Therefore, plaque purification may be needed to obtain a genetically identical clone. The ease of Gibson technology combined with the use of modified codons means a stable cDNA clone of dengue 2/3 virus can be generated. The sequences modified in this chimeric virus account for about one-fifth of the genome. As sequences at the 5' end of RNA did not change, the modification of the viral RNA sequence would not have any effect on the rate of translation initiation. However, the translation elongation rate may be altered (33). Kinetics results indicate that the use of modified codons in prM and E genes has a minimal effect on virus replication. Thus, the availability of a stable infectious cDNA clone of dengue 3 viruses would provide a valuable tool for generating mutants in order to gain a greater understating of replication, assembly, pathogenicity and immunogenicity of the virus.

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Conflicts of Interest

The authors declare no conflicts of interest.

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การปรับแต่งโคดอนของยีนพรีเอ็มและอีทำให้ได้โคลนซีดีเอ็นเอทั้งจีโนม ของเชื้อไวรัสแดงกึ่งของไวรัสลูกผสม 2/3 ที่คงตัวสามารถเพาะเลี้ยงได้ในอี โคลไ

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ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ,

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จากความพยายามในการสร้างเชื้อไวรัสแดงกึ่งลูกผสมที่มียีนของพรีเอ็มและอีจากเชื้อไวรัสแดงกึ่ง 3 และส่วนที่เหลือของจีโนมของเชื้อไวรัสแดงกึ่ง 2 สายพันธุ์ 16681-3pm พบว่าจีโนมของไวรัสลูกผสมที่เชื่อมต่อกันไม่สามารถประกอบออกมาให้อยู่ในรูปของโคลนซีดีเอ็นเอของไวรัสทั้งจีโนมที่คงตัวใน อี โคลไ ได้ คาดเดาว่าในส่วนยีนที่กำหนดการสร้างโปรตีนพรีเอ็มและอีที่ได้จากตัวเชื้อไวรัสน่าจะมีลำดับนิวคลีโอไทด์ที่ทำให้เกิดความเป็นพิษต่อแบคทีเรีย ผู้วิจัยจึงได้ทำการปรับเปลี่ยนรูปแบบการใช้โคดอนของเชื้อไวรัสให้เป็นแบบที่ถูกใช้บ่อยในจีโนมของคน ทำการเชื่อมชิ้นส่วนของยีนพรีเอ็มและอีที่ถูกปรับแต่งเข้าไปในซีดีเอ็นเอขนาดยาวเต็มสายของสายพันธุ์ 16681-3pm เพื่อแทนที่ในบริเวณที่เกี่ยวข้องกันในจีโนมของเชื้อไวรัสแดงกึ่ง 2 ด้วยวิธีกิบสันแอสซิมบลี ซีดีเอ็นเอขนาดยาวเต็มสายของเชื้อไวรัสลูกผสม D3 prMEopt/16681-3pm สามารถเพิ่มจำนวนได้ในอี โคลไ ผลการทรานสคริปโคลนดังกล่าวในหลอดทดลองได้ผลผลิตอาร์เอ็นเอขนาดยาวเต็มสายซึ่งสามารถสร้างไวรัสลูกหลานได้เมื่อใส่เข้าไปในเซลล์เพาะเลี้ยง จากผลการศึกษาทางจุลศาสตร์พบว่าการเพิ่มจำนวนของเชื้อไวรัสแดงกึ่งลูกผสม D3 prMEopt/16681-3pm ที่ถูกปรับโคดอนในยีนของพรีเอ็มและอีอยู่ในระดับใกล้เคียงกับเชื้อไวรัสที่สร้างโดยการเชื่อมในหลอดทดลอง การใช้โคดอนที่ถูกปรับเปลี่ยนร่วมกับวิธีกิบสันแอสซิมบลีทำให้ได้โคลนของซีดีเอ็นเอขนาดยาวเต็มสายของไวรัสลูกผสมที่มียีนพรีเอ็มและอีของเชื้อแดงกึ่ง 3 สายยาวที่สามารถเพาะเลี้ยงได้อย่างคงตัวใน อี โคลไ ซึ่งช่วยให้สามารถดัดแปลงลำดับเบสของเชื้อไวรัสเพื่อการศึกษาต่อไปได้ การมีโคลนของซีดีเอ็นเอดังกล่าวเป็นเครื่องมือสำคัญที่จะใช้ศึกษาบทบาทความสำคัญของนิวคลีโอไทด์และกรดอะมิโนในโปรตีนพรีเอ็มและอีของเชื้อแดงกึ่ง 3 ต่อการเพิ่มจำนวน การกระตุ้นภูมิคุ้มกัน และการก่อโรคของไวรัส รวมถึงมีบทบาทช่วยในการพัฒนาวัคซีนแดงกึ่ง **เชียงใหม่เวชสาร 2560;56(3):125-36.**

คำสำคัญ: เชื้อไวรัสแดงกึ่ง ซีโรไทป์ 3 ซีดีเอ็นเอ โคลน