MOLECULAR EPIDEMIOLOGY OF DENGUE VIRUS ISOLATED FROM SERA OF PATIENTS WITH SUSPECTED DENGUE FEVER IN BANGKOK, THAILAND SINCE 2006 ASSOCIATED WITH CONSTRUCTION AND CHARACTERIZATION OF INFECTIOUS CLONE DENGUE 4 1036 PDK40

By
MR. Kumchol CHAIYO

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy BIOLOGY
Department of BIOLOGY
Graduate School, Silpakorn University
Academic Year 2017

ระบาดวิทยาทางอณูโมเลกุลของเชื้อไวรัสเด็งกี่แยกจากซีรั่มของผู้ป่วยสงสัยโรคไข้เด็งกี่ในกรุงเทพมหานคร, ประเทศไทย ตั้งแต่ปีค.ศ. 2006 ร่วมกับการสร้างและการศึกษาคุณลักษณะโคลนติดเชื้อไวรัสเด็งกี่ 4 สายพันธุ์ 1036 PDK40

โดย
นายขุมชล ไชยโย

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บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2560

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By	Kumchol CHAIYO
Field of Study	BIOLOGY
Advisor	Jundee Rabablert

Graduate School Silpakorn University in Partial Fulfillment of the Requirements for the Doctor of Philosophy

..... Dean of graduate school
(Associate Professor Jurairat Nunthanid, Ph.D.)

Approved by

..... Chair person
(Associate Professor Chockpisit Thepsithar , Ph.D.)
..... Advisor
(Assistant Professor Jundee Rabablert , Ph.D.)
..... Co Advisor
(Professor Eeritus Dr. Sutee Yoksan , M.D., Ph.D)
..... External Examiner
(Dr. Sittiruk Roytrakul , Ph.D.)

56303801 : Major BIOLOGY
Keyword : DENGUE VIRUS, DENGUE FEVER, PHYLOGENETIC ANALYSIS

MR. KUMCHOL CHAIYO : MOLECULAR EPIDEMIOLOGY OF DENGUE VIRUS ISOLATED FROM SERA OF PATIENTS WITH SUSPECTED DENGUE FEVER IN BANGKOK, THAILAND SINCE 2006 ASSOCIATED WITH CONSTRUCTION AND CHARACTERIZATION OF INFECTIOUS CLONE DENGUE 4 1036 PDK40 THESIS ADVISOR : ASSISTANT PROFESSOR JUNDEE RABABLERT, Ph.D.

Dengue fever, dengue hemorrhagic fever and dengue shock syndrome are mosquito-borne infectious diseases. These diseases have been caused by dengue virus serotype 1, 2, 3, and 4 (DENV1-4), Family *Flaviviridae*, genus *Flavivirus* in sub-tropical and tropical regions. Different serotypes and genotypes of DENV may play an important role in disease severity among dengue patients. Up to date, dengue vaccine is no available. The aims of this study (i) to evaluate the molecular epidemiology of DENV isolates of patient sera in C6/36 cells by qRT-PCR, DNA sequencing and phylogenetic tree and (ii) to construct and evaluate biological markers of IC DEN4V 1036 PDK40.

qRT-PCR reveals 75 positive isolates consist of DENV1 (n=15), DENV2 (n=20), DENV3 (n=28) and DENV4 (n=12). DNA sequencing and phylogenetic tree analysis demonstrated genotype I of DENV1, genotype Asian I of DENV2 and genotype I of DEN4V. DEN3V consisted of genotype II (n=6) and genotype III (n=22). Report of dengue survey in Thailand revealed that DENV3 genotype II has been found since 1973, while genotype III has been found since 2008.

IC-DEN4V-1036-PDK40 was constructed by utilizing live attenuated (LAV) DEN4V 1036 PDK40. Three fragments of DEN4V 1036 PDK40 and 1 fragment of cloning vector were assembled by using the Gibson assembly method. RNA of IC-DEN4V-1036-PDK40 was synthesized and transfected into Vero cells. The rescued virus was amplified in Vero cells for 5 passages to achieve satisfactory viral titers. IC-DEN4V-1036-PDK40 showed attenuated characterization including small/pinpoint plaque size, temperature sensitivity and low growth efficiency in *Aedes. aegypti*. These results indicate safety of IC-DEN4V-1036-PDK40. This infectious clone might be useful for use as vaccine or a backbone for construct chimera vaccine.

ACKNOWLEDGEMENTS

This study was partially supported by grants from the National Vaccine Institute, Thailand (No. 2558.1/2 and 2559.1/6) and partially supported by the Department of Biology, Faculty of Science, Silpakorn University (Grant No. SRF-JRG-2559-04).

I would like to express my deepest and sincere gratitude to my major advisor, Assistant Professor Dr. Jundee Rabablert, Department of Biology, Faculty of Science, Silpakorn University for her continuous and constructive guidance and advice throughout the course of study.

I also would like to express my appreciation to my co-advisor Professor Dr. Sutee Yoksan, Center for Vaccine Development, Institution of Molecular Bioscience, Mahidol University for giving me a good advice and be guidance for experiment, suggestions and comments.

I would like to express my appreciation to Associated Professor Dr. Chockpisit Thepsithar for her kindness, valuable suggestions and comments.

I would like to special thank Dr. Sittiruk Roytrakul, Genome Institute, BIOTEC who was the external examiner of my thesis defense for him kindness, willingness, valuable suggestions and comments.

I would like to thank Professor Dr. Kulkanya Chokephaibulkit, Department of Pediatrics, Faculty of Medicine Siriraj Hospital and Professor Dr. Ampaiwan Chuansumrit Department of Pediatrics, Faculty of Medicine Ramathibodhi Hospital, Mahidol University for giving me samples.

I would like to thank Mr. Supoth Rajakam, Center for Vaccine Development, Institution of Molecular Bioscience, Mahidol University for virus isolation in cell culture.

Finally, I would like to express my deepest appreciation to my family for their love, cheerfulness, helpful, understanding, support, guidance and encouragement throughout my life.

Kumchol CHAIYO

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

Abbreviation	Term
AA	Amino acid
Ab	Antibody
ADE	Antibody-dependent enhancement
AFI	Acute febrile illness
Ag	Antigen
ASEAN	Association of South East Asia Nations
ATCC	American type culture collection
A260	Absorbance at wavelength 260 nanometers
A280	Absorbance at wavelength 280 nanometers
bp	Base pair(s)
C	Capsid
°C	Degree celcius
CHO	Carbohydrate
CMC	Carboxymethyl cellulose
CNS	Central nervous system
CPE	Cytopathic effect
cDNA	Complementary deoxynucleic acid
D	Domain
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEN	Dengue
DENV	Dengue virus
DEPC	Diethyl pyrocarbonate
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPIV	Dengue purified formalin-inactivated vaccine
DSS	Dengue shock syndrome

LIST OF ABBREVIATIONS

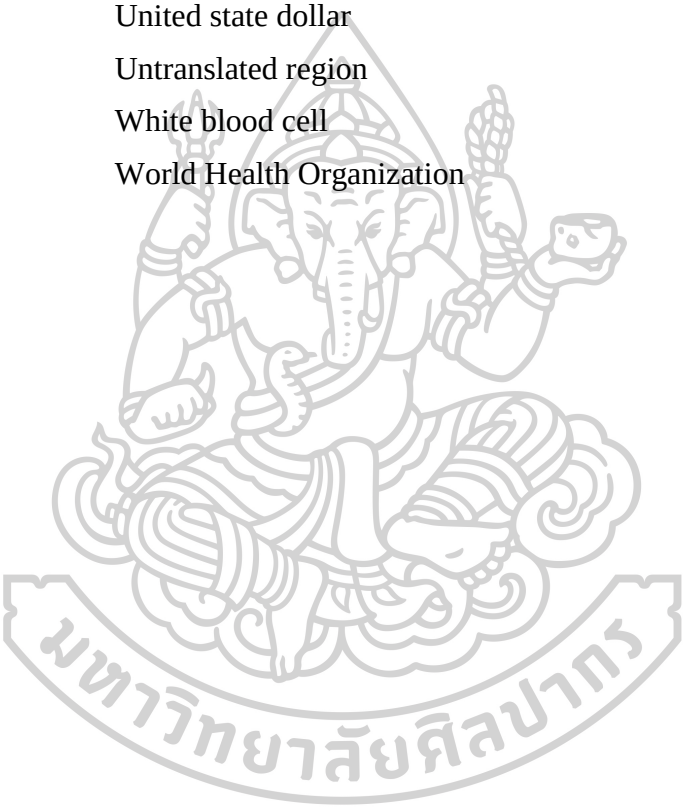
dNTP	Deoxynucleotide triphosphate
dpi	Day(s) post inoculation
ELISA	Enzyme-linked immunosorbent assay
E	Envelope
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FRhl	Fetal rhesus lung
g	Gram(s)
GAGs	Surface glycosaminoglycans
GMK	Green monkey kidney
GPI	Glycosyl-phosphatidylinositol
HI	Hemagglutination inhibition
Hr	Hour(s)
IC	Infectious clone
IFA	Indirect immunofluorescence assay
IFN	Interferon
IgM	Immunoglobulin M
IgG	Immunoglobulin G
JEV	Japanese encephalitis virus
kb	Kilobases
kDa	Kilodaltons
LAV	Live-attenuated vaccine
LB	Luria Bertani
M	Membrane
MEGA	Molecular evolutionary genetics analysis
MOI	Multiplicity of infection
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
µg	Microgram(s)

LIST OF ABBREVIATIONS

µl	Microliter(s)
µM	Micromolar(s)
NC	Negative control
ND	Not done
NS	Nonstructural
NT Ab	Neutralizing antibody
ng	Nanogram(s)
nm	Nanometer(s)
OD	Optical density
ORF	Open reading frame
pH	Potential of hydrogen
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDK	Primary dog kidney
PRNT	Plaque reduction neutralization test
pfu	Plaque forming unit
pmol	Picomol(s)
prM	Premembrane
psi	Pounds per square inch
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
rNS1	Recombinant nonstructural protein 1
rDENV	Recombinant dengue plasmid
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerized chain reaction
SEA	South-East Asia
sec	Second(s)

LIST OF ABBREVIATIONS

sNS1	Secreted nonstructural protein 1
TBE	Tris Borate Ethylenediaminetetraacetic acid
TLR	Toll-like receptor
TS	Temperature sensitivity
U	Unit(s)
US$	United state dollar
UTR	Untranslated region
WBC	White blood cell
WHO	World Health Organization



กิตติกรรมประกาศ

วิทยานิพนธ์นี้

ได้รับทุนอุดหนุนการทำวิทยานิพนธ์ จากเงินงบประมาณแผ่นดิน

(หมวดเงินอุดหนุนทั่วไป) ของบัณฑิตวิทยาลัย

ประจำปีเงินประมาณ ๒๕๕๙ (ครั้งที่ ๒)

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

CHAPTER I
INTRODUCTION

Dengue virus (DENV) belongs to the genus *Flavivirus*, family *Flaviviridae*. There are four antigenically distinct DENV serotypes; DEN1V, DEN2V, DEN3V and DEN4V. (Guzman et al., 2016). DENV is transmitted to humans through the bite of infected *Aedes* mosquitoes, particularly *Ae. aegypti* and *Ae. albopictus* (Hugo et al., 2014). Infection with any of the four DENV may cause a wide spectrum of clinical features ranging from nearly asymptomatic disease, an undifferentiated febrile illness, dengue fever, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS). DENV affects 50 - 200 million people and leads to about 20 thousand deaths annually in tropical and subtropical regions of the world. The mortality rate of patients with severe dengue diseases is about 1-2.5% (Martina, Koraka and Osterhaus, 2009; Guzman et al., 2016). In Thailand 2015, a total of 142,925 dengue cases (morbidity rate was 222.56/100,000 population) with 147 deaths (mortality rate was 0.23/100,000 population) were reported (BOE, 2016). In endemic area, co-circulation of multiple DENV serotypes were shown (Holmes et al., 2009). In addition, each serotype of DENV shows phylogenetically distinct genotypes (Klungthong et al., 2008; Teoh et al., 2013). Genotype and clade replacements in DENV serotypes are associated withthe prevalence of dengue disease (Zhang et al., 2005).

The *Flavivirus* genome consists of single-stranded positive sense RNA approximately 11 kilobases (kb) long that is capped at the 5 end and lacks a 3 polyadenylated tail. DENV genome is translated to be a single polyprotein and post-translationally cleaved into three structural proteins: capsid (C), premembrane (prM) and envelope (E); and seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Guzman et al., 2016).

Live-attenuated viruses are widely used for the prevention of human viral diseases. Attenuation of virulent wild viruses has generally been accomplished by serial passages of virus in cells from non-natural hosts. The late Profressor Natt Bhamarapravati and Profressor Sutee Yoksan developed live-attenuated (LAV) DEN4V 1036 PDK48 vaccine. DEN4V (1036 parental strain) was isolated from a patient with dengue fever in 1976 (Angsubhakorn et al., 1988; Bhamarapravati and Sutee, 2000). DEN4V showed plaque size reduction early in PDK passages. The change in this biological attribute was first noted fewer than 10 passages. There was no evidence of plaque size reversion when DEN4V was passaged serially. DEN4V PDK passage 40-60 were temperature sensitive at 39°C and did not produce CPE in LLC-MK2 cells. However, these late passages could replicate in Peripheral blood mononuclear cell (PBMC) cultures and cloud produce neurovirulence in suckling mice. The average survival time in suckling mice of DEN4V PDK60 virus was 12.4 days while that of the parental virus was 8.4 days. (Yoksan, Bhamarapravati and Halstead, 1986). DEN4V PDK 40 and PDK 50 viruses had reduced primate (Angsubhakorn et al., 1988). In a phase II clinical trial, LAV DEN4 1036 PDK48 showed safety and induced immunogenicity in Thai volunteers (Bhamarapravati and Sutee, 2000). This DEN4V 1036 PDK48 virus has proved to be highly immunogenic

and non reactogenic. However, the replicating feature of LAV is accompanied by concerns of potential reactogenicity in vaccinees, particularly in immunocompromised individuals, genetic instability leading to reversion to virulence (Kinney and Huang, 2001). Lee et al [2011] also demonstrated that LAV DEN4V replicated in Vero and MRC5 cells at 10 passages showed genetic reversion (attenuation to virulence).

Infectious, genome length cDNA clones (single-genome-length clones or in-vitro-ligated subclones) permit the efficient, directed genetic engineering of mutations into the viral genome and analyses of the phenotypic effects of the mutations in the clone-derived viruses (Rice et al., 1989; Kinney et al., 1997; Durbin et al., 2001). The Gibson assembly is a novel methodology for the construction of *Flavivirus* infectious clones (Santos et al., 2013; Siridechadilok et al., 2013; Bordat, Houvenaghel and German-Retana, 2015). This method joins multiple overlapping DNA fragments using 3 different enzymes: (i) a T5 exonuclease which creates 3′ overlapping regions, (ii) thermostable DNA polymerase for gap filling and (iii) T4 DNA ligase (Gibson et al., 2009). DEN4V 1036 PDK40 is selected to construct and evaluate biomarkers of infectious clone. Vero cell is chosen from among those certified for human use by the Bureau of Biologics, US Food and Drug Administration

In this study, we evaluated (i) the molecular epidemiology of DENV isolated from patient sera, Bangkok, Thailand, during 2006 to 2015 and (ii) construction of infectious clone DEN4V 1036 PDK40 and undertaken a phenotypic characterization which suggests that this clone could be a safe addition for vaccine development.

CHAPTER II
OBJECTIVES

Part A: To evaluate the molecular epidemiology of DENV isolates of patient sera in C6/36 cells by qRT-PCR, DNA sequencing and phylogenetic tree

1. To develop qRT-PCR to detect DENV.
2. To detect DENV isolates of patient sera in C6/36 cells using qRT-PCR.
3. To study phylogenetic relationship of DENV isolates of patient sera in C6/36 cells using DNA sequencing and phylogenetic tree.

Part B: To construct and evaluate biological markers of IC DEN4V 1036 PDK40

1. To construct IC DEN4V 1036 PDK40
2. To evaluate biological markers of IC DEN4V 1036 PDK40 *in vitro* and *in vivo*.

CHAPTER III
LITERATURE REVIEWS

1. Dengue virus

DENV is one of the most important human arboviruses. DENV is transmitted to human by *Aedes* mosquitoes, mainly *Ae. aegypti* and *Ae. albopictus* (Guzman et al., 2010). The incidence of dengue is associated with geographical expansion of *Aedes* mosquitoes vector (WHO, 1997;2009). DENV belongs to family *Flaviviridae* (genus *Flavivirus*). DENV consists of 4 genetically and antigenically serotype; DEN1V, DEN2V, DEN3V and DEN4V (Guzman and Harris, 2015). Each serotype consists of 4-5 genotypes (Klungthong et al., 2008). Infection with any of the four DENV may cause a wide spectrum of clinical features ranging from nearly asymptomatic disease to severe disease (Whitehorn and Simmons, 2011).

1.1. Dengue viral genome and replication

DENV is icosahedral enveloped virus with positive single-strand RNA (+ssRNA) genome. The virion of DENV is spherical particle, 40-50 nm in diameter with lipid envelope (Modis et al., 2003). DENV has a 11 kb single-stranded positive-sense RNA genome. DENV genome is translated to be a single polyprotein and post-translationally cleaved into three structural proteins: capsid (C), premembrane (prM) and envelope (E); and seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (**Figure 1)** (Guzman and Harris, 2015).

DENV transmits to human through mosquito bite. During mosquito feeding, DENV is inoculated into the dermis and epidermis, and some virus is also injected directly into the bloodstream **(Figure 2)** (Johnston, Halliday and King, 2000; Marovich et al., 2001). First target cells of DENV after mosquito bite are Langerhans cells, dermal and interstitial dendritic cells (Durbin et al., 2008). DENV infects host cells by binding to host cells receptor on the surface and endocytosis via receptor mediated endocytosis in a clathrin-dependent manner (Acosta, Castilla and Damonte, 2008). Infected cells migrate to lymph nodes and spread the virus to monocytes and macrophages. Then, the virus is disseminated via the lymphatic system and circulation system and become primary viremia. The primary viremia resulted in infection of circulation monocyte (Durbin et al., 2008) and liver, spleen and bone marrow macrophage (Jessie et al., 2004; Blackley et al., 2007). Upon acidification of the virus-containing endosome, E protein fuses dengue virus membrane to vesicular membrane of the host cells and release viral genome to host cells cytoplasm (Rey et al., 1995; Mendes et al., 2012). The dengue genome serves as mRNA for translation of the viral polyprotein. The polyprotein is cleaved by host protease and viral protease to become three structural proteins, C (core), PrM (membrane), E (envelope) and seven non-structural (NS) proteins (Brinton, 2002). The viral RNA replication occurs in viral replication complex inside vesicle package. Negative sense viral RNA is synthesized

to act as template for positive sense viral genome. Newly synthesized viral genome forms complex with capsid proteins and become nucleocapsid. The viral nucleocapsid buds into endoplasmic reticulum (ER) lumen acquire lipid bilayer, viral E and prM proteins call as immature virions. Immature virions are transport to trans-Golgi network by secretory pathway (Welsch et al., 2009). The viral particles become mature infectious particles by cleavage of prM by Furin-mediated proteolysis in trans-Golgi network (Stadler et al., 1997). The mature infectious virus particles are released by exocytosis and ready to infect the new cells **(Figure 1)**.

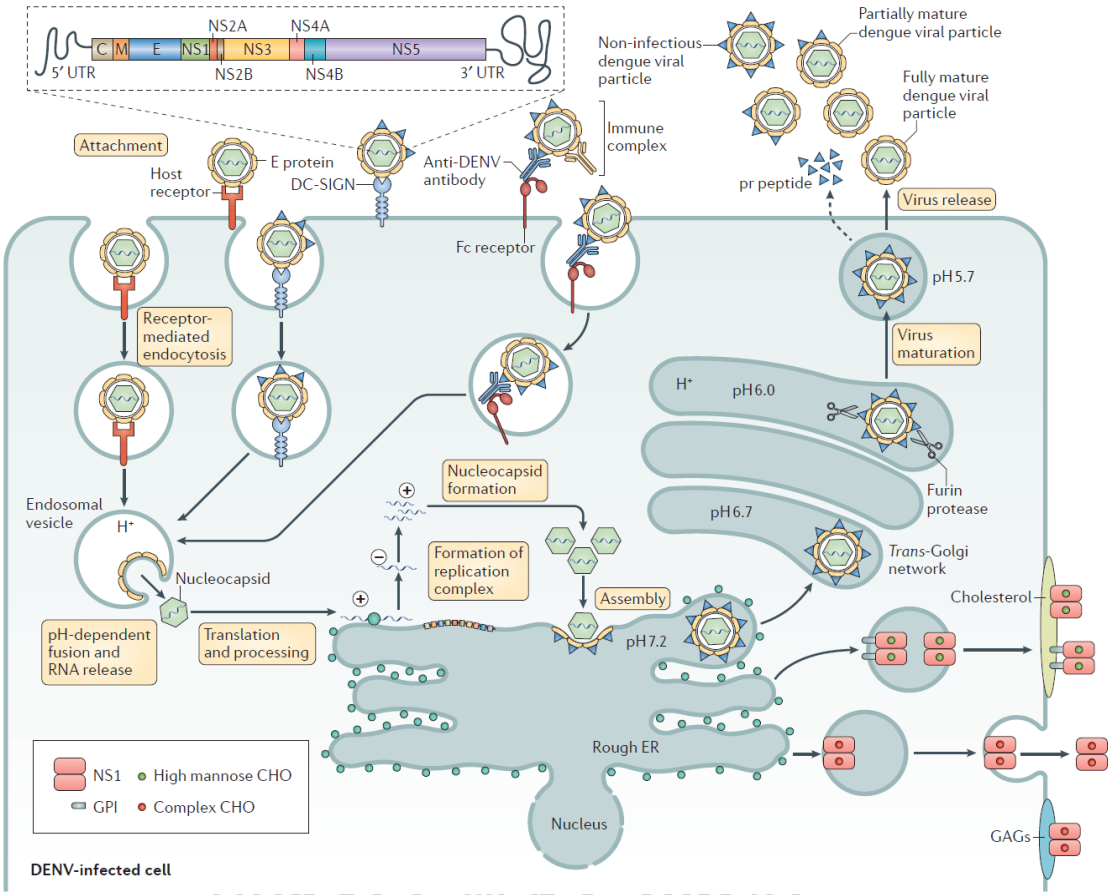


Figure 1 DENV life cycle.Mature virus binds envelope (E) to receptors on host cell. Virus enters host cell through receptor-mediated endocytosis. Low pH environment inside endosome during endosome maturation induces rearrangement of E protein and facilitates fusion of viral membrane to endosomal membrane. After membranes fusion, nucleocapsid is released to host cell cytoplasm. Nucleocapsid disassembles to release viral genome. The viral genome is translated into a single polyprotein, and then cleaved with NS2B, NS3 and host protease into individual proteins. Site of replication locates on endoplasmic reticulum (ER). Nonstructural proteins form replication complex and initiate transcription at site of replication. Initially, NS1 is associated with ER membrane and modified by addition of high mannose carbohydrate (CHO) moieties. Some NS1 acquires glycosyl-phosphatidylinositol (GPI). Membrane-bound NS1 and GPI-anchored NS1 translocate to cell surface or secreted from host cells. Surface NS1 are associated with cholesterol and glycosaminoglycans (GAGs) on cell surface. The structural proteins including prM and E are associated in ER membrane. Nucleocapsid buds into ER lumen, acquires other structural proteins on membrane, and forms immature particle. Immature virus particle is trafficked through secretory pathway. Th low pH of trans-Golgi network cause rearrangement of prM and E. prM protein is cleaved by furin protease and become mature M protein. The mature virus particle is released from host cell and ready to infect new host cells. The immature and partially mature virus particle are unable to infected new host cells (Guzman et al., 2016).

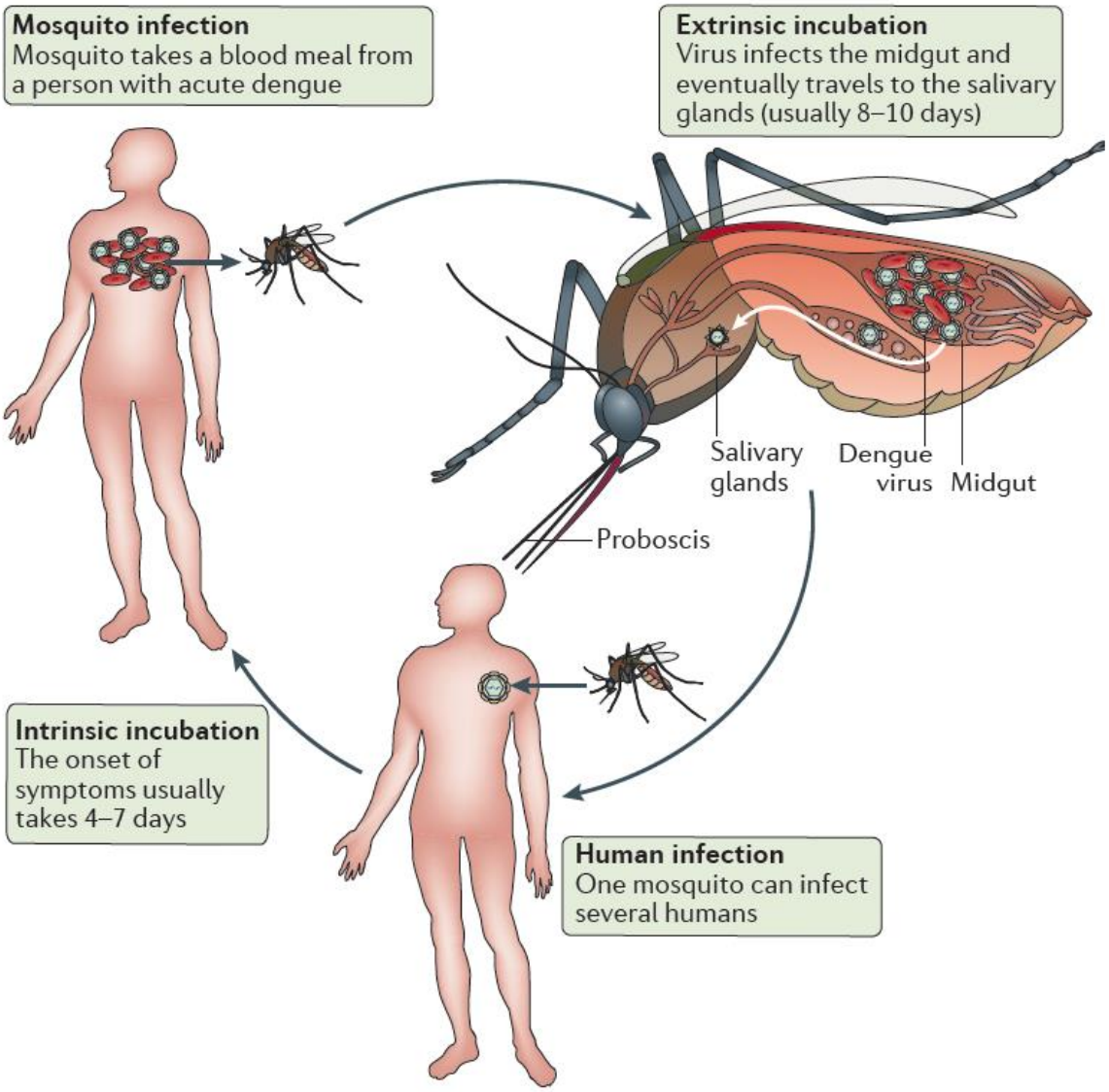


Figure 2 DENV cycle in humans and mosquitoes.*Aedes* mosquito feeds blood from person with DENV viremia. DENV infect mosquito midgut cells and others, and then disseminate to salivary gland. After 8-10 days of extrinsic incubation period, an infected mosquito can transmit DENV to humans. Infected human usually takes 4-7 days for onset of symptoms and had DENV viremia. Both symptomatic and asymptomatic person can transmit virus to mosquitoes(Guzman et al., 2016).

1.2. Virus structural proteins and functions

1.2.1 Capsid protein

Capsid (C) protein or nucleocapsid is a a highly basic protein (~11 kDa). C contains estimated 25% of basic amino acids. The structure of C is homodimeric containing 4 alpha-helices (Ma et al., 2004). The internal hydrophobic residue region is flanking with charged residue region both N terminal and C terminal (Khromykh and Westaway, 1996). An internal hydrophobic region mediates membrane association of C protein (Markoff, Falgout and Chang, 1997). C-terminal hydrophobic tail serves as a signal peptide for ER translocation of prM (Stocks and Lobigs, 1998). The charged residue regions play a role to binding with 5' UTR and 3' UTR of the viral RNA genome (Khromykh and Westaway, 1996). Capsid protein plays a role in virion assembly, to encapsidate the viral RNA genome and become nucleocapsid (Ma et al., 2004).

1.2.2 Membrane protein

Membrane (M) protein is glycosylated protein. Membrane protein has 2 forms, precursor (prM; 26 kDa) and mature (M; ~19 kDa). prM is translocated into the ER by a signal peptide, hydrophobic tail of C (Stocks and Lobigs, 1998). The prM forms heterodimer with Envelope. The N-terminal region of prM protein has N-linked glycosylation site and six conserved cysteine residues (Ruiz-Linares et al., 1989). prM assists Envelope protein folding in ER (Courageot et al., 2000). prM plays a role in prevention of E from structural rearrangement and fusion during virion transit through the Golgi apparatus (Heinz et al., 1994). The acidity during virus releasing process, prM protein is induced a global rearrangement and proteolytic cleavaged by furin-liked protease, resulting in disulphide bond free and unglycosylated (Yu et al., 2008). After cleavage, the pr peptide does not immediately disassociate from the virus particle. Virion releases pr peptide after exposure to neutral pH of extracellular space. This process prevents the immature particle from fusion within the Golgi (Yu et al., 2009).

1.2.3 Envelope protein

Envelope (E) protein is a dimeric glycosylated protein (~53kDa). E protein is the major protein on the surface of *Flavivirus* virions. E protein is conserve structural protein which has 12 conserved cysteine residues that forms 6 disulphide bonds (Mandl et al., 1989). E protein consists of 3 domains; N-terminal structural central domain or domain I (DI), fusion domain or domain II (DII) and putative receptor binding domain or domain III (DIII). DIII projects slightly from the virion surface and might be involved in receptor binding; it is a major target of neutralizing antibodies (Sukupolvi-Petty et al., 2007; Volk et al., 2007; Sukupolvi-Petty et al., 2010). E protein plays a role in attachment to host cells and fusion with host cell membrane. During endocytosis, conformation of E protein is changed due to low pH. Dimeric E protein dissociates into monomeric subunit then form fusogenic trimer (Kimura and Ohyama, 1988; Nayak et al., 2009). The trimer E protein become contacts by DIII shift and rotate, resulting in bend and apposes of host cell membrane and viral membrane. Finally, the viral membrane is fuse with host cell membrane (Modis et al., 2004).

1.3. Virus nonstructural proteins and functions

1.3.1 Nonstructural protein 1

Nonstructural protein 1 (NS1) is translocated into the ER during synthesis (~46 kDa). NS1 protein consists of two conserve N-link glycosylation position and 12 conserves cysteine residues that form disulide bonds (Lee, Crooks and Stephenson, 1989). After synthesis, NS1 forms highly stable homodimers and associates to membranes. Intracellular NS1 localizes at sites of viral RNA synthesis and plays an essential role in viral RNA replication (Mackenzie, Jones and Young, 1996). Dengue virus is use a cellular glycosyl-phosphatidylinositol (GPI) linkage pathway to express a GPI-anchored NS1 proteins on the surface of infected mammalian cells (Jacobs et al., 2000). NS1 is retained within a secretory-derived compartment, expressed on the surface of infected cells, and secreted from mammalian cells. Secreted NS1 (sNS1) forms soluble, hexameric lipoprotein particles of ~10 nm that appear as three dimers held together in a barrel coniguration (Flamand et al., 1999). sNS1 accumulates to high levels in human sera and tissues and can be used to diagnose *Flavivirus* infections at an early stage (Alcon-LePoder et al., 2006). sNS1 is highly antigenic and induce a strong humoral response. NS1-specific antibody can direct complement-mediated lysis of infected cells (Chung et al., 2006) and triggers endothelial permeability and vascular leak that leads to shock (Beatty et al., 2015). Furthermore, NS1 inhibit the classical pathway of complement fixation by binding to and increasing the turnover of complement factor C4 (Avirutnan et al., 2011). NS1 clearly plays an important role in *Flavivirus*-specific humoral responses.

1.3.2 Nonstructural protein 2A

Nonstructural protein 2A (NS2A) is a small membrane spanning hydrophobic protein (~22 kDa). NS2A localizes at sites of RNA replication and interacts with replicase components NS3, NS5, and the 3' UTR of genome RNA (Mackenzie et al., 1998). NS2A protein is an important role in virus assembly by transport RNA to subcellular compartment, a location for assembly (Liu, Chen and Khromykh, 2003). Moreover, NS2A protein can modulates the host interferon expression (Munoz-Jordan et al., 2003).

1.3.3 Nonstructural protein 2B

Nonstructural protein 2B (NS2B) is a small membrane-associated hydrophilic protein (~14 kDa). NS2B has central hydrophilic region around 40 residues, flanked with hydrophobic region. The hydrophobic region is found to mediate with host cells membrane (Clum, Ebner and Padmanabhan, 1997). NS2B bind with NS3 and function as a cofactor of NS3 protein for proteolytic process of viral polyprotein (Falgout, Miller and Lai, 1993).

1.3.4 Nonstructural protein 3

Nonstructural protein 3 (NS3) is a large multifunctional protein (~70 kDa). NS3 consist of serine protease domain residue in N-terminal 167 amino acids of NS3 (Bazan and Fletterick, 1989) and C-terminal helicase domain (Sampath et al., 2006). NS3 utilizes NS2B as cofactor for activate serine protease activity for viral replication (Falgout, Miller and Lai, 1993). Viral protease enzyme is a critical protein for viral replication. The viral protease enzyme is cleaves viral

polyprotein at 2A/2B, 2B/3, 3/4A, 4A/4B and 4B/5 junctions and to generate the C terminal of mature capsid (Yamshchikov and Compans, 1994; Bera, Kuhn and Smith, 2007). The C-terminal helicase domain is play a role in RNA replication including viral RNA binding (Cui et al., 1998), RNA helicase–nucleoside triphosphatase (NTPase) (Kuo et al., 1996), RNA triphosphatase (RTPase) (Wengler and Wengler, 1993) and helicase activity (Li et al., 1999). Furthermore, NS3 protein may exhibit some RNA polymerase activity (Raviprakash et al., 1998). NS3 has been shown to induce apoptosis, through activation of caspase-8 (Prikhod'ko et al., 2002; Prikhod'ko et al., 2004). NS2B-3 serine protease can down-regulate the activation of type I IFN in human dendritic cells (Rodriguez-Madoz et al., 2010).

1.3.5 Nonstructural protein 4A

Nonstructural protein NS4A (NS4A) is a small hydrophobic protein (~16kDa). C-terminal of NS4A acts as signal peptide for NS4B for translocation into ER lumen. NS4A/NS4B junction is cleavaged by viral NS2B-NS3 serine protease (Preugschat and Strauss, 1991). Interaction between NS4A and NS1 is playing an important role in viral replication (Lindenbach and Rice, 1999). NS4A is also purposed to be a part of viral porin proteins. NS4A, NS2A and NS2B of JEV found to altered membrane permeability and growth inhibit of host cells. The author suggests that these proteins form pore which induce cytopathic effect of host cells (Chang et al., 1999).

1.3.6 Nonstructural protein 4B

Nonstructural protein NS4B (NS4B is small hydrophobic protein (~27 kDa). NS4B is transmembrane protein locate on site of replication and in the nucleus (Westaway et al., 1997). Another function of NS4B is antagonizes the innate immune response via, blocking STAT1 in IFN signaling pathway and results in increase viral production (Munoz-Jordan, 2005). NS4B is reported to enhance helicase activity of NS3 by form complex with NS3 and subsequence trigger NS3 to disassociate from ssRNA (Umareddy et al., 2006).

1.3.7 Nonstructural protein 5

Nonstructural protein NS5 (NS5) is largest highly conserve multifunctional protein (~103 kDa) of DENV. N-terminal and C-terminal have methyltranferase (MTase) and RNA-dependent RNA polymerase (RdRP) function, respectively (Perera and Kuhn, 2008). NS5 MTase play a role in methylates the 5′ cap of viral RNA genome (Ray et al., 2006). NS3 and NS5 function together during RNA capping (Issur et al., 2009). In addition, NS5 MTase also methylates internal adenosine residue of viral RNA (Tan et al., 1996). The C-terminal domain of NS5 contains conserved RdRP motifs. NS5 RdRP activity is play a central role in viral replication. The polymerase activity of NS5 has been confirmed by recombinant NS5 proteins that capable to replicate viral and other RNA template (Raviprakash et al., 1998). In addition, monoclonal antibodies against NS5 protein can be block RNA polymerase activity of dengue virus (Bartholomeusz and Wright, 1993). The crystal structure of dengue NS5 protein shares a similar structure to other RdRP molecule (Perera and Kuhn, 2008). In infected cells, NS5 RdRP synthesized negative-sense viral RNA and forms replicative intermediate, dsRNA, with positive-sense viral RNA (Chu and Westaway, 1985). The negative-sense viral RNA is used as template for viral RNA

genome production. Dengue NS5 protein has been report to form complex with NS3 and stimulate NTPase activity of NS3 (Cui et al., 1998).

Table 1 Summary of DENV proteins, with modification of Guzman (2016).

Protein	Size (kDa)	Key features	Main functions
C	11	Homodimer basidic protein Hydrophobic protein flanks with charged region	Virus structure Viral replication
prM	26	C-terminal hydrophobic tail	Stabilize E during transit of the virions through the secretory pathway.
E	53	Dimers dissociate into monomeric subunits,	Receptor binding Fuse viral membrane to cellular endoplasmic membrane Host range tropism
NS1	46	Can be endoplasmid reticulum-anchored, membrane-associated or secreted (sNS1)	Intracellular NS1 is involved in early viral RNA replication sNS1 activates the innate immune system and is implicated in vascular leakage
NS2A	22	Hydrophobic integral membrane protein	Involved in RNA replication
NS2B	14	Small hydrophobic protein	Co-factor for NS3
NS3	70	Maltifunctional protein with several catalytic domains	Involved in nucleoside triphosphatase and helicase function during RNA synthesis
NS4A	16	Hydrophobic integral membrane protien	Required for formation of replication vesicles
NS4B	27	Small hydrophobic protein	Suppresses IFNβ and IFNγ signaling
NS5	105	Largest and most highly conserved DENV protein	Involved in RNA synthesis Involved in blockages of IFN system

2. Epidemiology

2.1. Global burden of disease

During eighteenth and nineteenth centuries DENV circulate throughout tropical and subtropical area. Globalization enhance rapid spread and introduction of new virus strain, serotype or genotype to multiple area. The incident of DENV infection dramatically increased since 1950s **(Figure 3)**. DENV began spread in South-East. The population growth, regional economic growth, modern transportation, expansion of mosquito vector accelerates widespread of DENV (Halstead, 1992; Simmons et al., 2012; Guzman, 2014; Messina et al., 2014; Gubler, 2015). Nowadays, tropical and subtropical regions become hyperendemic area with co-circulation of all 4 serotypes of DENV (Halstead, 1992; Guzman, 2014) **(Figure 4).**

Over 3.6 billion people were affected by DENV (Beatty, Letson and Margolis, 2009). Annually, there are 390 million DENV infection, 2 million cases of severe dengue disease and 21,000 deaths. Asia shown highest incidence of DENV infection. Most of dengue cases are children between 5 to 15 years old. However, the demographic profile of dengue case differs in each country (Gubler, 2011).

2.2. Dengue in Asia

Disruption of ecosystems, increased troop movement and rapid urbanization after World War II facilitated the spread of DENV in Asia. Discarded water storage containers for domestic purposes, surplus war equipment and other mechanized debris all served as ideal breeding habitats for *Ae. aegypti*. By 1945, Cambodia, Philippines, Thailand and Vietnam; countries that were already endemic for dengue, became hyperendemic (Halstead, 2006). Isolation of all serotypes in the 1940s and 1950s in these areas led to an assumption of their earlier existence (Sabin, 1952). DHF emerged in Manila, Philippines, in 1954 (Hammon, Rudnick and Sather, 1960), then in Thailand in 1958 and in Malaysia, Cambodia, Singapore and Vietnam in the 1960s (Gubler, 2002). In India, the first virologically proven epidemic occurred in Kolkata and the East Coast in 1963–64. By 1988, DHF was starting to simmer in various parts of India (Gupta et al., 2012). Cases of DHF were also reported in Karachi, Pakistan, in 1994 (Chan et al., 1995). It has been estimated that Asia bears 70% of the global dengue burden, a figure to which India alone is calculated to contribute 34% (Bhatt, 2013). As India is the largest trading hub in South Asia, it is likely to be the major disseminating source of infection for neighbouring countries like Bangladesh, Bhutan, Maldives, Nepal, and Pakistan. In Bangladesh, DF was documented from the mid-1960s to the mid- 1990s, but an outbreak of DHF was reported in 2000 (Rahman et al., 2002). Bhutan (Dorji et al., 2009) and Nepal (Pandey et al., 2004) reported epidemics only as recently as 2004.

Approximately, two thirds of the global population that is exposed to dengue resides in the Asia-Pacific region (WHO, 2012). Of these, around 1.3 billion people live in ten dengue- endemic countries of Southeast Asia where dengue is one of the most common causes of hospitalization and fatalities in children (Shepard, Undurraga and Halasa, 2013). The rate of severe dengue in the region is 18 times higher than that in the Americas (Shepard, Undurraga and Halasa, 2013). A total of 187,333 dengue cases from the region were reported to WHO in 2010 (Ferreira, 2012). According to WHO, dengue risk territories are Bangladesh, Bhutan, Brunei, Cambodia, Hong Kong, India, Indonesia, Laos, Macau, Malaysia, Myanmar, Nepal, Pakistan,

Philippines, Singapore, Sri Lanka, Taiwan, Thailand, and Vietnam (WHO, 2017). It is apparent that 11 countries in the WHO Southeast Asia region (Bangladesh, Bhutan, India, Indonesia, Maldives, Myanmar, Nepal, North Korea, Sri Lanka, Thailand and East Timor) have become hyper-endemic, with regular reporting of dengue cases since 2000 with the exception of North Korea. The highest ever combined totals of clinical cases (Higa, 2011) and deaths (1982) were recorded in 2010 (Ferreira, 2012).

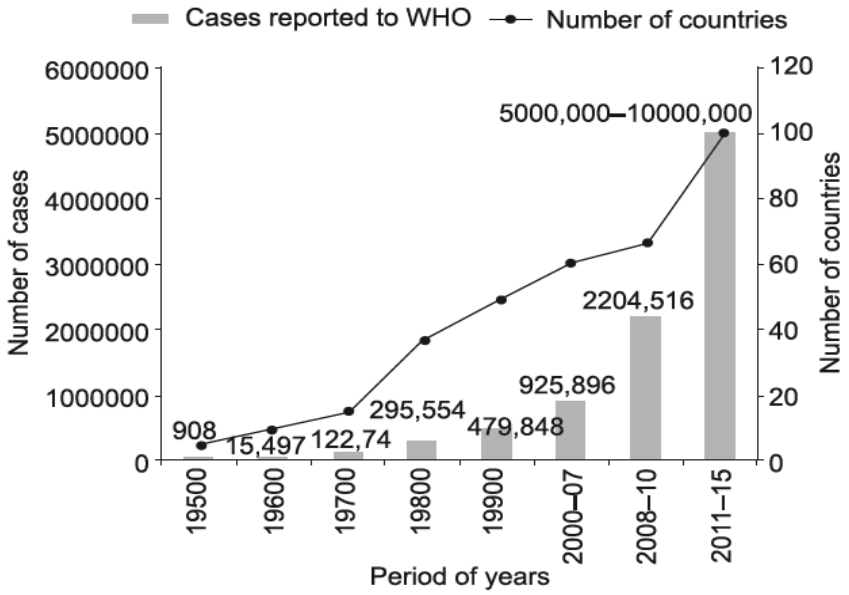


Figure 3 Number of dengue cases reported to the World Health Organization .[With minor modification; Source: Disease Surveillance and Epidemiology, WHO Southeast Asia Regional Office (SEARO).

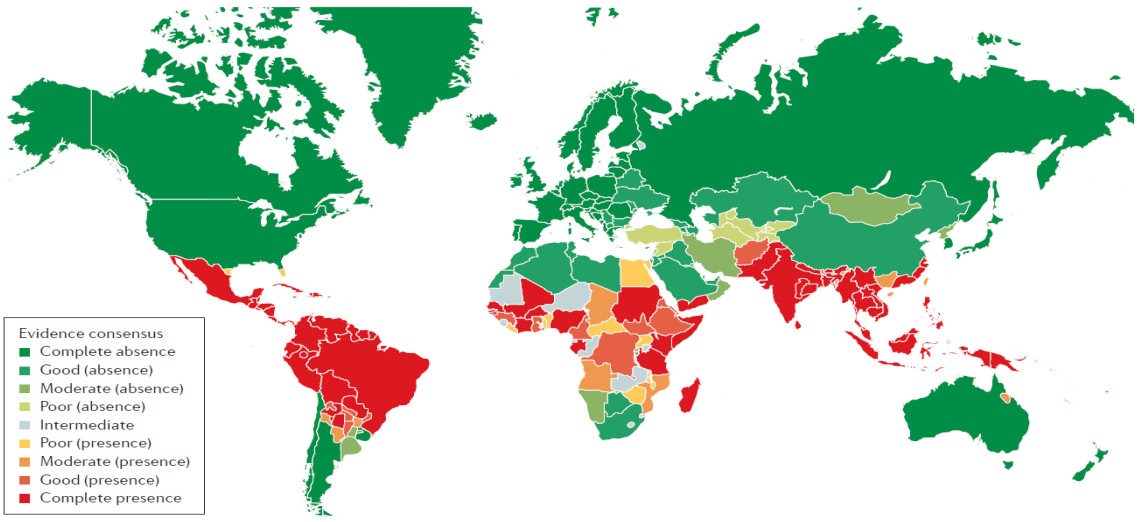


Figure 4 The suitability of different regions for DENV transmission The global evidence consensus, risk and burden of dengue is shown with evidence consensus on complete absence (dark green) through to complete presence (dark red) of dengue. (Guzman et al., 2016).

2.3. Phylogenetic tree analysis

2.3.1. DEN1V

Phylogenetic studies classified DEN1V into five genotypes, namely, I, II, III, IV, and V (Allicock et al., 2012). The genotypes I, II, and V were observed in Thailand. Genotype I is a major genotype whereas genotype II (Halstead and Simasthien, 1970) and genotype V are minor genotype (Zhang et al., 2005). Genotype I, V and IV have been geographically dispersed in SEA (Zhang et al., 2005; Klungthong et al., 2008), Pacific island region (Yamanaka et al., 2011; Sasmono et al., 2015) and Latin America region (Raghwani et al., 2011; Villabona-Arenas and Zanotto, 2013), respectively.

2.3.2. DEN2V

Phylogenetic studies classified DEN1V into five genotypes, namely, Asian I, Asian II, American, Asian/American and Cosmopolitan (Allicock et al., 2012). The genotypes Asian I, Asian/American and Cosmopolitan were observed in Thailand. Asian I genotype is a major genotype whereas Asian/American and Cosmopolitan are minor genotypes (Twiddy et al., 2002; Zhang et al., 2006). Asian I, Asian/American and Cosmopolitan genotype have been geographically dispersed in SEA (Zhang et al., 2006; Klungthong et al., 2008), Latin America (Nogueira, de Araujo and Schatzmayr, 2007) and Pacific island (Kotaki et al., 2016), respectively.

2.3.3. DEN3V

Phylogenetic studies have classified DEN3V into five genotypes, namely, I, II, III, IV, and V (Lanciotti et al., 1994). The genotypes II and III were observed in Thailand. Genotype II is a major genotype whereas genotype III is a minor genotype (Lanciotti et al., 1994; Zhang et al., 2005; Klungthong et al., 2008; Huang et al., 2012; Chen, 2013). Genotype I, II and III have been geographically dispersed in Pacific island (Sasmono et al., 2015), SEA (Lanciotti et al., 1994) and Latin America (Martins et al., 2014), respectively.

2.3.4. DEN4V

Phylogenetic studies have classified DEN3V into three genotypes, namely, I, II and III (Lanciotti et al., 1994). All genotypes were observed in Thailand (Klungthong et al., 2008). Genotype I is a major genotype whereas genotype II and genotype III are minor genotypes (Wang et al., 2000; Klungthong et al., 2004; Klungthong et al., 2008). Genotype I has been geographically dispersed in SEA (Lanciotti et al., 1994). Genotype II has been geographically dispersed in SEA (Haryanto et al., 2016) and Latin America (Villabona-Arenas et al., 2014).

3. Clinical signs of dengue disease

3.1. Signs and symptoms

Dengue is a dynamic illness, despite its short duration (no more than 1 week in nearly 90% of cases). Its clinical expression can change as the days go by and can also worsen suddenly. Dengue illness can evolve into three phases: the acute febrile phase observed in most of the patients and the critical and the recovery (convalescent) phases **(Table 2)**

Fever occurs during the acute febrile stage and is generally the first clinical manifestation of illness with a variable intensity. It is associated with headache and vomiting, as well as body pains. In children, fever is frequently the only clinical manifestation or is associated with rash and/or unspecific digestive symptoms. The pharynx can become reddened, but other signs and symptoms of the respiratory system are not frequent or clinically significant. Slight abdominal pain and diarrhea can occur; diarrhea more frequently occurs in patients who are <2 years of age and in adults. In general, compared with children, adolescents and adults show a 'flu-like syndrome' (including malaise, headache and body pains) with more prominent digestive symptoms than respiratory symptoms, if any. During the febrile stage, leukocyte counts are usually decreased. Petechiae (small spots on the skin caused by broken capillaries) or ecchymosis (large subcutaneous bleeding spots) can be present, with or without thrombocytopenia. After 2–5 days, these symptoms can be followed by rapid clinical deterioration. Most patients with dengue recover after defervescence; however, the clinical state of some patients worsens when the fever drops. Thus, the period during which the fever subsides indicates the beginning of the critical phase.

The critical phase coincides with the leakage of plasma that can lead to shock, which is characterized by coldness in the teguments, weak pulse, delayed capillary filling, tachycardia, oliguria and hypotension. Shock is caused by low blood volume (hypovolemia). At the beginning, not all clinical signs of shock are observed, and, in this setting, shock can be detected by a narrowing of the differential arterial tension or pulse pressure (a difference of ≤20 mmHg between the maximum or systolic arterial tension and the minimum or diastolic arterial tension). At this stage, patients usually have a flushed face, a warm trunk, cold and clammy extremities, diaphoresis (sweating), slow venous filling, restlessness, irritability, pain in the upper and middle abdomen and decreased urinary output. In addition, patients might also exhibit signs of impaired hemostasis, including scattered petechiae on the forehead and extremities, spontaneous ecchymosis, easy bruising and bleeding at venipuncture sites, and circumoral and peripheral cyanosis (blue skin discoloration). Gastrointestinal bleeding occurs in <10% of patients and usually follows a period of uncorrected hypotensive shock. Patients with shock also experience rapid and potentially labored breathing, a weak pulse and have a rapid heartbeat that sounds 'thready'. Finally, their livers are usually firm, tender and can become enlarged to 4–6 cm below the costal margin, the hematocrit level is increased and the platelets which were decreasing progressively reach their lowest count. In those who recover, this critical phase lasts for 24–36 hours and is followed by a rapid convalescence can involve complications, such as encephalopathy, bradycardia, ventricular extarsystoles and, rarely, myocarditis and encephalitis (Guzman et al., 2016).

Table 2 Key clinical terms (WHO, 1997)

Phase	Characterization
Acute phase	Characterized by high fever that is driven by high viral loads (viremia)
Critical phase	Characterized by plasma leakage into the abdominal and pleural cavities, which becomes evident at the end of the febrile (acute) stage of illness (days 3–6)
	Warning signs that announce shock are usually present
Convalescent phase	Involves both cessation of plasma leakage and reabsorption of leaked fluids
Dengue or dengue fever	A nonspecific febrile illness that is characterized by fever and the presence of two or more other symptoms, such as headache, rash, retro-orbital or ocular pain and myalgia
	Most patients have a satisfactory resolution without signs of severity or warning signs (referred to as dengue without warning signs according to the 2009 WHO classification)
Dengue haemorrhagic fever	Characterized by increased vascular permeability, plasma leakage, bleeding, thrombocytopenia and fever (according to the 1997 WHO classification)
	The term and concept are not included in the revised 2009 WHO classification nor are they recommended for triage of patients because it is not necessarily associated with severity, among other reasons
Dengue with warning signs	At the end of the febrile period, some patients have signs or symptoms that are suggestive of important fluid loss associated with capillary leakage (for example, severe abdominal pain), announcing the imminence of shock and indicating that fluid replacement is urgently required (according to the 2009 WHO classification)
Severe dengue	Circulatory shock or respiratory distress associated with severe plasma leakage, severe bleeding or severe organ involvement (frequently myocarditis, encephalitis and severe hepatitis) with or without shock or bleeding (according to the 2009 WHO classification)

3.2. WHO 1997 case classification systems

Early clinical diagnosis of DENV infection cannot be differentiated from other acute febrile illnesses (AFI) (Thai et al., 2010). Symptoms of DENV infection range from inapparent febrile illness to severe and fatal hemorrhagic infection (Gubler, 1998).

The mortality rate of young children higher than older children and adults. Clinical manifestation by DENV infection is classified to 4 symptoms consist of undifferentiated fever, dengue fever, dengue hemorrhagic fever and dengue shock syndrome with the latter classified as grade I, II, III, or IV. The disease has three phases consists of an initial febrile phase, a critical phase around the time of defervescence, a spontaneous recovery phase. The symptoms of initial febrile phase are characterized by high fever, headache, vomiting, myalgia, and joint pain. On days 3-7, a critical phase is associated with hemoconcentration, hypoproteinemia, pleural effusions, vascular leakage, hemorrhage, severe abdominal pain, tender hepatomegaly, high hematocrit and a decreasing of platelet count. In a spontaneous recovery phase, the symptom is reversed to a regular level in 48 to 72 hours (Whitehorn and Simmons, 2011). Infection with any of the four serotypes causes a similar clinical presentation that may vary in severity. Incubation period of DENV infection is 3 to 7 days. A study reported that the skin is flushed and a positive tourniquet test was early diagnosed dengue in febrile patients (Tantawichien, 2012). A definitive diagnosis of dengue severity could be made only with the development of thrombocytopenia and plasma leakage, which usually occurs only 1–2 days before the onset of shock (Kalayanarooj et al., 1997).

3.2.1. Undifferentiated fever

Undifferentiated fever (UF) of DENV infection is described by person with acute febrile illness and laboratory confirmed DENV infection (Thai et al., 2010). Undifferentiated fever of DENV infection is usually occurring in primary infection and maybe occur in secondary infection. Clinical symptoms are indistinguishable from other viral infections (Malavige and Ogg, 2012). DENV is considered to be one of the major causes of undifferentiated fever (Pradutkanchana et al., 2003) and caused one-third of all cases of acute undifferentiated non-malarial fever in an area of Vietnam (Phuong et al., 2006).

3.2.2. Dengue fever

The clinical features of DF frequently depend on the age of patients. Infants and young children may have an undifferentiated febrile disease with maculopapular rash. Older children and adults may have a mild febrile syndrome, high fever, severe headache, pain behind the eyes, muscle and bone or joint pains, nausea and vomiting, and rash. Recovery may be associated with prolong fatigue and depression, especially in adults. DF may be accompanied by bleeding complication, such as gingival bleeding, gastrointestinal bleeding, and hematuria (WHO, 1997;2009). In Hong Kong, Chuang and colleagues found that 123 cases from 126 cases of patients were infected with DF and more than one third of patients had gastro-intestinal and upper respiratory complaints (Chuang et al., 2008).

3.2.3. Dengue haemorrhagic fever

The clinical features of DHF is characterized by four major clinical manifestations consist of high fever, hemorrhagic phenomena, hepatomegaly and circulatory failure. Common hemorrhagic manifestations include skin hemorrhages such as petechiae, purpuric lesions, and ecchymosis. A thrombocytopenia and hemoconcentration are constant findings in DHF. Hemoconcentration, indicating plasma leakage, is almost always present in DHF. The positive tourniquet test, which indicates that the patients has increased capillary leakage help for diagnosis of medical technician (Gubler, 1998).

3.2.4. Dengue shock syndrome

DSS is usually characterized by severe vascular leakage, disordered hemostasis, a rapid, weak pulse with narrowing of the pulse pressure (20 mmHg), restlessness and progresses to death if treatment is not appropriate (WHO, 1997).

3.3. WHO 2009 case classification systems

According to the 2009 WHO clinical classification, a patient can have dengue with or without warning signs or severe dengue, highlighting that severity is considered as the second step of the same disease. In other words, dengue can be considered to be a single disease entity that is both systemic and dynamic

There are somewhat competing views in the field as to the optimal approach for the clinical classification of patients with dengue and the identification of warning signs of severe disease, and several reviews and position papers regarding the usefulness of the 2009 WHO system compared with the 1997 WHO system have been published (Horstick, 2014). Prospective clinical studies developed in Asian and Latin-American countries have concluded that the 2009 WHO dengue classification system may be better at detecting severe DENV infection cases (Zakaria, 2014). Others have argued that the revised 2009 WHO classification has a high sensitivity for identifying severe dengue and is easy to apply (Prasad et al., 2013); some consider the 2009 system to be promising from both research and clinical perspectives (van de Weg et al., 2012). Indeed, the 2009 classification system has greater discriminatory power for detecting patients who are at risk of progression to severe disease and those who need hospitalization than the 1997 classification (Lovera, 2014). Furthermore, the 2009 system is simple to use for triage and case management according to disease severity, even in primary care settings (Gibson, 2013), and for disease surveillance. It also reflects the natural course of dengue illness from mild to severe disease and covers all clinical manifestations (Pamplona, 2014). A formal expert consensus was reached in La Habana, Cuba, in 2013 with dengue experts from the Americas, where a decrease in disease lethality after the introduction of the revised classification was evident (Guzman et al., 2016).

That said, through the analysis of retrospective data, some investigators have found that warning signs are not as useful in adults as they are in children (Thein et al., 2013), and have argued that the current recommended predictors of severe dengue are, therefore, limited (Premaratna et al., 2013). Others have put forward that there is a need for a more precise definition of warning signs to enable optimal triaging for accurate identification of patients who require hospitalization (Hadinegoro, 2012). In addition to these critiques, one study described that both the 1997 and the 2009 WHO

classification systems show high sensitivity but lack specificity (Chaterji, 2011), and that the 2009 system requires refined definitions of severe bleeding and organ impairment to improve its clinical relevance (Gan, 2013). A major ongoing clinical study, coordinated by one of the three large European Union-funded consortia that are currently working on dengue research themes, might address some of these issues (Jaenisch, 2014). Finally, since the introduction of the revised criteria, a high number of patients have been admitted to hospital or placed under clinical observation during dengue epidemics. This increase is probably owing more to traditional hospital-based methods of managing patients with dengue than to the 2009 WHO classification system, a conclusion that is supported by the fact that this increase in clinical intervention can be alleviated through the participation of trained primary care health units, which the WHO is trying to facilitate (Guzman et al., 2016).

Although the 2009 WHO classification is more applicable to clinical and epidemiological purposes than the 1997 classification, debate continues regarding its usefulness for pathogenesis research (Simmons, 2015). In particular, some have argued that the dengue fever, DHF and DSS classifications were more capable of correctly identifying cases of plasma leakage than the 2009 system, and that this identification served as a useful predictor of disease severity that was directly related to the main underlying model of pathogenesis. However, in a separate study, the same authors concluded that the 1997 system misclassified a substantial proportion of patients (Srikiatkhachorn, 2010). Specifically, only 68% of patients who were in need of clinical intervention were classified as having DHF and, therefore, in using this system, it could be inferred that 32% of severe cases would be missed. One of these studies has been analyzed by a group of experts (Akbar, 2012), who concluded that the revised classification reflects clinical severity in real time, which is something that clinicians have wanted for some time, and with its simplified structure will facilitate effective triage and patient management and also allow collection of improved comparative surveillance data.

4. Laboratory diagnosis

DENV compose of four antigenically distinct serotypes (DEN1V, DEN2V, DEN3V, and DEN4V) which share some antigens within group and with other *Flavivirus* such as Japanese encephalitis virus, West Nile virus, Yellow fever virus. In endemic area, DENV co-circulates with two or more *Flavivirus* which show similar clinical presentation. The clinical findings are not useful for differentiate dengue infection with other febrile illness. The accurate laboratory diagnosis of DENV infection is important for clinical care, case confirmation to differentiate dengue infection with other infection and for the clinical management (Guzman and Kouri, 2004). The common laboratory diagnosis of DENV infection is based on virus isolation (Lam et al., 1986), serodiagnosis (Vaughn et al., 1998) and molecular technique (Lanciotti et al., 1992)**.** Serology is the most widely used in routine diagnosis (Guzman and Kouri, 2004). Sensitivity of each method depends on period of patient's illness (Guzman et al., 2016).

4.1. Virus isolation

Virus isolation can be performing only in early stage of the disease, during viremia period **(Figure 5).** Serum is sample of choice when perform virus isolation but other sample such as plasma, leukocytes, whole blood, and autopsy tissue also can be used. Due to DENV is heat-labile, must be storage sample in 4ºC for short-term and -80°C for long-term. DENV was first isolated in 1942 by Sasamu. Blood samples of dengue patients in Nagasaki–Sasebo region of Japan were taken within 48 hours after onset of fever and were inoculated intracerebrally (IC) into suckling white mice. The symptoms of infected mice were debility, tremor or paralysis Hotta (Hotta, 1952). In 1960s, suckling mice became as standard method for isolation of DENV. In 1970s, Standard method for DENV isolation was developed into mosquito inoculation. Four mosquito species have been used: *Toxorhynchites splendens*, *Tx. amboinensis*, *Ae. albopictus*, and *Ae. aegypti*. The both sexes of mosquito are susceptible to DENV infection (Rosen and Gubler, 1974). After 4-14 day incubation, DENV was detected by indirect immunofluorescence assay (IFA) (Rosen et al., 1985). Mosquito inoculation is high sensitivity but required hardwork, need of expert. Cell culture inoculation is widely used for routine DENV isolation. Three mosquito derived cell lines have high susceptibility for DENV infection: AP-61 from *Ae. pseudoscutellaris*, C6/36 from *Ae.* albopictus and TRA- 284 from *Tx. amboinensis*. The C6/36 cell is the most widely used (Gubler et al., 1984). Mammalian cell line is also commonly used for virus isolation include BHK-21 (baby hamsters kidney), LLC-ML2 and Vero (monkey kidney). The disadvantage of mammalian cell is the lower effective for dengue infection than the mosquito cell lines (Liu and Wu, 2004). Virus identification can do by indirect immunofluorescent technique (IFA). Serotype-specific monoclonal antibody is used for antigen detection in infected cell line, mosquito or mouse brain tissue. IFA is currently used for identification of infected-cell culture (Henchal et al., 1983).

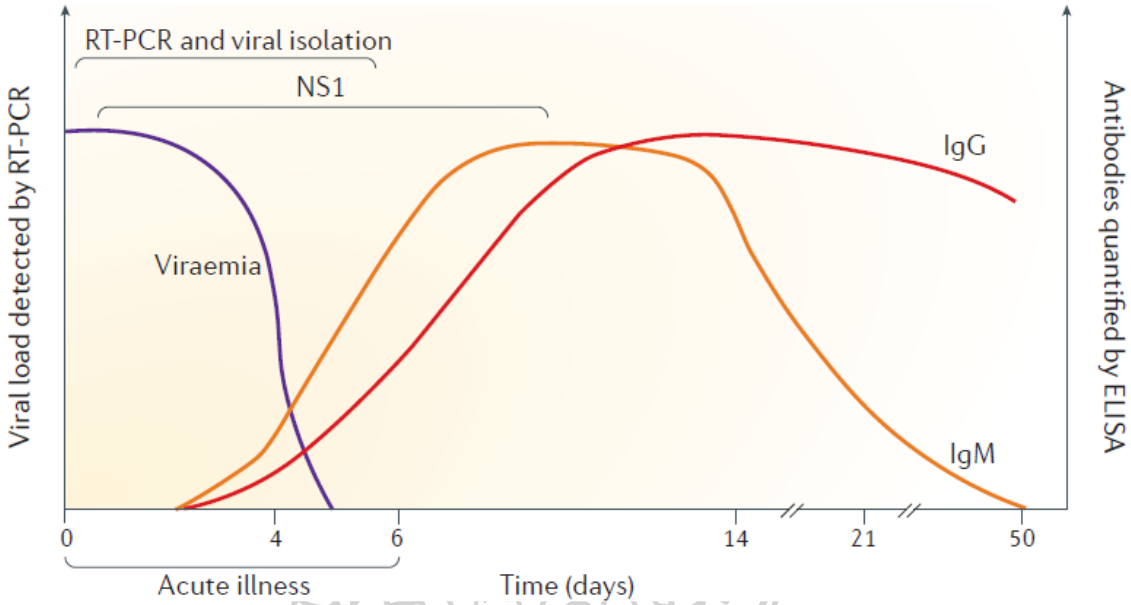


Figure 5 Dengue diagnosis. Viraemia, non-structural 1 (NS1) antigen and antibodies change over time; thus, different diagnostic tests will be appropriate depending on the stage of infection. ELISA, enzyme-linked immunosorbent assay (Guzman et al., 2016).

4.2. RT-PCR

Genome detection is the important diagnostic tools of DENV infection. In 1987, Henchol and colleagues developed slot-blot nucleic acid hybridization using radioactive-labeled cDNA probe to detect dengue 2 virus (Henchal et al., 1987). Polymerase chain reaction (PCR)-based method is the most widely used due to rapid, high sensitivity and high specificity. In 1991, Henchal and colleagues developed RT-PCR to diagnostic DENV infection in acute serum (Henchal et al., 1991). In 1992, Lanciotti, et al. developed nested RT-PCR technique that increases sensitivity and specificity of assay. The researcher design primer for first-round PCR, locate at C/prM of viral RNA genome, to amplify 511 bp PCR product. PCR product from first-round PCR is diluted 1:100 and used as template for second round PCR. In the second round PCR, serotype-specific primers is used for amplify each serotype into difference PCR product size (Lanciotti et al., 1992)**.**

4.3. qRT-PCR

Real-time Polymerase Chain Reaction (Real-time PCR) or quantitative real time polymerase chain reaction (qPCR) used to amplify and simultaneously quantify the target DNA. Frequently, real-time polymerase chain reaction is used for detect and quantify gene expression from small amounts of RNA. The RNA sample is reverse transcribed to cDNA and then amplify by PCR. The common methods of quantification of Real-time PCR are; (1) the use of fluorescent dyes that intercalate with double-stranded DNA such as SYBR green, and (2) modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA; hydrolysis probe and hybridization probe **(Figure 6)** (van der Velden et al., 2004).

SYBR green technique is the most frequently used dye in nonspecific detection systems. SYBR green I is a dye which detects PCR products by the DNA-intercalating dye SYBR Green I. The dye binds to the minor groove of dsDNA, During the consecutive PCR cycles, the amount of double-stranded PCR product will exponentially increase, and therefore more SYBR Green I dye can bind and emit its fluorescence (at 520 nm) (van der Velden et al., 2004).

The real-time RT-PCR assay (qRT-PCR) is the quantitative methods for DENV detection and typing. The fluorogenic-probe-based 5’ exonuclease assay (Taqman) was applied to monitoring the target PCR product amplification. Many qRT-PCR methods were developed both simplex and multiplex platform. These protocols vary in target site on viral RNA genome (Warrilow et al., 2002; Johnson, Russell and Lanciotti, 2005; Kong et al., 2006). The advantages of this assay are rapid, quantitative, high sensitivity and high specificity. The real-time RT-PCR assay can be used to quantitate the viremia in the patients with acute phase of fever to predict the severity of the disease (Vaughn et al., 2000).

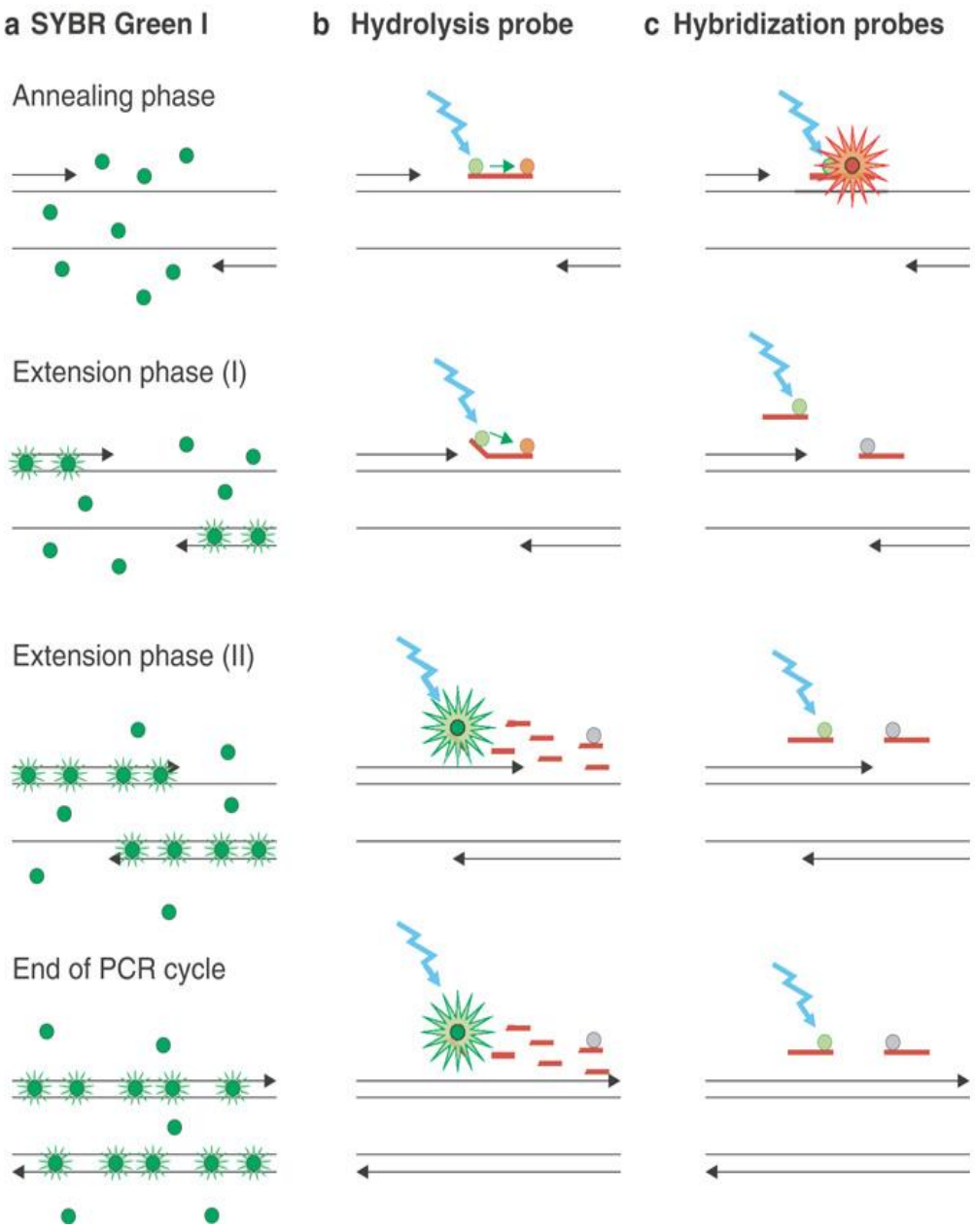


Figure 6 Principles of qPCR techniques. (a) SYBR green I qPCR, (b) Hydrolysis probe qPCR and (c) Hybridization probe qPCR (van der Velden et al., 2004).

5. Dengue vaccine

5.1. Inactivated vaccine

Vaccination with inactivated vaccines ideally should induce a balanced immune response without the viral interference (where in the replication of one virus can inhibit the generation of a balanced immune response against all four serotypes as it can interfere with the replication of the other serotypes) that can occur with live attenuated vaccines. In addition, there is no risk of viral replication or reversion to wild-type virus. Inactivated vaccines are less effective in inducing long-lasting immunity than live attenuated vaccines, so multiple doses and adjuvants are needed for optimal immunogenicity in unprimed individuals. A dengue inactivated vaccine might be useful as part of a heterologous prime–boost vaccine regimen (Yauch and Shresta, 2014).

A dengue purified formalin-inactivated vaccine (DPIV) is being developed and has been shown to be immunogenic in rhesus macaques. A phase I trial began in 2011, and two phase I trials of a tetravalent candidate began in 2012 in a dengue-primed population and in a non-endemic area (Yauch and Shresta, 2014).

5.2. Live attenuated vaccine

Live attenuated vaccines have numerous advantages, including the ability to induce an immune response that mimics the response to natural infection, the induction of robust B cell and T cell responses and the ability to confer lifelong immune memory. Live attenuated vaccines can be produced at relatively low cost and might be effective after one dose (Yauch and Shresta, 2014). Early dengue vaccine efforts focused on passaging wild-type DENV strains through various types of primary cells or cell lines, including primary dog kidney (PDK) and African green monkey kidney (GMK) cells. Passaging of DENV in vitro renders it less virulent in humans and was investigated in two series of work.

In the first series, vaccine strains from each serotype obtained by passage through PDK cells or primary GMK cells were selected and tested in monovalent, bivalent, trivalent and tetravalent vaccinations in Thai adults (Bhamarapravati and Sutee, 2000). Of the tetravalent recipients, only one of ten seroconverted to all four serotypes, and neutralizing antibody responses were directed primarily against DEN3V. Subsequently, several tetravalent vaccine formulations were tested and the dominant neutralizing antibody response was still against DEN3V (Sabchareon et al., 2002; Sabchareon, 2004). Following on from these studies, the DEN3V vaccine strain was re-derived genetically, grown in Vero cells and tested in volunteers (Sanchez, 2006). All recipients had adverse reactions and the trial was halted (Yauch and Shresta, 2014).

In the second series, different formulations of the tetravalent vaccine were tested in monkeys and *Flavivirus*-naive adults and children (Simasathien et al., 2008). The formulations were improved to reduce the reactogenicity and increase the immunogenicity (Thomas, 2013). These new formulations were safe and moderately effective, and the authors recommend that studies in a larger number of adults and then in children are warranted (Yauch and Shresta, 2014).

Another attenuation strategy is the targeted mutagenesis of 3' UTR regions of DENV RNA. The viral 3' UTR is approximately 450 nucleotides long and

comprises four defined domains: domain A; domains A2 and A3, which seem to work as enhancers for viral RNA replication; and domain A4 and the 3´ stem loop, which are essential elements for viral replication (de Borba, 2015). The deletion was created by the removal of nucleotides 172–143 from the 3' UTR. This deletion, designated Δ30, has been shown to attenuate DEN1V and DEN4V in rhesus monkeys and to inhibit dissemination of DENVs in mosquitoes (Whitehead, 2003b;2003a). Monovalent and tetravalent preparations have been given to human volunteers and produced good immune responses (Durbin, 2011). A phase I trial investigated a single dose of four different formulations of a live tetravalent vaccine in *Flavivirus*-naive volunteers. The vaccines were well tolerated, produced no severe adverse events and only one dose induced a good neutralizing antibody response in 75–90% of the individuals (Durbin, 2011). One of these tetravalent DENV vaccines was licensed to several vaccine developers (Kirkpatrick et al., 2015) and entered large-scale phase III efficacy trials in Brazil following a small human challenge trial conducted in the United States. A single dose of the dengue vaccine TV003 fully protected 21 vaccinated volunteers against infection in a virus challenge study, whereas 20 unvaccinated controls all developed an infection (Kirkpatrick, 2016).

In addition, a candidate tetravalent dengue vaccine (called CYD-TDV) has been developed, via the insertion of the prM and E genes of the four DENV serotypes into the genetic backbone of the 17D yellow fever vaccine virus (Guy et al., 2015). Two ChimeriVax phase III trials were conducted in >30,000 children in five Asian and five American countries. Overall efficacy in the Asian trial was 56.5% and 60.8% in the American trial (Capeding, 2014; Villar, 2015). In addition, a reduction in severe complications was reported with a vaccine efficacy of >80% against DHF. These vaccines seem to boost immune responses and protect individuals who have had one previous DENV infection and are, therefore, at risk of ADE. However, these vaccines failed to protect seronegative individuals against clinical infection with all four DENV serotypes, and a group of young vaccinated children had higher rates of hospitalized breakthrough DENV infections than controls (Hadinegoro, 2015). Children who were ≤5 years of age when vaccinated experienced a DENV disease resulting in hospitalization at five times the rate of controls. The etiology of disease in placebo and vaccinated children that results in hospitalization during a DENV infection, while clinically similar, are of different origin. The implications of the observed mixture of DENV protection and enhanced disease in CYD vaccinees is under study (Halstead and Russell, 2016). CYD-TDV seems to protect people who have been infected once and, accordingly, are at risk of severe disease. But, conversely, it puts people who were susceptible to a first infection at risk of severe disease. Even so, the vaccine is approved in Mexico, the Philippines and Brazil.

Another vaccine construct has been developed by substituting the prM and E genes of DEN2V PDK-53 with those of wild-type DEN1V, DEN3V or DEN4V (Huang, 2003). Three different formulations of these tetravalent vaccine (DENVax) were tested in monkeys, and all vaccinated monkeys developed neutralizing antibodies against all four serotypes after one or two doses (Osorio et al., 2011). On the basis of these results, phase I and phase II trials were carried out to evaluate different vaccination regimens, formulations and alternative routes of immunization (Osorio, 2014). The vaccine was well tolerated in children and adults 1.5–45 years of age, irrespective of prior dengue exposure; mild injection-site symptoms were the most

common adverse events. DENVax induced a neutralizing antibody response and seroconversion to the four DENVs, as well as cross-reactive T cell-mediated responses that could be necessary for a broad protection against dengue illness (Osorio, Wallace and Stinchcomb, 2016). Currently, phase III trials of the vaccine have been initiated in several Asian countries.

Following on from live attenuated vaccines, another generation of vaccine candidates, including subunit vaccines, inactivated vaccines, DNA vaccines and viral vector vaccines, is being launched.

5.3. Subunit vaccine

The advantages of protein vaccines compared with live attenuated vaccines are that they are safe, the induction of a balanced immune response to the four DENV serotypes should be feasible and the immunization schedule can be accelerated, reducing the risk of incomplete immunity and the potential for ADE. However, these vaccines require the use of adjuvants and multiple doses to achieve optimal immunogenicity, and they may not be as efficient as live attenuated vaccines at inducing long-lasting immunity (Yauch and Shresta, 2014).

The protein target of subunit vaccine development for dengue has been the E glycoprotein, as the majority of neutralizing epitopes on the DENV virion are located in this protein. Recombinant E has been produced using *Escherichia coli*, baculovirus and insect cells, yeast and mammalian cells (Simmons et al., 2001; Guzman, 2003; Kuwahara and Konishi, 2010). Truncated recombinant E subunits (80E) of each serotype were obtained in a *Drosophila melanogaster* Schneider 2 cell expression system and were found to induce neutralizing antibody responses in mice and in non-human primates (Clements, 2010). A phase I trial of the DEN1V-80E vaccine candidate has been completed (Coller et al., 2011) and a phase I trial of a tetravalent formulation began in 2012. The subunit vaccine might be an important component in a prime–boost vaccine regimen.

Domain III-capsid (DIII-C) is a novel candidate vaccine containing viral fragments that might potentially induce neutralizing antibodies and cell-mediated immunity. DIII-C has been evaluated in Balb/c mice and Vervet monkeys (Marcos et al., 2013; Suzarte et al., 2015). In animal models, DIII-C has been shown to induce a serotype-specific immune response in terms of both antiviral antibodies and cellular immune response with partial protective efficacy (Costa, 2006). This candidate is at an advanced stage of preclinical development.

CHAPTER IV
MATERIALS AND METHODS

Part A: To evaluate the molecular epidemiology of DENV isolates of patient sera in C6/36 cells by DNA sequencing and phylogenetic tree

1. Viruses and cell culture

DEN1V (16007-strain), DEN2V (16681-strain), DEN3V (16562-strain), DEN4V (1036-strain) and DEN4V (1036 PDK40-strain) were donated from Professor Dr. Sutee Yoksan, Center for Vaccine Development, Institute of Molecular Bioscience, Mahidol University. The viral titers were approximate 1×10^5 pfu/ml. These viruses were used as the viral nucleic acid/positive standard in this study.

C6/36 cells were cultured in Modified Eagle Medium, (MEM, GIBCO, NY, USA) containing 10% Fetal bovine serum (FBS, GIBCO), 2 mM L-glutamine (GIBCO), 1% sodium bicarbonate (Sigma-Aldrich, MO, USA) and 1% non-essential amino acid (GIBCO). Cells were incubated at 32°C.

2. Patient sera

A total of 326 patient sera with suspected dengue were collected during 2006-2015 donated by Professor. Dr. Sutee Yoksan, Center for Vaccine Development, Institute of Molecular Bioscience, Mahidol University.

3. Virus isolation in C6/36 cells

A total of 100 µl of patient sera were seeded in completed-monolayer C6/36 cells in T25 cm^2 containing completed medium (MEM+10% FBS). The infected cells were incubated in 32°C for 3 days. The DENV-infected supernatants were inoculated into new monolayer C6/36 cells for 3 days. The virus isolates were stored at -80°C prior to molecular detection.

4. RNA extraction

Viral RNA was extracted from samples using E.Z.N.A viral RNA mini kit (Omega biotek, USA), in accordance with the manufacturer's instructions. Briefly, 150 µl of samples were mixed with 560 µl carrier-added QVL buffer and incubated for 10 mins. After lysis, the samples were mixed with absolute ethanol. The mixture was transferred to spin column. The sample-filled columns were centrifuged and washed with VHB buffer and RWB2 buffer. RNA was eluted with 50 µl of DEPC water. The eluted RNA was stored at -80 °C until use.

5. cDNA synthesis

The cDNA was synthesized using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA), in accordance with the manufacturer's instructions. Briefly, in a total volume of 20 µl, 13 µl of viral RNA was mixed with 100 pmol random hexamer, 0.5 mM dNTP, RT buffer, and Maxima H Minus Enzyme. The RT-PCR mixture was incubated 10 mins at 25 °C, 30 min at 50 °C and

5 mins at 85 °C. cDNA synthesis was conducted on Tpersonel 48 (Biometra, Germany). The cDNA was stored at -20°C until use.

6. PCR and cloning of DEN1V-4 plasmids

Serotype-specific primer set was designed by Johnson et al. [2005] and used to amplify each serotype of DENV **(Table 3).** DENV 1-4 was amplified using KAPA Taq ReadyMix PCR Kit (KAPA biosystems, Massachusetts, USA), in accordance with the manufacturer's instructions. Briefly, in a total volume of 20 μl, 2 μl of cDNA was mixed with 0.5 μM forward primer, 0.5 μM reverse primer, KAPA Taq ReadyMix and nuclease-free water. The PCR mixture was incubated 2 mins at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 55 °C and 30 sec at 72 °C. PCR was conducted on Tpersonel 48. Molecular sizes of PCR products were determined using 2% agarose gel electrophoresis with SYBR safe (Thermo Scientific).

Positive PCR product of each serotype was subsequently cloned to pCR2.1 vector (Thermo Scientific; **Figure 7**), in accordance with the manufacturer's instructions. Briefly, in a total volume of 6 μl, 4 μl of fresh PCR product was mixed with salt solution and TOPO vector. The mixture was incubated for 5 mins at room temperature. The plasmids were placed on ice and proceeded to transformation.

7. Transformation of DEN1V-4 plasmids

Plasmid was transformed to *E. coli* DH5α (RBC bioscience, Taiwan), in accordance with the manufacturer's instructions. Briefly, 3 μl of plasmid was mixed with competent cells by vortex. The mixture was placed on ice for 10 mins and then spreaded on Luria-Bertani (LB) agar (Becton Dickinson, New Jersey, USA) containing 100 μg/ml ampicillin (General drugs house, Thailand), X-gal (Thermo Scientific) and IPTG (Thermo Scientific). Plate was incubated overnight at 37 °C. White colonies were subcultured on LB agar and proceeded to confirmation by colony PCR.

8. Colony PCR

M13 forward sequencing primer (-20; GTAAAACGACGGCCAGT) and M13 reverse sequencing primer (-24; AACAGCTATGACCATG) were used for confirmation of insert in recombinant *E. coli*. Selected colonies were mixed to PCR mastermix and proceeded to PCR protocol as described above. Positive colony was subsequently proceeded to plasmid extraction.

Table 3 Primer and probe sets used in DENV qRT-PCR.

Name	Nucleotide sequences	Genome position	Fluorophore
DEN1V F	CAAAAGGAAGTCGTGCAATA	8936-8955	
DEN1V C	CTGAGTGAATTCTCTCTACTGAACC	9023-9047	
DEN1V probe	CATGTGGTTGGGAGCACGC	8961-8979	FAM/BHQ-1
DEN2V F	CAGGTTATGGCACTGTCACGAT	1426-1447	
DEN2V C	CCATCTGCAGCAACACCATCTC	1482-1504	
DEN2V probe	CTCTCCGAGAACAGGCCTCGACTTCAA	1454-1480	HEX/BHQ-1
DEN3V F	GGACTGGACACACGCACTCA	701-720	
DEN3V C	CATGTCTCTACCTTCTCGACTTGTCT	749-775	
DEN3V probe	ACCTGGATGTCGGCTGAAGGAGCTTG	722-747	TR/BHQ-2
DEN4V F	TTGTCCTAATGATGCTGGTCG	884-904	
DEN4V C	TCCACCTGAGACTCCTTCCA	953-992	
DEN4V probe	TTCCTACTCCTACGCATCGCATTCCG	939-960	Cy5/BHQ-3

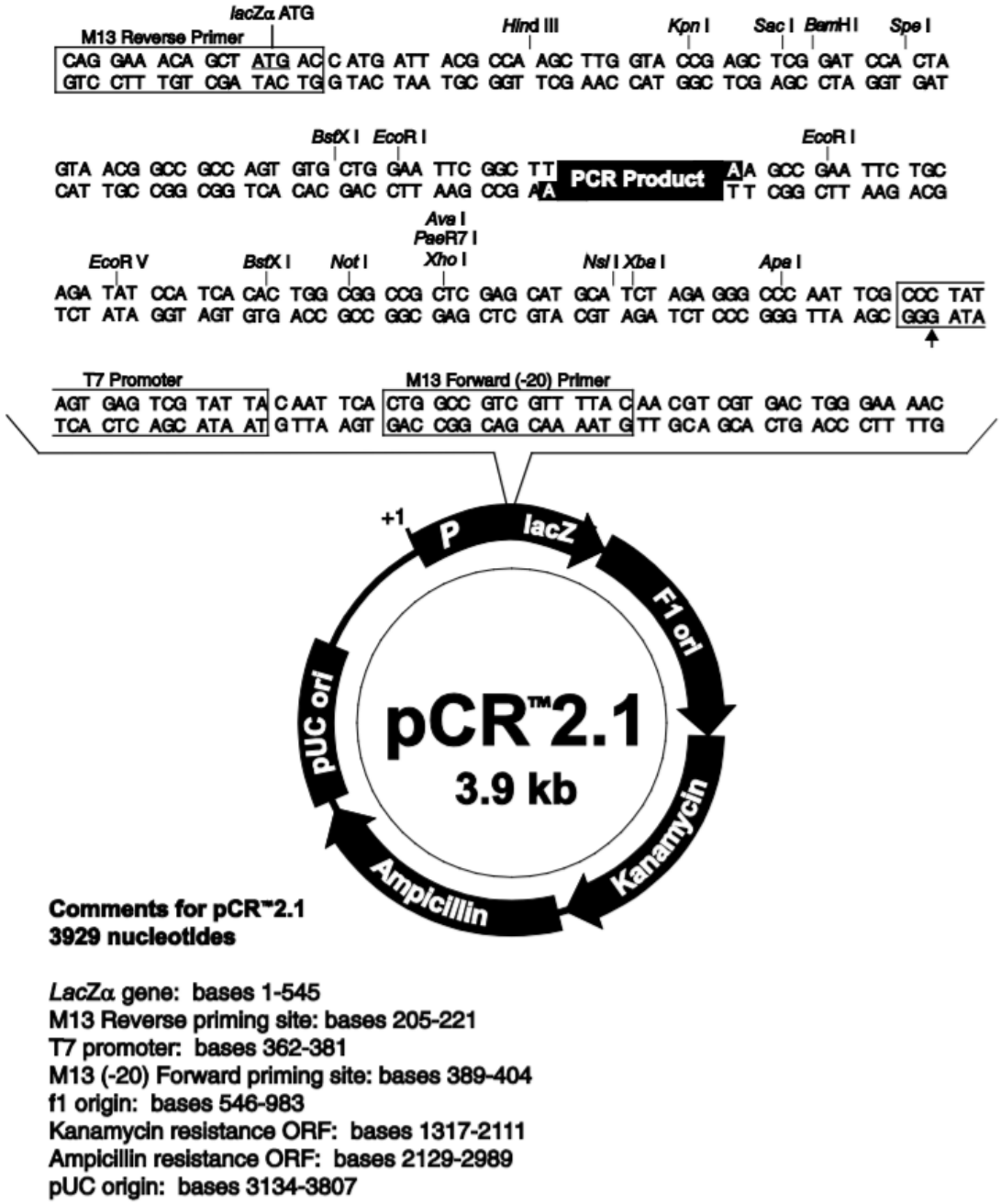


Figure 7 Topo pCR2.1 plasmid

9. Plasmid extraction

Plasmid was extracted from *E. coli* using QIAprep Spin Miniprep Kit (Qiagen, Germany), in accordance with the manufacturer's instructions. Briefly, selected colony was subcultured to LB broth containing 100 μg/ml ampicillin and incubated 18 h at 37 °C in a shaking incubator. Cell pellet was harvested by centrifuged for 5 mins at 13,000 RPM. Cell pellet was resuspensed in P1 buffer containing RNase A. The mixture was mixed with P2 and P3 buffer and then centrifuged for 10 mins at 13,000 RPM. The mixture was transferred to spin column. The sample-filled columns were centrifuged and washed with PB buffer. Plasmid was eluted with 50 μl of EB buffer. The eluted plasmid was stored at -20 °C until use.

10. DNA sequencing and sequence analysis

PCR product or plasmid was sequenced by the Sanger method (First BASE Laboratories, Malaysia). DNA sequences were manipulated using BioEdit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). Basic Local Alignment Search Tool (BLAST; https://www.ncbi.nlm.nih.gov/BLAST/) was used to analyzed the sequence.

11. Standard curve preparation

DENV 1-4 plasmids were quantified by spectrophotometer using Nanodrop. Copy no. of plasmid was calculated by following equation:

DNA (copy) = (6.02×10^{23} (copy/mol) × DNA amount (g)/(DNA length (bp) × 660 (g/mol/bp)) (Lee et al., 2006).

DENV 1-4 plasmids were 10-fold serial diluted in nuclease-free water (10^4-10^1 copy no./μl). Each dilution of DENV 1-4 plasmids was used as standard curve in qRT-PCR. Standard plasmids were stored at -20 °C until use.

12. qRT-PCR

qRT-PCR was performed following the literature protocol of Johnson et al. (2005) Briefly, viral RNA was extracted from samples. Maintenance medium was used as negative control. Serotype-specific primer and probe set (**Table 3)** were used for detect DENV genome in infected supernatant. Viral RNA was amplified using KAPA PROBE FAST Universal One-Step qRT-PCR Master Mix Kit (KAPA biosystems), as followed manufacturer's instruction). Briefly, in a total volume of 20 μl, 5 μl of sample RNA was mixed with 50 pmol (each) of DENV 1-4 primers, 9 pmol of each DENV 1-4 probe, KAPA PROBE FAST qPCR Master Mix, KAPA RT Mix and PCR grade water. qRT-PCR was conducted on Chromo4 (Bio-Rad, California, USA). The qRT-PCR mixture was incubated 5 mins at 42 °C. 5 mins at 95 °C followed by 40 cycles of 3 sec at 95 °C and 30 sec at 60 °C with data acquisition. Positive results were determined according to the amplification cycle at which the fluorescence will detect above the threshold cycle (C_T) relative fluorescence unit (RFU). Viral titers were quantified by comparison with a DENV 1-4 standard curve and were presented as copies/μl.

13. DENV 1-4 envelope RT-PCR and DNA sequencing

Serotype-specific E primers were used **(Table 4)** to amplify envelope gene of DENV 1-4 (1.5 kb). cDNA was amplified using Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific), as followed manufacturer's instruction). Briefly, in a total volume of 100 µl, 4 µl of cDNA was mixed with 50 pmol (each) of forward and reverse primers, Phusion Flash PCR Master Mix and nuclease-free water. The PCR mixture was incubated 10 sec at 98°C, followed by 30 cycles of 1 sec at 98°C, 5 sec at 55 °C and 30 sec at 72 °C. Molecular sizes of PCR products were determined using 1% agarose gel electrophoresis with SYBR safe. PCR products were sequenced and analyzed as descripted above.

14. Phylogenetic tree construction

Each serotype of DENV E sequence data from GenBank were included in this study (Appendix B). Sequence multiple alignments were performed using ClustalW (Thompson, 1994). The phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software (Tamura et al., 2013). Neighbour-joining tree was constructed with 1,000 bootstrap replicates.

Table 4 Primer set used in the DENV E RT-PCR and DNA sequencing.

DENV serotype	Name	Primer sequence 5′→3′	Position
DEN1V	DG1 (E1)-F	AGT-AGA-GAC-TTG-GGC-TCT-GA	802
	DG1 (E2)-R	CCA-GTT-GAT-TAC-ACA-TCC-CG	2443
DEN2V	DG2 (E1)-F	CAG-CTG-TCG-CTC-CTT-CA	914
	DG2 (E2)-R	GCT-CTA-GAT-CGG-CCT-GCA-CCA-T	2423
DEN3V	DG3 (E1)-F	GCC-CAT-TAC-ATA-GGC-ACT-TCC	857
	DG3 (E2)-R	ACA-CA**Y**-CCC-ATG-TCA-GCT-TG	2427
DEN4V	DG4 (E1)-F	CTC-TTG-GCA-GGA-TT**Y**-ATG-GC	843
	DG4 (E2)-R	CAC-TCC-ATG-ACA-CCA-CAC-AAC-C	2460

Part B: To evaluate biological marker of constructed D4 1036 PDK40 infectious clone

15. Virus and cell cultures

Live-attenuated DEN4V 1036 PDK40 were donated from Professor Dr. Sutee Yoksan, Center for Vaccine Development, Institute of Molecular Bioscience, Mahidol University. The viral titers were approximate 1×10^5 pfu/ml.

Vero and LLC-MK2 cells were cultured in Dulbecco's Modified Eagle Medium, (DMEM, GIBCO) containing 10% FBS, 2 mM L-glutamine, 1% sodium bicarbonate, 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO). Cells were incubated at 37°C in the presence of 5% CO_2.

16. Mosquitoes

Ae. aegypti mosquitoes were F100 progeny from eggs oviposited by adults collected as larvae in a low socioeconomic sector of Chachoengsao province, Thailand. *Ae. aegypti* mosquitoes were fed with sugar meal (10% sucrose + 2% vitamin B) in cage ($30\times30\times30$ cm^3) at 28°C, relative humidity 70-80%, 16 hours of light and 8 hours of dark. Female 3 days-old *Ae. aegypti* were used for replication experiments.

17. DEN4V 1036 PDK 40 genome sequencing

Viral RNA was extracted from DEN4V 1036 PDK40 using E.Z.N.A viral RNA minikit (Omega), in accordance with the manufacturer's instructions. The cDNA was synthesized using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Thirteen fragments of PCR product were amplified using Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) with 13 pairs of DEN4 walking primers on Tpersonel 48 (Biometra). Molecular sizes of PCR products were determined using 1% agarose gel electrophoresis with SYBR safe visualization (Thermo Scientific). PCR products were purified using QIAquick Gel Extraction Kit (Qiagen) and sequenced by the Sanger method (First BASE Laboratories, Malaysia). DNA sequences were analyzed using BioEdit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). For DEN4V references, DEN4V 1036 (GenBank accession no. LQ250785) and DEN4V 1036 PDK48 (GenBank LQ250787) were used.

18. Construction of infectious clone DEN4V 1036 PDK40

Three fragments of DEN4V consisted of (i) fragment 1, DEN4V position 1-4,071 (4071 bp); (ii) fragment 2, DEN4V position 4,043-9,064 (5,022 bp) and (iii) fragment 3, DEN4V position 9,041-10,648 (1,608 bp). Three fragments of viral genome and a fragment of RBC TA cloning vector (RBC bioscience) were amplified using primer sets shown in **Table 5.** The T7 promoter sequence (AAT-ACG-ACT-CAC-TAT-AGG-G) was added in primer upstream of fragment 1. M13F and M13R sequences were used as overlapping sequence between DEN4V genome and the vector. All 4 fragments were assembled into a circular plasmid, using NEBuilder HiFi DNA Assembly mastermix (New England Biolab, NEB, Massachusetts, USA). The plasmid was transformed into *E. coli* NEB10 beta (NEB) using heat shock method. *E. coli* were spread on LB agar with 100 µg/ml ampicillin and incubated overnight at 37°C. Colonies were randomly selected and screened for inserts using colony PCR. Colonies were grown overnight in LB broth with 100 µg/ml ampicillin. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen) and quantified using Nanodrop 1000 (Thermo

Scientific) and subjected to DNA sequencing. Cloning errors were corrected by PCR-based site-directed mutagenesis. Plasmid DEN4V-1036-PDK40 was linearized using ScaI-HF restriction enzyme (restriction site: AGTACT, NEB). Linearized plasmid was in vitro transcribed using T7 RiboMAX Express Large Scale RNA Production System (Promega, Wisconsin, USA) at 37°C for 2 hrs. Uncapped RNA was purified and treated with DNase using QIAamp Viral RNA Mini Kit and RNase-Free DNase Set (Qiagen). Uncapped RNA was capped m7Gppp5´N using Vaccinia Capping System (NEB). One µg of capped RNA was transfected into Vero cells (1×10^5 cells) using Lipofectamine 3000 (Thermo Scientific) and incubated at 37°C. After overnight incubation, maintenance medium was added. DEN4V-infected cells or supernatants were harvested at day 10 post-transfection. For mock-transfection, cells were transfected with lipofectamine without plasmid. DEN4V-infected cells were determined using indirect immunofluorescence assay (IFA) as described below. DEN4V-infected supernatants were quantified by plaque assay and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The rescued DEN4V was designed as IC-DEN4V-1036-PDK40.

Table 5 Primer sets used for construction of infectious clone.

Fragment	**Name**	**Sequence 5′→3′**	**Amplicon size**	**Position on DEN4V genome**
1	M13F-T7-1F	TGT-AAA-ACG-ACG-GCC-AG**T-AAT-ACG-ACT-CAC-TAT-AGG-G**AG-TTG-TTA-GTC-TGT-G	4,071	1-4071
	1036 4071R	TGA-GTG-CTG-TTA-TTT-CTA-CCC-AAT-G		
2	1036 4043F	GTC-TCA-TTG-GGT-AGA-AAT-AAC-AG	5,022	4043-9064
	1036 9064R	TCT-CTG-CCA-AAC-CAG-TGA-TCT-TC		
3	1036 9041F	TGA-AGA-TCA-CTG-GTT-TGG-CAG-AG	1,608	9041-10,648
	10648-M13-R	CAG-GAA-ACA-GCT-ATG-ACC-***AGT-ACT***-AGA-ACC-TGT-TGG-ATC-AAC		
4	M13R-F	GGT-CAT-AGC-TGT-TTC-CTG	2,725	M13R-M13F
	M13F-R	ACT-GGC-CGT-CGT-TTT-ACA		

Bold characters indicate the T7 promoter sequence

Bold and Italic characters indicate the restriction site of *ScaI*

19. Indirected immunofluorescence assay

IC-DEN4V-1036-PDK40 infected cells were spotted on slides and fixed with cold acetone. Mouse anti-DEN4 monoclonal antibody (1-H-10, ATCC, Virginia, USA) and goat anti-mouse monoclonal antibody conjugated with fluorescein isothiocyanate (FITC, Thermo Scientific) were added for the detection of DEN4 antigen. Slides were inspected under a UV microscope (series BX60, Olympus, Japan).

20. Plaque assay in LLC-MK2 cells

Monolayer LLC-MK2 cells were cultured in 6 well plates. IC-DEN4V-1036-PDK40 infected supernatants were 10-fold diluted with maintenance medium. Two hundred microliter of each dilution were added to LLC-MK2 cells. For mock-infection, cells were infected with maintenance medium. Cells were incubated at 37°C for 90 min. Overlay media containing DMEM, 2% carboxymethyl cellulose (CMC, Sigma-Aldrich) and neutral red was added to each well. Cells were incubated at 37°C with 5% CO_2 for 7 days. The plaque size was recorded and plaques were counted and presented as pfu/ml.

21. Temperature sensitivity in LLC-MK2 cells

Temperature sensitivity of viral replication was estimated as published previously (Kinney et al., 1997). Monolayer LLC-MK2 cells were infected with DEN4V 1036 or IC-DEN4V-1036-PDK40 infected supernatant. Mock- or DENV-infected cells were incubated for 8 days at 37°C and 39°C, respectively. Infected supernatants were kept in -80°C prior to use. Viral titer was quantified by plaque assay. Temperature sensitivity was calculated by the following equation:

Temperature sensitivity value = (viral titer at 39°C/viral titer at 37°C) ×100.

Temperature sensitive virus shows sensitivity values below 1.00 or decrease viral titer 90% after cultured at 39°C.

22. Replication kinetics in Vero cells

DEN4V 1036 or IC-DEN4V-1036-PDK40 were added to cells at a multiplicity of infection (MOI) of 0.01. Vero cells (1×10^6 cells) were incubated at 37°C for 90 min. Four ml of maintenance medium were added to each well. Infected cells were incubated at 37°C. Infected supernatants were harvested daily for 8 days and kept at -80°C prior to use. Viral titer was quantified by plaque assay in LLC-MK2 cells as described above.

23. Replication kinetics in *Ae. aegypti*

Female 3-days old *Ae. aegypti* mosquitoes were intrathoracically inoculated with 100 pfu in 0.34 µl of DEN4V 1036 or IC-DEN4V-1036-PDK40. A total of 30 mosquitoes was infected with each virus sample. Mock-infected or infected-mosquitoes were reared at 28°C for 14 days. The mosquitoes were harvested at 1, 3, 5, 7, 11 and 14 days post-incubation (dpi) and kept at -80°C until use. Viral titer from mosquitoes were quantified by qRT-PCR. One hundred microliter of PBS containing 20% FBS were added to each mosquito. Infected-mosquitoes were crushed using micropestle in 1.5 ml microcentrifuge tube. Debris was discarded by centrifuge for 10 mins at 13,000 rpm, 4°C. Mosquito suspension was proceeded to RNA extraction and quantified viral titer by qRT-PCR.

24. Quantification of DEN4V in *Ae. aegypti* by qRT-PCR

qRT-PCR was performed following the literature protocol of Johnson et al. (2005) Briefly, viral RNA was extracted from infected-supernatant or mosquito suspension. Maintenance medium or mock-infected mosquitoes were used as negative control. Viral RNA was amplified using KAPA PROBE FAST Universal One-Step qRT-PCR Master Mix Kit (KAPA biosystems). DEN4V-specific primers (forward primer: TTG-TCC-TAA-TGA-TGC-TGG-TCG and reverse primer: TCC-ACC-TGA-GAC-TCC-TTC-CA) and probe (Cy5-TTC-CTA-CTC-CTA-CGC-ATC-GCA-TTC-CG-BHQ3) were mixed with component supplemented by kit. qRT-PCR was conducted on Chromo4 (Bio-Rad, California, USA). qRT-PCR cycling condition was followed manufacturer's instruction with annealing temperature at 60°C. The assay was performed in triplicated. Viral titers were quantified by comparison with a DEN4V RNA standard curve and were presented as copies/µl

CHAPTER V
RESULTS

Part A: To evaluate the molecular epidemiology of DENV isolates of patient sera in C6/36 cells by DNA sequencing and phylogenetic tree

1. DENV plasmid preparation

PCR products of DEN1V, DEN2V, DEN3V and DEN4V were detected by 2% agarose gel electrophoresis. The result revealed that molecular sizes of DEN1V, DEN2V, DEN3V and DEN4V were 112, 79, 75 and 109 bp, respectively **(Figure 8).**

PCR products of DEN1V, DEN2V, DEN3V and DEN4V were cloned to pCR2.1 using NS5, C, prM and M region, respectively. Colony PCR were screened for recombinant DENV (rDENV). The result revealed that molecular sizes of rDEN1V, rDEN2V, rDEN3V and rDEN4V were 312, 279, 275 and 309 bp, respectively **(Figure 9).**

2. Standard curve of multiplex DEN1V-4 qRT-PCR

rDENV 1-4 plasmids were quantified using Nanodrop. Concentration of rDEN1V, rDEN2V, rDEN3V and rDEN4V were 108.3, 150.2, 83.7 and 120.4 ng/µl, respectively. The copy No. of rDEN1V, rDEN2V, rDEN3V and rDEN4V were 2.5×10^{10}, 3.5×10^{10}, 2.0×10^{10}, and 2.8×10^{10} copy no./µl. Standard curve of DENV 1-4 were constructed from 10^4 to 10^1 copy no./µl **(Figure 10 to 13).** The coefficiency regression (R^2) of DEN1V, DEN2V, DEN3V and DEN4V standard curve were 0.959, 0.984, 0.997 and 0.995, respectively **(Figure 10 to 13).** Limit of detection of DEN1V, DEN2V, DEN3V and DEN4V were 10 copy no./µl.

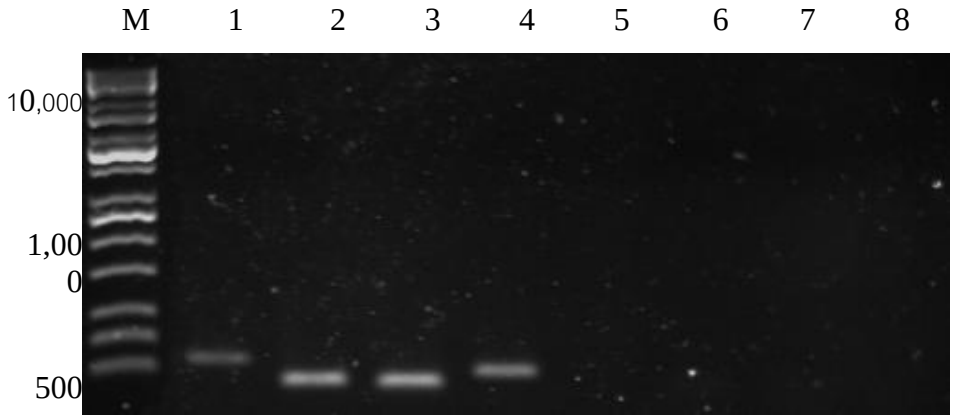


Figure 8 Agarose gel electrophoresis of DENV PCR product. Lane M; Universal ladder (KAPA biosystems), Lane 1; DEN1V (112 bp), Lane 2; DEN2V (79 bp), Lane 3; DEN3V (75 bp), Lane 4; DEN4V (109 bp), Lane 5; DEN1V negative control, Lane 6; DEN2V negative control, Lane 7; DEN3V negative control and Lane 8; DEN4V negative control.

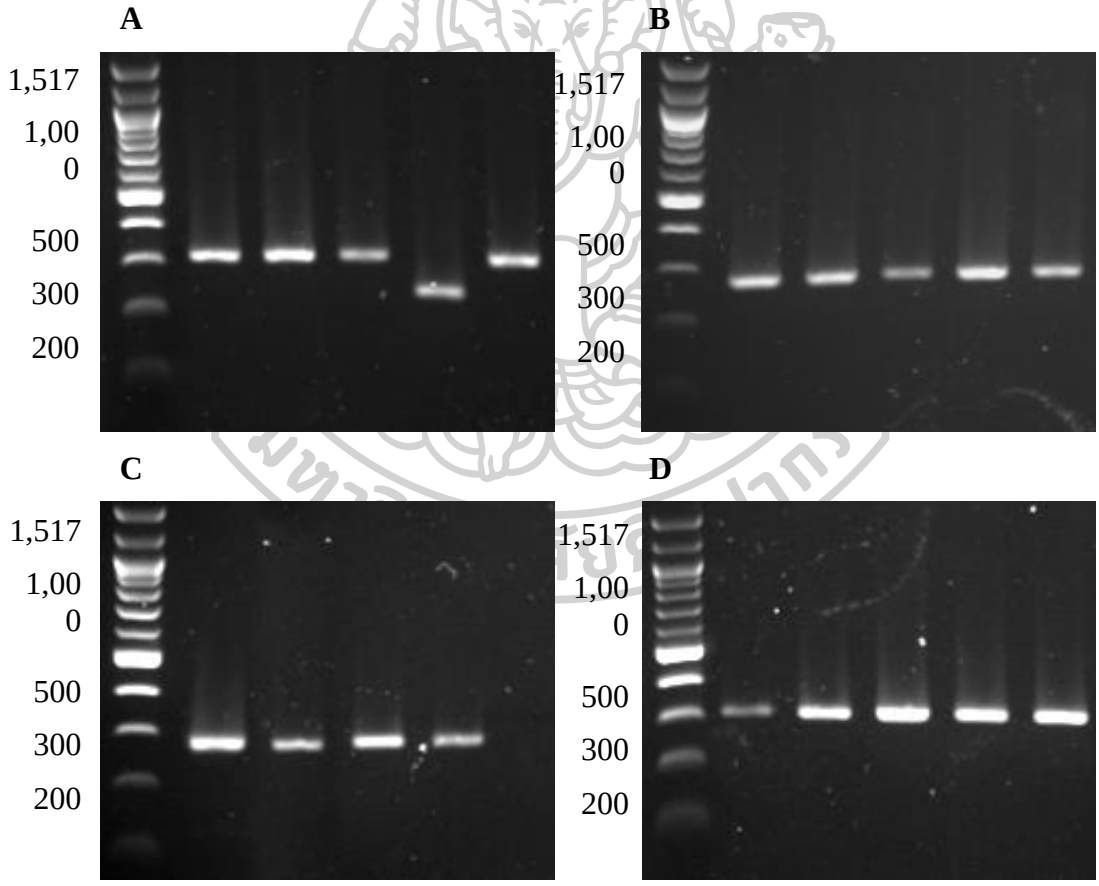
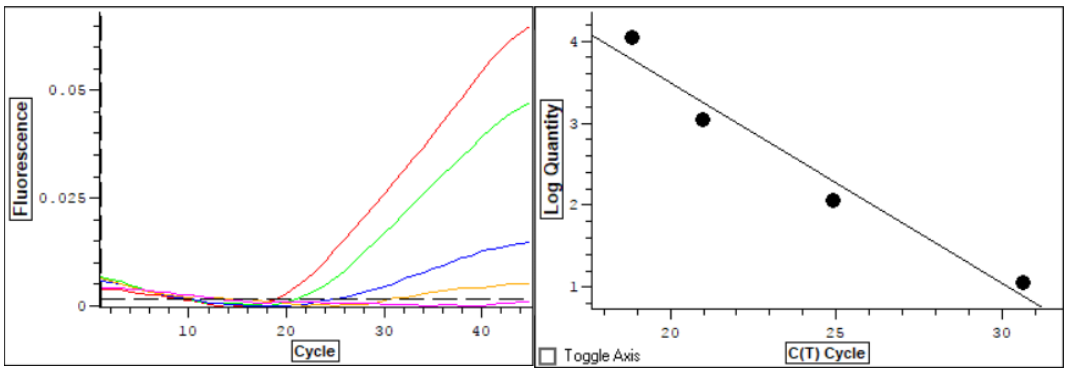


Figure 9 Agarose gel electrophoresis of rDENV PCR product. A; rDEN1V (312 bp), B; rDEN2V (279 bp), C; rDEN3V (275 bp) and D; rDEN4V (309 bp). 100 bp DNA ladder (NEB) was used as ladder.

A B

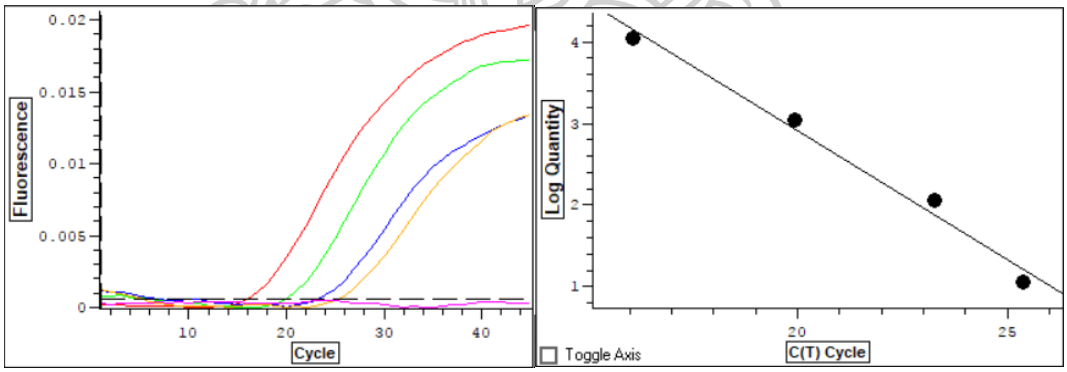


$y = -0.2444x + 8.38$

$R^2 = 0.959$

Figure 10 Amplification curve (A) and standard curve (B) of DEN1V by qRT-PCR.Graph indicated 10-fold serial dilution of rDEN1V (16007 strain) with concentration 10^4 to 10^1 copies/µl. Standard linear equation and R^2 were calculated from Ct of each dilution.

A B

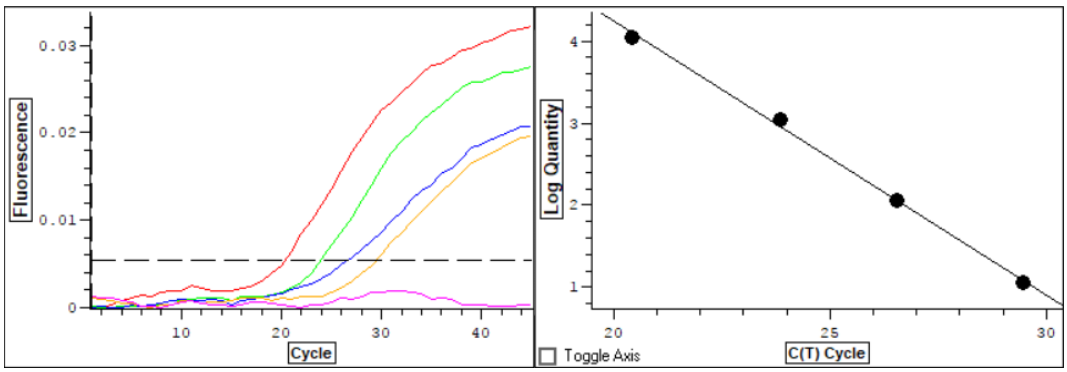


$y = -0.3156x + 9.23$

$R^2 = 0.984$

Figure 11 Amplification curve (A) and standard curve (B) of DEN2V by qRT-PCR.Graph indicated 10-fold serial dilution of rDEN2V (16681 strain) with concentration 10^4 to 10^1 copies/µl. Standard linear equation and R^2 were calculated from Ct of each dilution.

A B

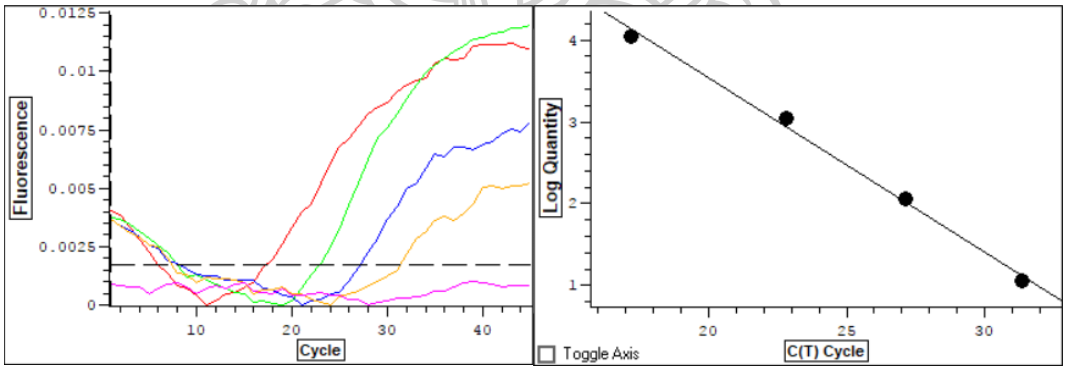


$y = -0.335x + 10.95$

$R^2 = 0.997$

Figure 12 Amplification curve (A) and standard curve (B) of DEN3V by qRT-PCR.Graph indicated 10-fold serial dilution of rDEN3V (16562 strain) with concentration 10^4 to 10^1 copies/µl. Standard linear equation and R^2 were calculated from Ct of each dilution.

A B



$y = -0.213x + 7.80$

$R^2 = 0.995$

Figure 13 Amplification curve (A) and standard curve (B) of DEN4V by qRT-PCR.Graph indicated 10-fold serial dilution of rDEN4V (1036 strain) with concentration 10^4 to 10^1 copies/µl. Standard linear equation and R^2 were calculated from Ct of each dilution.

3. qRT-PCR of DENV isolates

Seventy-five of 326 patient sera (23%) isolated in C6/36 cells were detected by qRT-PCR. DEN3V was the most predominant serotype (n=28, 37.3%). This was followed by DEN2V (n=20, 26.7%), DEN1V (n=15, 20.0%), and DEN4V (n=12, 16.0%) as shown in **Table 6.** Viral titers were ranged from 7.75×10^2 to 2.26×10^6 copy no./µl **(Table 7)**.

Table 6 Summary of qRT-PCR results.

qRT-PCR				
DEN1V	DEN2V	DEN3V	DEV-4	Total
15 (20.0%)	20 (26.7%)	28 (37.3%)	12 (16.0%)	75

Table 7 DENV qRT-PCR compared with RT-PCR.

No.	Sample	qRT-PCR	RT-PCR	DENV serotype
1	06-129	4.14×10^5	+	3
2	06-177	4.12×10^5	+	1
3	06-429	3.21×10^5	+	3
4	11-69	9.62×10^4	+	1
5	11-1193	5.21×10^5	+	1
6	11-1194	1.33×10^5	+	4
7	11-1212	9.62×10^5	+	1
8	11-1230	1.21×10^5	+	3
9	11-1236	3.13×10^5	+	2
10	11-1253	5.25×10^4	+	2
11	11-1373	3.33×10^3	+	4
12	11-1380	8.95×10^3	+	2
13	11-1387	5.25×10^3	+	2
14	11-1404	1.25×10^4	+	4
15	11-1414	2.85×10^4	+	2
16	11-151	3.13×10^5	+	2
17	11-1569	7.25×10^4	+	2
18	11-1590	5.85×10^5	+	1
19	11-1657	5.95×10^4	+	2
20	11-1660	2.51×10^5	+	1
21	11-1666	7.89×10^4	+	4
22	11-1688	9.51×10^3	+	2
23	11-1694	7.75×10^5	+	2
24	11-1695	3.23×10^3	+	2
25	11-1707	4.75×10^5	+	2
26	11-172	7.75×10^4	+	1
27	11-265	3.45×10^5	+	1
28	11-372	1.25×10^5	+	3
29	11-586	2.45×10^5	+	3
30	11-606	3.11×10^4	+	1

Table 7 DENV qRT-PCR compared with RT-PCR (continued).

No.	Sample	qRT-PCR	RT-PCR	DENV serotype
31	11-941	6.75×10^4	+	2
32	11-976	9.21×10^4	+	1
33	12-436	5.65×10^4	+	2
34	13-1328	1.05×10^5	+	3
35	13-1329	3.57×10^5	+	3
36	13-37	2.45×10^5	+	2
37	13-45	5.85×10^5	+	2
38	14-141	1.05×10^5	+	3
39	14-164	2.45×10^5	+	3
40	14-170	3.45×10^5	+	3
41	14-177	3.45×10^5	+	3
42	14-182	4.15×10^5	+	3
43	15-1046	4.12×10^5	+	2
44	15-1048	2.45×10^5	+	1
45	15-1052	4.57×10^5	+	3
46	15-1053	1.24×10^5	+	3
47	15-1057	3.16×10^5	+	2
48	15-1066	4.27×10^5	+	3
49	15-1068	5.14×10^5	+	3
50	15-1072	1.27×10^5	+	3
51	15-1074	3.65×10^4	+	4
52	15-1075	1.15×10^6	+	1
53	15-1082	7.75×10^5	+	2
54	15-1084	2.06×10^5	+	3
55	15-1090	2.17×10^5	+	3
56	15-1091	1.05×10^5	+	3
57	15-1092	2.25×10^5	+	3
58	15-1093	3.17×10^5	+	3
59	15-1105	3.22×10^4	+	4
60	15-1252	2.12×10^3	+	2

Table 7 DENV qRT-PCR compared with RT-PCR (continued).

No.	Sample	qRT-PCR	RT-PCR	DENV serotype
61	15-1303	2.17×10^5	+	3
62	15-1305	1.84×10^4	+	4
63	15-1306	7.75×10^2	+	4
64	15-1307	1.84×10^5	+	4
65	15-1310	2.26×10^6	+	1
66	15-1312	2.31×10^4	+	3
67	15-1313	2.85×10^5	+	3
68	15-1315	8.54×10^4	+	4
69	15-1316	1.05×10^5	+	3
70	15-1317	2.55×10^5	+	3
71	15-1319	2.65×10^5	+	3
72	15-1321	3.11×10^4	+	1
73	15-1322	2.85×10^3	+	4
74	15-1323	3.65×10^5	+	1
75	15-1324	1.84×10^5	+	4

4. DENV E gene RT-PCR

To evaluate the genotype within serotype, we performed genotyping based on E gene. The full-length E gene was generated for 75 isolates of all four serotypes. RT-PCR of E gene was performed and PCR products were subsequecnly sequenced. All of DENV-positive samples were able to amplify E gene. Agarose gel electrophoresis of each serotype showed 1,642, 1,510, 1,571 and 1,618 bp of DEN1V, DEN2V, DEN3V and DEN4V, respectively **(Figure 14).**

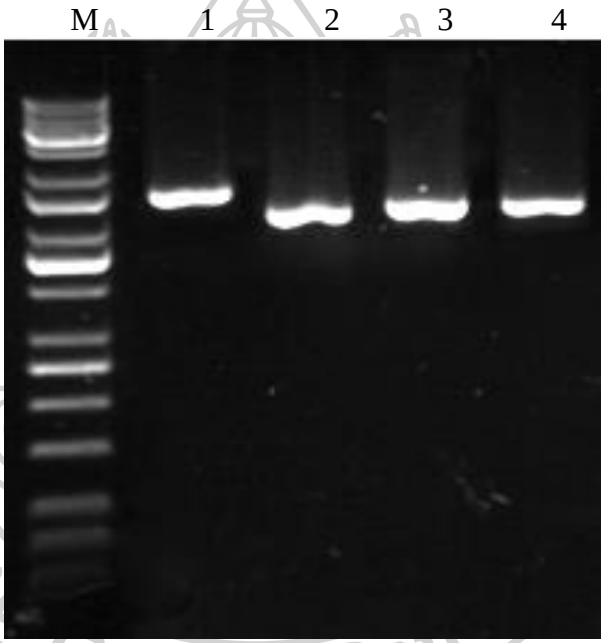


Figure 14 Agarose gel electrophoresis of DENV E gene. Lane M; 100 bp DNA marker (Solis Biodyne), Lane 1; DEN1V (1,642 bp), Lane 2; DEN2V (1,510 bp), Lane 3; DEN3V (1,571 bp) and Lane 4; DEN4V (1,618 bp).

5. Phylogenetic tree analysis

Phylogenetic analysis of E gene sequence revealed only one genotype of DEN1V (n=15), DEN2V (n=20) and DEN4V (n=12)., but 2 genotypes of DEN3V (n=28), as shown in **Table 8.** Genotype I of DEN1V consists of the samples 2006-2015 **(Figure 15).** Genotype Asian I of DEN2V consists of the samples 2011-2015 **(Figure 16).** Genotype I of DEN4V consists of the samples 2011-2015 **(Figure 17).** In case of DEN3V consists of 2 genotypes, genotype II (n=6) and genotype III (n=22) consist of the samples 2006-2015 **(Figure 18).**

Table 8 Genotypic summary of isolated DENV.

	DEN1V	**DEN2V**	**DEN3V**		**DEN4V**	**Total**
Genotype	GI	Asian I	GII	GIII	GI	
Number	15	20	6	22	12	75

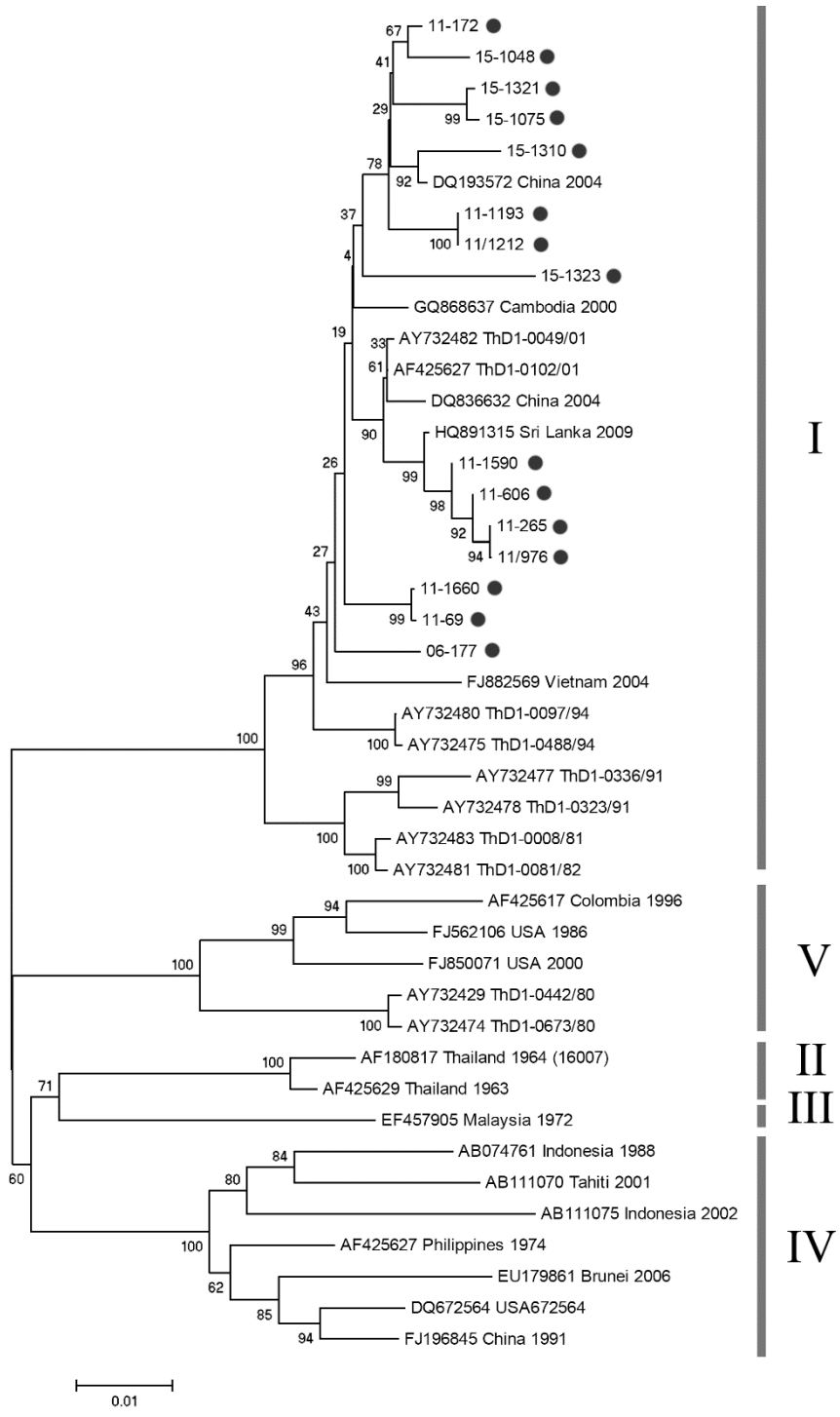


Figure 15 Neighbor joining tree of DEN1V. Black dot denotes DENV used in this study. Scale bar indicates evolutionary distance.

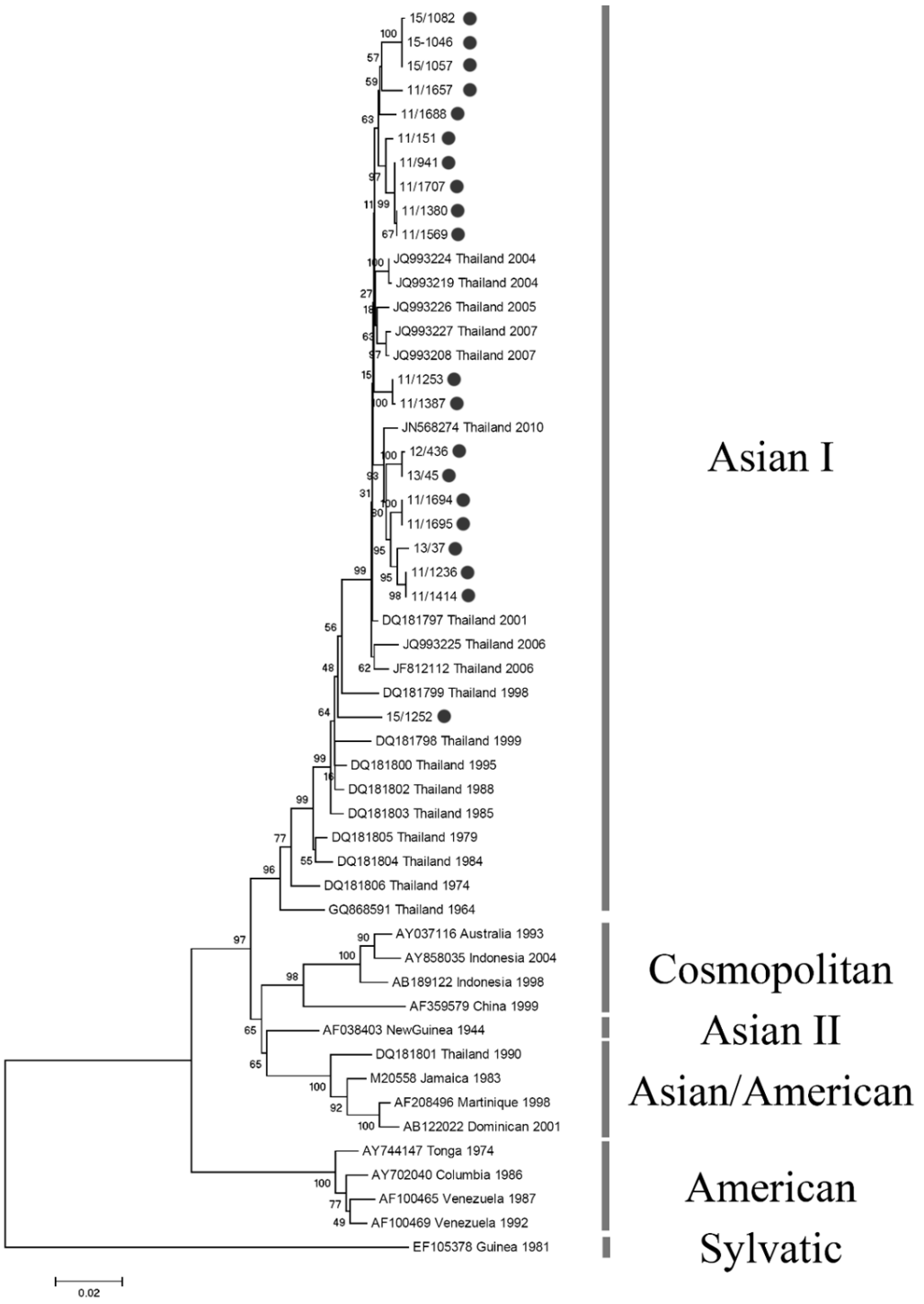


Figure 16 Neighbor joining tree of DEN2V.Black dot denotes DENV used in this study. Scale bar indicates evolutionary distance.

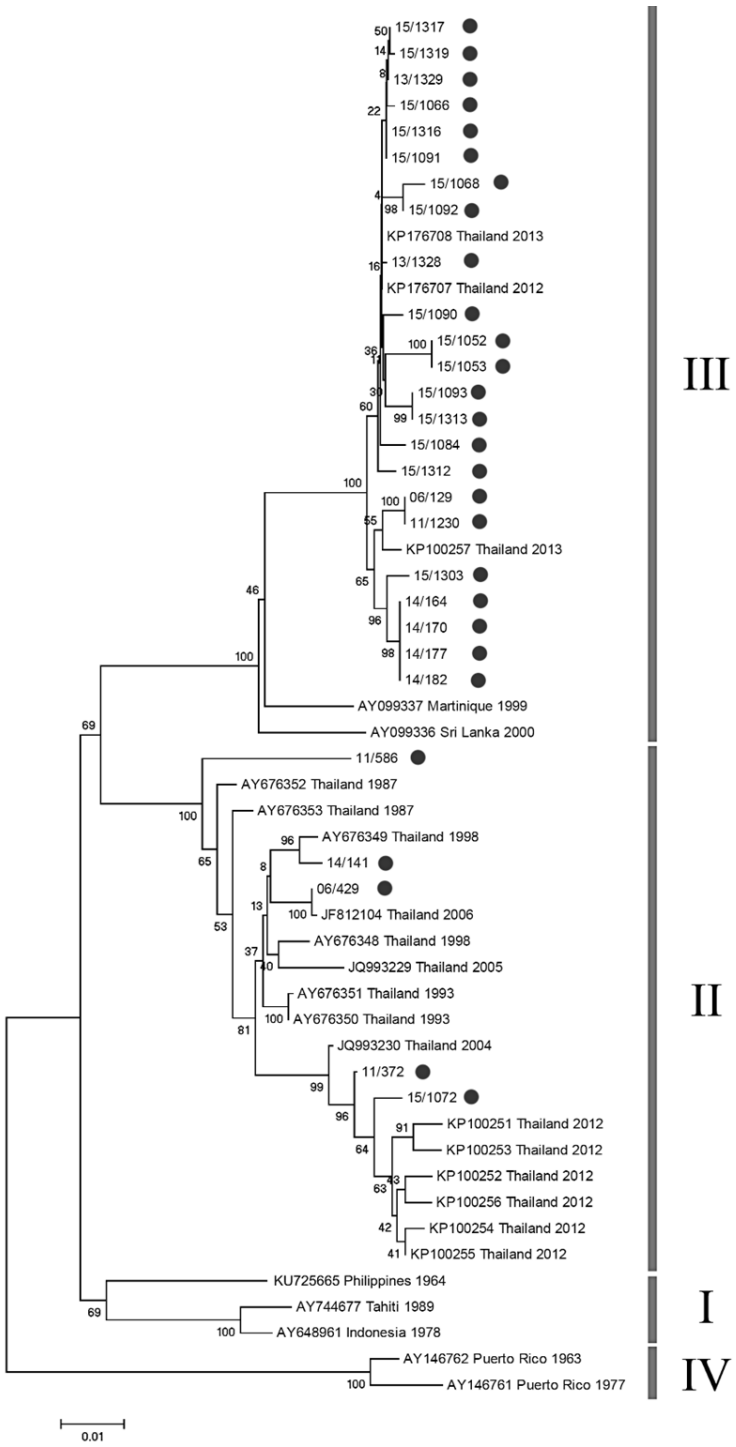


Figure 17 Neighbor joining tree of DEN3V.Black dot denotes DENV used in this study. Scale bar indicates evolutionary distance.

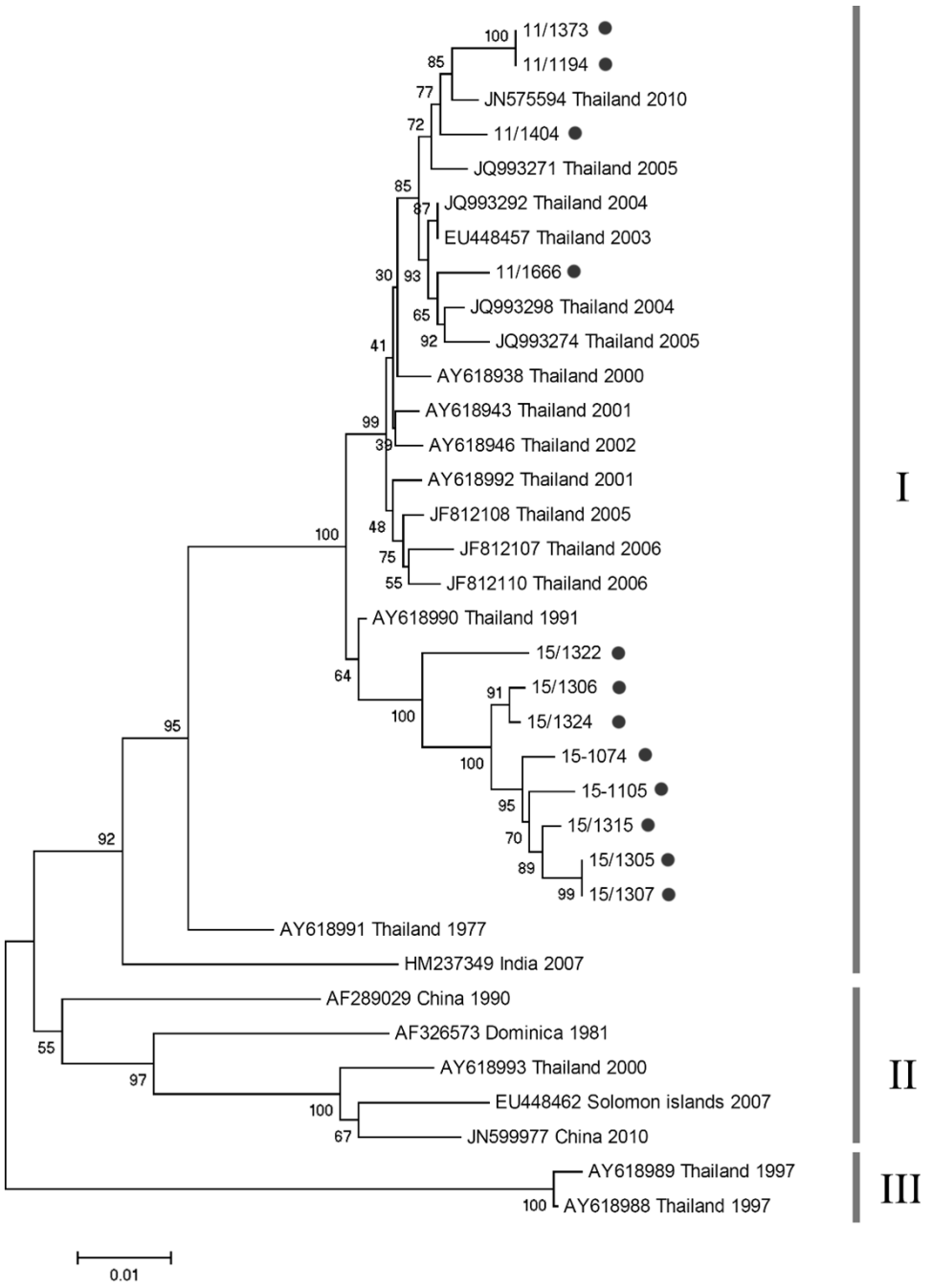


Figure 18 Neighbor joining tree of DEN4V.Black dot denotes DENV used in this study. Scale bar indicates evolutionary distance.

Part B: To evaluate biological marker of constructed D4 1036 PDK40 infectious clone

To characterize a virus at the molecular and biological level, it is necessary to produce an infectious clone. Current molecular tools that permit rapid genetic analyses should be employed for quality control to document the genetic authenticity of vaccine viruses. In this study, we report the full genome sequence of LAV DEN4V 1036 PDK 40 and a constructed infectious clone, DEN4V 1036 PDK40 (IC-DEN4V-1036-PDK40). A phenotypic characterization of this clone was also performed to demonstrate safety properties for its inclusion in a vaccine.

6. DEN4V 1036 PDK40 genome sequence

To analyze the whole genome sequence of LAV DEN4V 1036 PDK40 (10,648 bp), Sanger DNA sequencing method was used. We have found that DEN4V 1036 PDK40 had 7 missense mutations whereas DEN4V 1036 PDK48 had 6 missense mutations which are different from DEN4V 1036. The only mutation which is different between PDK48 and PDK 40 is the substitution of Val by Met at aa position 463 in the E. The other 6 missense mutations were found in concordance between DEN4V 1036 PDK40 and DEN4V 1036 PDK48 as shown in **Table 9.** Sequence alignment of genome and coding sequence were shown in Appendix C, respectively.

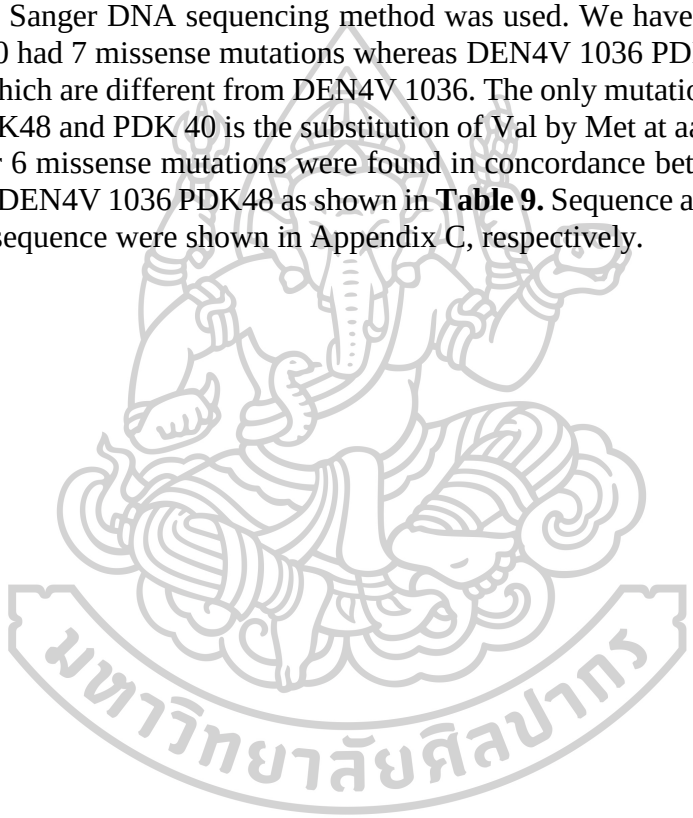


Table 9 Comparision between genome sequence of DEN4V 1036, PDK40 and PDK48

Nucleotide position	**Nucleotide**			**Amino acid**			**Protein position**	**Polyprotein position**
	1036*	**PDK-48***	**PDK40**	**1036***	**PDK-48***	**PDK40**		
1971	G	A	A	Glu	Lys	Lys	E-345	624
2325	G	G	A	Val	Val	Met	E-463	742
3182	G	C	C	Gln	His	His	NS1-253	1027
6660	C	T	T	Leu	Phe	Phe	NS4A-95	2187
6957	A	A/T	T	Ile	Ile/Phe	Phe	NS4B-44	2286
7162	T	C	C	Leu	Ser	Ser	NS4B-112	2354
7546	C	C/T	T	Ala	Ala/Val	Val	NS4B-240	2366
7623	G	T/G	**G**	Asp	Tyr/Asp	Asp	NS5-21	2508

*DEN4V 1036 (GenBank accession no. LQ250785) and DEN4V 1036 PDK48 (GenBank LQ250787)

7. Construction of infectious clone DEN4V 1036 PDK40

To characterize a virus at the molecular and biological level, it is necessary to produce an infectious clone. Current molecular tools that permit rapid genetic analyses should be employed for quality control to document the genetic authenticity of vaccine viruses. In this study, we report the full genome sequence of LAV DEN4V 1036 PDK 40 and a constructed infectious clone, DEN4V 1036 PDK40 (IC-DEN4V-1036-PDK40). A phenotypic characterization of this clone was also performed to demonstrate safety properties for its inclusion in a vaccine.

To construct an infectious clone DEN4V 1036 PDK 40, Gibson's assembly method was used (Gibson et al., 2009; Gibson, 2011). Three fragments of DEN4V genome and one fragment of vector were amplified by PCR. Agarose gel electrophoresis of each fragment showed 4,071, 5,022, 1,608 and 2,725 bp of fragment 1, 2, 3 and 4, respectively **(Figure 19).** A total of 4 fragments were assembled using Gibson's assembly method **(Figure 20A).** The 14 kb plasmid was transformed into E. coli NEB10-Beta. DNA sequencing was used to confirm the insertion and correction of full-length DEN4V genome. The insertion was confirmed by full-length PCR (1-10648) as shown in **Figure 20B.** Viral RNA of pDEN4V-1036-PDK40 was transfected to Vero cells by lipofection. To confirm the presence of DEN4V, IFA and qRT-PCR were used.

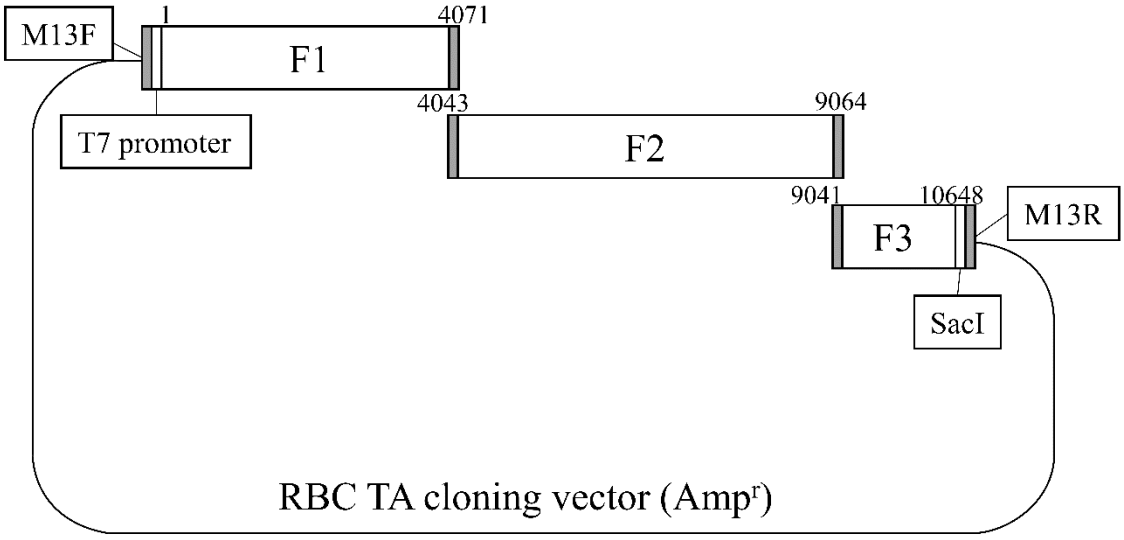


Figure 19 Construction of IC-DEN4V-1036-PDK 40. Three fragments of DEN4V genome and 1 fragment of vector were amplified. A total of 4 fragments were assembled to plasmid named pDEN4V-1036-PDK40 as described under Materials & Methods. The full-length DEN4V genome was placed under control of the T7 promoter for in vitro transcription. Gray bars indicate overlapping regions between the fragments.

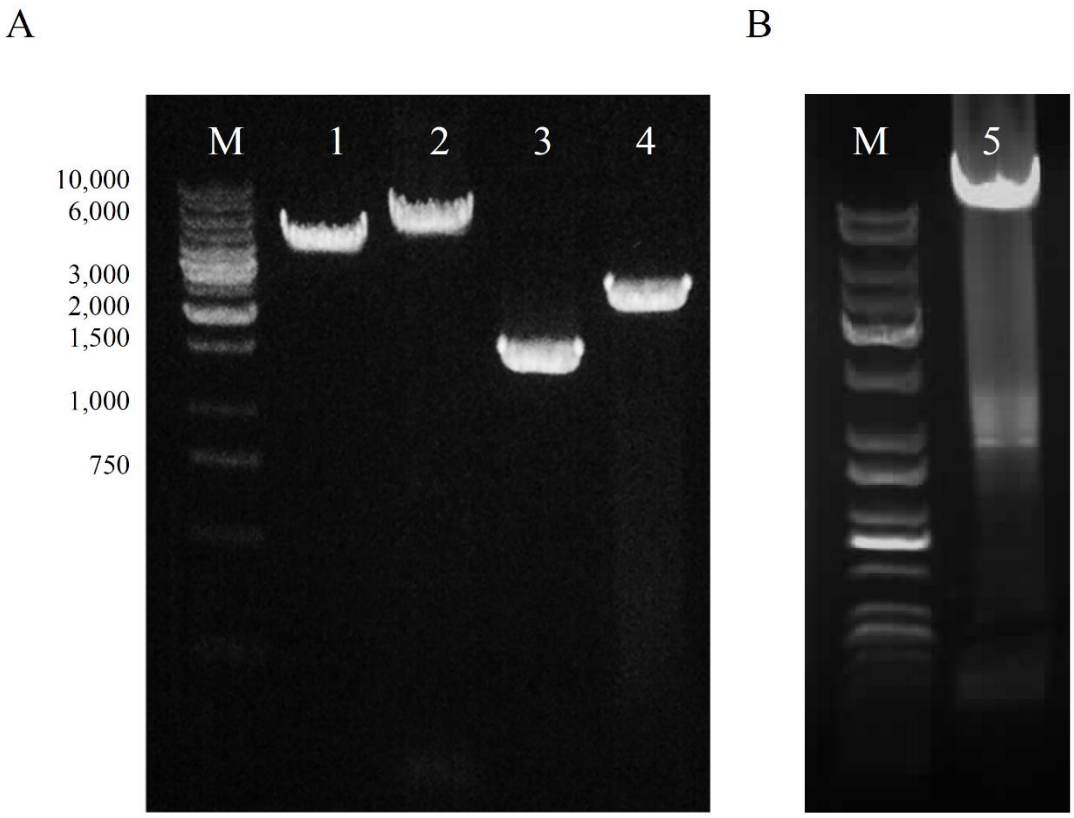


Figure 20 Agarose gel electrophoresis of 3 fragments of DEN4V and 1 fragment of vector.(A) Lane M; KAPA universal DNA ladder size 100-10,000 bp, Lane 1; Fragment 1 (4,071 bp), Lane 2; fragment 2 (5,022 bp), Lane 3; fragment 3 (1,608 bp) and Lane 4; fragment 4 (2,725 bp) and (B) Lane M; KAPA universal DNA ladder size 100-10,000 bp, Lane 5; full-length DEN4V genome (10,648 bp).

8. Presentation of IC-DEN4V-1036-PDK40 in Vero cells by IFA and qRT-PCR

IFA demonstrated that positive cells exhibited bright-green apple cytoplasmic fluorescence **(Figure 21A)** whereas negative cells exhibited dull green or yellow in the cytoplasm **(Figure 21B)** at 10 dpi. qRT-PCR revealed that IC-DEN4V-1036-PDK40-infected supernatant had a viral titer of 1×10^5 copies/µl at 10 dpi **(Figure 21C).**

For a further phenotypic characterization of the viral clone, plaque size, temperature sensitivity in LLC-MK2 cells and replication kinetics in Vero cells and *Ae. aegypti* of IC-DEN4V-1036-PDK40 were analyzed.

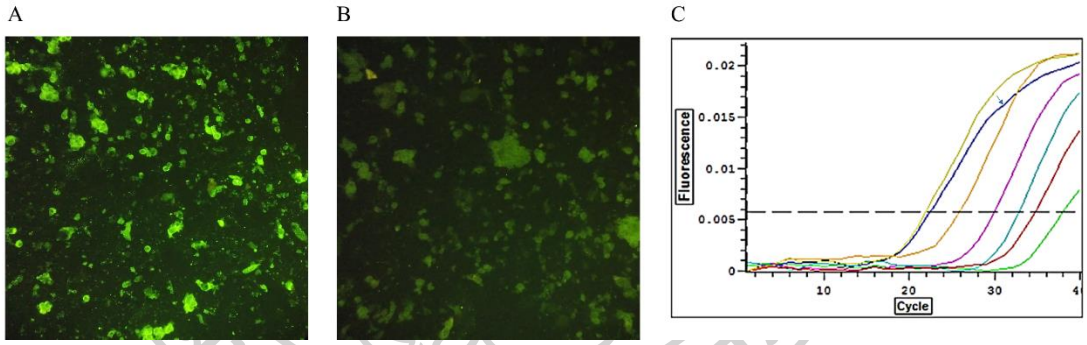


Figure 21 Presence of DEN4V in Vero cells at days 10 after transfection, confirmed by IFA (A) mock-transfected Vero cells (B) and qRT-PCR (C).

9. Plaque size of IC-DEN4V-1036-PDK40 in LLC-MK2 cells

IC-DEN4V-1036-PDK40 produced pinpoint plaques (≤1 mm) in LLC-MK2, while DEN4V 1036 produced medium (2-3 mm) and large plaques (≥5 mm) **(Figure 22).**

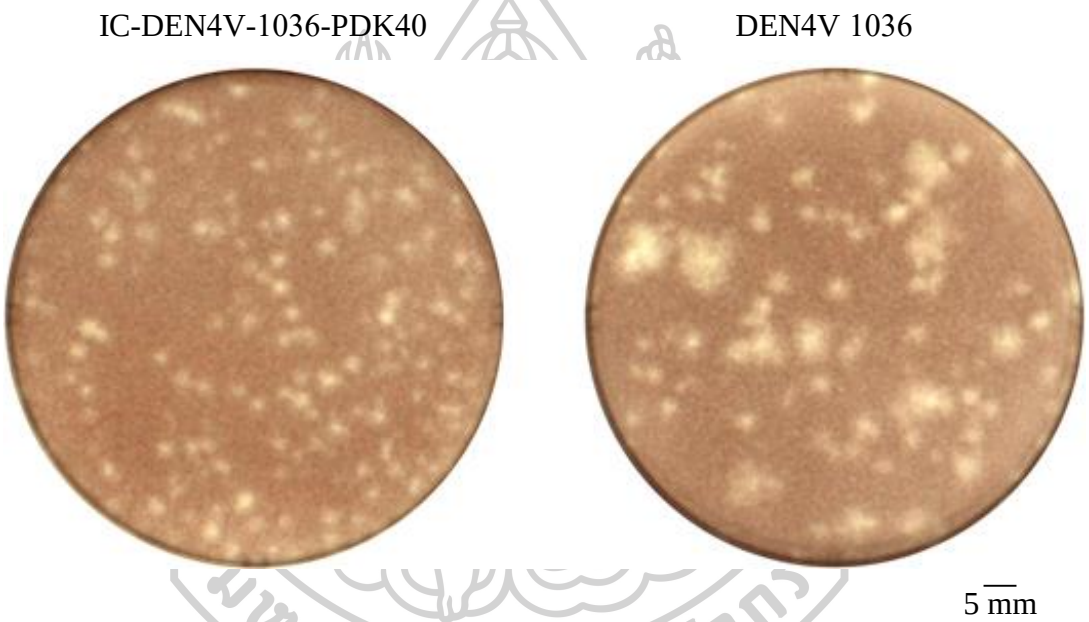


Figure 22 Plaque sizes in LLC-MK2 cells of IC-DEN4V-1036-PDK40 and DEN4V 1036.Scale bar = 5 mm.

10. Temperature sensitivity of IC-DEN4V-1036-PDK40 in LLC-MK2 cells

The clone also exhibited a temperature sensitive phenotype as this virus was completely unable to replicate in LLC-MK2 cells at 39°C. DEN4V1036 had a 90% reduction of viral titer at 39°C relative to the titer at 37°C **(Figure 23).** IC-DEN4V-1036-PDK40 and DEN4V 1036 showed a titer of 2.5×10^7 and 2.9×10^7 PFU/ml at 37°C, 8 dpi. DEN4V 1036 showed a titer of 3.2×10^7 PFU/ml.

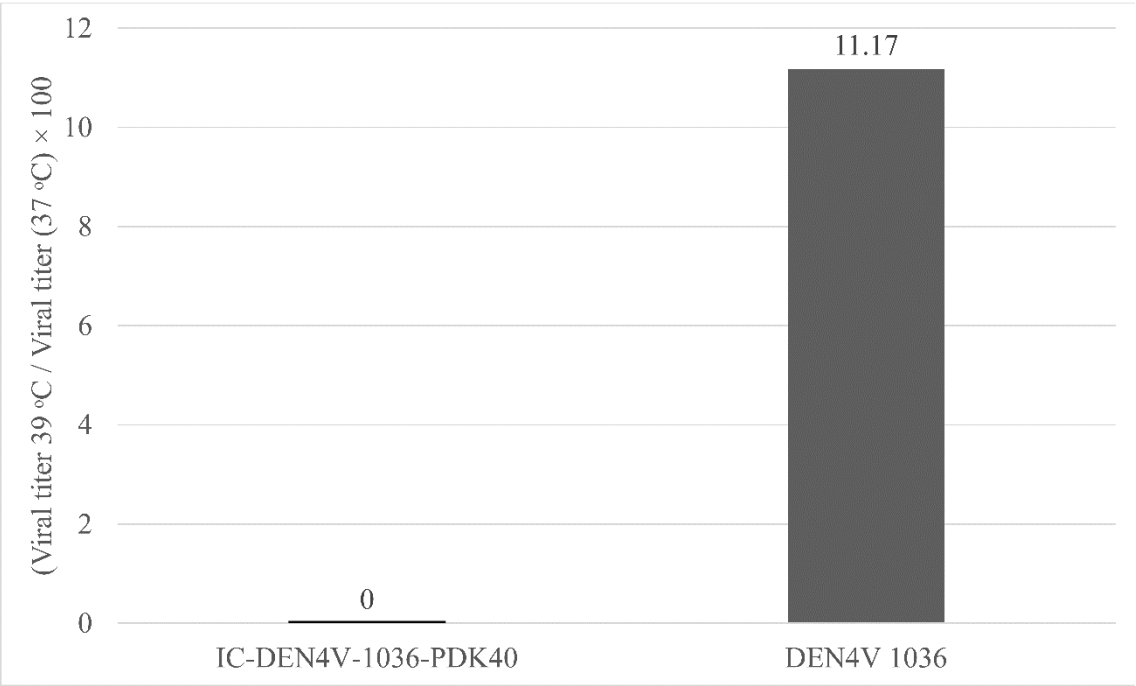


Figure 23 Temperature sensitivity in LLC-MK2 cells of IC-DEN4V-1036-PDK40 and DEN4V 1036. The temperature sensitivity value was calculated by (viral titer at 39°C/viral titer at 37°C) × 100

11. Replication kinetics of IC-DEN4V-1036-PDK40 in Vero cells

IC-DEN4V-1036-PDK40 and DEN4V 1036 showed a peak titer of 2.3×10^7 and 3.4×10^7 pfu/ml at 7 dpi, respectively **(Figure 24).** These results would indicate that IC-DEN4V-1036-PDK40 and DEN4V 1036 exhibit similar replication patterns in Vero cells.

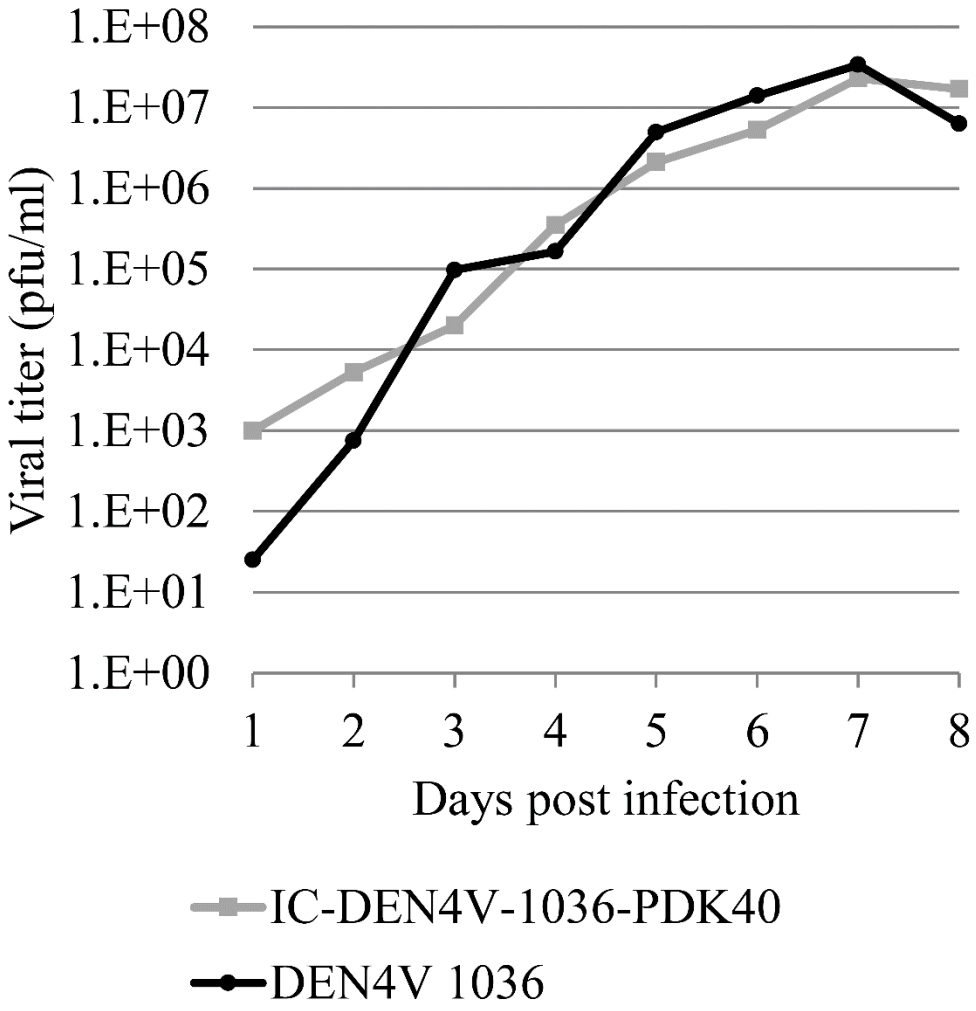


Figure 24 Replication kinetics in Vero cells of IC-DEN4V-1036-PDK40 compared with DEN4V 1036.DEN4V was detected by plaque assay (pfu/ml).

12. Replication kinetics of IC-DEN4V-1036-PDK40 in *Ae. aegypti*

To study the time-course of replication of IC-DEN4V-1036-PDK40 in *Ae. aegypti*, qRT-PCR was used. IC-DEN4V-1036-PDK40 showed a peak titer of 1.3×10^7 copies/µl at 11 dpi and declined to be 5.3×10^5 copies/µl at 14 dpi. On the other hand, DEN4V 1036 showed a peak titer of 5.6×10^7 copies/µl at 14 dpi **(Figure 25).** These results suggest that IC-DEN4V-1036-PDK40 has a lower replication efficiency when compared to DEN4V 1036.

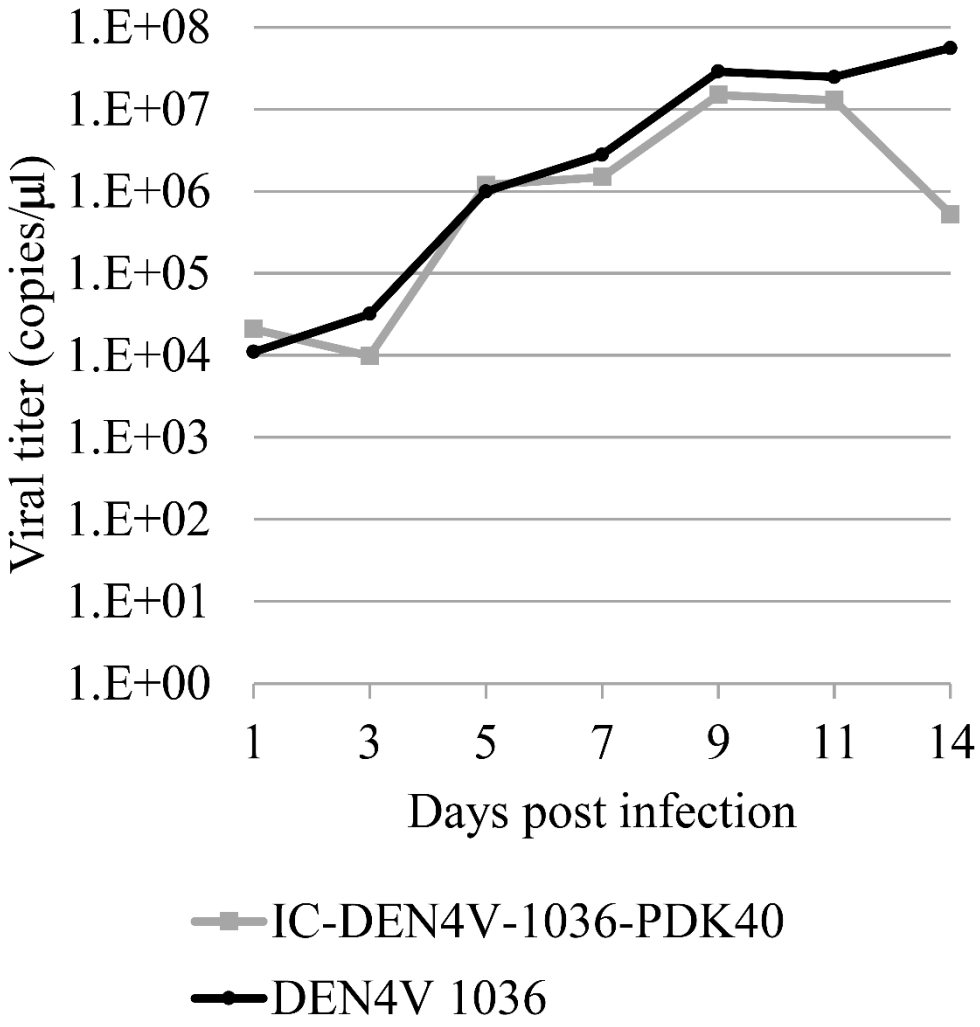


Figure 25 Replication kinetics in *Ae. aegypti* of IC-DEN4V-1036-PDK40 compared with DEN4V 1036. DEN4V were detected by qRT-PCR (copies/µl).

CHAPTER VI
DISCUSSION

Part A: To evaluate the molecular epidemiology of DENV isolates of patient sera in C6/36 cells by DNA sequencing and phylogenetic tree.

Thailand is one of the DENV hyperendemic area. All four serotypes of DENV have been spread to all provinces of Thailand (BOE, 2016). Currently, only limited DENV genetic information is available in Thailand. Molecular epidemiology of each DENV serotypes may aid in the better management of dengue (Lestari et al., 2017). In this study, we evaluated molecular epidemiology of 75 DENV isolates in Bangkok, Thailand during 2006-2015. We found DEN3V was the predominant serotype during 2015 **(Table 6)**. This result was consistenced with report of Bereau of Epidemiology, MOPH, Thailand (BOE, 2016). The envelope (E) genes of the DENV isolates (n=75) were sequenced by DNA sequencing. Phylogenetic relationship was analyzed using neighbor joining tree with 1000 bootstap. Genotype of DENV was classified based on Klungthong et al (2008).

DEN1V consists of 5 genotypes; I, II, III, IV and V. In Thailand, genotype I is a major genotype whereas genotype II (Halstead and Simasthien, 1970) and genotype V are minor genotype (Zhang et al., 2005; Klungthong et al., 2008). In this study, we found only DEN1V genotype I **(Figure 10)** was correlated to genotype I of DEN1V from previous reports (Zhang et al., 2005; Klungthong et al., 2008). These results indicated that this genotype is circulated in Bangkok. Circulating genotype I has been predominated in South-East Asia such as Cambodia (Shu et al., 2009), Indonesia (Yamanaka et al., 2011; Sasmono et al., 2015), Laos (Dubot-Peres et al., 2013), Malaysia (Ng et al., 2015), Myanmar (Ngwe Tun et al., 2016; Kyaw et al., 2017), Singapore (Schreiber et al., 2009; Lee et al., 2012) and Vietnam (Shu et al., 2009).

DEN2V consists of 5 genotypes; Asian I, Asian II, Asian/American, Cosmopolitan and American (Klungthong et al., 2008). In this study, we found only Asian I genotype **(Figure 11)** was correlated to Asian I genotype of DEN2V previous reports (Zhang et al., 2006; Klungthong et al., 2008). These results indicated that Asian I genotype is circulated in Bangkok. However, Cosmopolitan genotype was reported in 1969 and 1998 (Twiddy et al., 2002; Zhang et al., 2006) and Asian/American genotype was reported in 1980-1991 (Zhang et al., 2006; Klungthong et al., 2008). These results indicated that this genotype is circulated in Bangkok. Circulating Asian I genotype has been predominated in South-East Asia such as Cambodia (Huang et al., 2012). Laos (Huang et al., 2012; Ernst et al., 2015), Myanmar (Ngwe Tun et al., 2016; Kyaw et al., 2017) and Vietnam (Vu et al., 2010).

DEN4V consists of 3 genotypes; I, II and III. In this study, we found only genotype I **(Figure 13)** was correlated to genotype I of DEN4V from previous reports (Wang et al., 2000; Klungthong et al., 2004; Klungthong et al., 2008). In addition, genotype II and III was reported during 1997-2001 (Klungthong et al., 2004), Predominant genotype I has been reported in South-East Asia such as Cambodia (Tuiskunen et al., 2011), Myanmar (Ngwe Tun et al., 2016; Kyaw et al., 2017) and Vietnam (Takamatsu et al., 2015). However, Genotype II was firstly reported in Thailand since 2012 (Kittichai et al., 2015). Genotype II has been predominantly circulating in Indonesia (Haryanto et al., 2016), Malaysia (Holmes et al., 2009), Singapore (Lee et al., 2012).

DEN3 V consists of 4 genotypes; genotype I, II, III and IV (Klungthong et al., 2008). We found genotype II and III **(Figure 12).** Predominant genotype III were shown whereas genotype II has been predominant in Thailand since 1970s (Lanciotti et al., 1994; Zhang et al., 2005; Klungthong et al., 2008; Chen, 2013). Predominant genotype III has been circulated in Thailand and Laos since 2008 (Huang et al., 2012; Lao et al., 2014). Predominant genotype III has been circulated in South-East Asia such as Laos (Lao et al., 2014), Myanmar (Kyaw et al., 2017), Singapore (Lee et al., 2012) and Vietnam (Phu Ly et al., 2015). In addition, genotype II has beed circulated in South-East Asia such as Laos (Lao et al., 2014), Malaysia (Fong et al., 2004), Myanmar (Shu et al., 2009; Thant et al., 2015), Singapore (Lee et al., 2012) and Vietnam (Huang et al., 2007).

Previous reports demonstrated that co-circulation of DENV serotype 1, 2, 3 and 4 caused DF, DHF and DSS. Up to date, we found that the co-circulation of multiple genotypes and genotype replacement increased dengue cases in Thailand.

Part B: To evaluate biological marker of constructed D4 1036 PDK40 infectious clone

Over the past decade, intensive efforts were dedicated to the development of tetravalent DENV vaccines which would offer effective protection against all 4 circulating serotypes (Bhamarapravati and Sutee, 2000; Blaney et al., 2005; Raviprakash et al., 2008; Guy et al., 2011). These attempts have been made use of live-attenuated viruses such as DEN4V 1036 PDK48, to produce vaccine candidates which are currently undergoing phase I and II clinical trials (Bhamarapravati and Sutee, 2000; Sabchareon et al., 2002). This vaccine has safety and induced humoral and cell-mediated immunity (Bhamarapravati and Sutee, 2000; Rabablert et al., 2000).

In this study, we described the construction and phenotypic characterization of a DEN4V infectious clone which exhibits an attenuated phenotype indicating its potential safety for the inclusion in a chimeric dengue vaccine.

We identified 7 different missense mutations between DEN4V 1036 and DEN4V 1036 PDK40. These missense mutations were E-345$_{(Glu \rightarrow Lys)}$, E-463$_{(Val \rightarrow Met)}$, NS1-253$_{(Gln \rightarrow His)}$, NS4A-95$_{(Leu \rightarrow Phe)}$, NS4B-44$_{(Ile \rightarrow Phe)}$, NS4B-112$_{(Leu \rightarrow Ser)}$ and NS4B-240$_{(Ala \rightarrow Val)}$ **(Table 9).** Six of 7 mutations of DEN4V 1036 PDK40 were correlated to mutations found in DEN4V 1036 PDK48 (Kinney and Huang, 2001)). In addition, DEN4V 1036 PDK48 has 3 mixed genotypes **(Table 9)**. The mixed genotypes found in LAV were probably the result of an evolution process by which the virus adapted to mammalian cells (Butrapet et al., 2000). The proportion of mixed genotypes for each position was unclear and needed further investigation by high-throughput genome sequencing.

Two of missense mutation found in E. The DEN E function as receptor binding domain. E consists of 3 domains (I, II, III) (Modis et al., 2003; Chin, Chu and Ng, 2007). Residue E-345 is located in domain III (DIII) which plays an important role in binding to host receptor (Chin, Chu and Ng, 2007). An E-345$_{(Glu \rightarrow Lys)}$ mutant virus had low neurovirulence in newborn mice and induced neutralizing antibodies in rhesus monkeys (Lin et al., 2014). One additional missense mutation in E was E-463$_{(Val \rightarrow Met)}$ which locates in the transmembrane anchor domain of the E (Fritz et al., 2011). This domain plays a role in a conformation change of envelope in low-pH endosome (Rey, 2003).

One of missense mutation found in NS1 which plays an essential role in viral RNA replication (Mackenzie, Jones and Young, 1996). NS1 consists of N-terminal and C-terminal tail. Residue NS1-253 $_{(Gln \rightarrow His)}$ is located at C terminal (Edeling, Diamond and Fremont, 2014). In addition to viral replication, this region is involved in endothelial cell cross-reactivity via molecular mimicry (Wan et al., 2008).

One of missense mutation found in NS4A which form complex with other viral membrane protein (NS2A, NS2B and NS4B) and serves as the scaffold for the replication complex formation (Lee et al., 2015). NS4A is also purposed to be a part of viral porin proteins. NS4A, NS2A and NS2B of JEV found to altered membrane permeability and growth inhibit of host cells. The author suggests that these proteins form pore which induce cytopathic effect of host cells (Chang et al., 1999).

Three of missense mutation found in NS4B which plays a role in viral replication (Westaway et al., 1997). NS4B consists of 5 TMD; TMD1-5 (Miller,

Sparacio and Bartenschlager, 2006). Residue NS4B-44$_{(Ile \rightarrow Phe)}$, NS4B-112$_{(Leu \rightarrow Ser)}$ and NS4B-240$_{(Ala \rightarrow Val)}$ are located in TMD1, TMD3 and TMD5. NS4B-112$_{(Leu \rightarrow Ser)}$ and NS4B-240$_{(Ala \rightarrow Val)}$ were observed in 5-FU mutagenized DEN4V 2A strain (Blaney et al., 2001; Blaney et al., 2002). Site-directed mutagenesis of these 2 mutation resulted in larger plaque size, higher viral replication efficiency and viral yield in Vero cells (Blaney et al., 2003). In addition, WRAIR LAV DEN4V 341750 PDK20 contains mutation NS4B-240$_{(Ala \rightarrow Val)}$. In contrast, NS4B-112 of this LAV was mutated from Ser/leu to Leu (Kelly et al., 2010). However, the importance of each missense mutations is still unclear and site-directed mutagenesis studies would be required to elucidate the functional role of specific residues.

In this work, we constructed DEN4V 1036 PDK40 infectious clone using the Gibson assembly method. After transfection in Vero cells, IC-DEN4V-1036-PDK40 was rescued as evidenced by positive results of IFA and qRT-PCR **(Figure 21).**

Selection of DENV vaccine was shown by plaque assay (Eckels et al., 1976), where smaller plaque sizes indicated attenuated viruses (Goh et al., 2016). As shown in **Figure 22**, IC-DEN4V-1036-PDK40 had a comparatively small plaque sizes, while DEN4V 1036 had a larger plaque sizes, a result which would correlate to previous reports in the literature (Halstead and Marchette, 2003). Serial passages of DEN4V 1036 in PDK cells resulted in small plaque sizes. DEN4V 1036 PDK5, 10, 20 and 30, but not PDK50 showed CPE in LLC-MK2 cells (Halstead and Marchette, 2003). In this study, IC-DEN4V-1036-PDK40 did not show CPE in Vero cells, thus suggesting that phenotypic characterization of IC-DEN4V-1036-PDK40 was similar to LAV DEN4V 1036 PDK48.

Dengue-infected patients present commonly temperatures of 39-41°C. Temperature sensitivity at 39-41°C is a crucial biomarker for DENV vaccine (Eckels et al., 1976; Halstead and Marchette, 2003). We found that IC-DEN4V-1036-PDK40 had temperature sensitivity when compared to DEN4V 1036. The result was consistent with previous report that LAV DEN4V showed temperature sensitivity (Halstead and Marchette, 2003). Temperature sensitivity of IC-DEN4V-1036-PDK40 indicated that this virus might be safe to use as vaccine.

Vero cells are widely accepted as continuous cell lines for vaccine development (Barrett et al., 2009). The use of Vero cells for production of live-attenuated viruses may preserve desirable biologic properties of viruses derived during serial passage in PDK cells (Halstead and Marchette, 2003). Therefore, we used Vero cells for transfection, amplification and replication of IC-DEN4V-1036-PDK40. We found that IC-DEN4V-1036-PDK40 showed a high replication efficiency in Vero cells equal to DEN4V 1036 at the same MOI. Rapid replication in HuH-7 cells of LAV has been previously observed with DEN2V 16681 PDK53 (Kinney et al., 1997; Goh et al., 2016). On the contrary, LAV DEN3V 16562 PGMK30 had a lower replication efficiency than DEN3V 16562 in HuH-7 cells (Goh et al., 2016). The association between replication efficiency and attenuation is still unclear, however, a high replication efficiency of IC-DEN4V-1036-PDK40 in Vero cells would be of advantage for vaccine production as high viral titers can be achieved.

Replication in the mosquito vector demonstrates the capability of virus to infect the host either through natural infection or vaccination. Crippled replication of

live attenuated DEN vaccine viruses in mosquitoes is desirable to ensure a low probability of transmission of the vaccine virus (Kinney and Huang, 2001). Route of inoculation is also an importance factor for LAV DENV to replicate in mosquito vector. Artificial oral transmission models showed a restricted capability to infect and disseminate LAV DENV in the mosquito vector (Khin et al., 1994; Jirakanjanakit et al., 1999). Intrathoracic inoculation allows LAV DENV to infect and replicate in *Ae. aegypti* and *Tx. splendens*. In this study, the growth efficiency of IC-DEN4V-1036-PDK40 for replication in *Ae. aegypti via* intrathoracic inoculation was less than one of DEN4V 1036. The lower capability to replicate in mosquito vector of LAV were observed in LAV DEN2V 16681 PDK53 (Khin et al., 1994), LAV DEN3V 16562 PGMK30 FRhL3 (Jirakanjanakit et al., 1999) and chimeric yellow fever/DENV (CYD) 1-4 (Johnson et al., 2004). This result indicated that IC-DEN4V-1036-PDK40 was relatively unlikely to undergo frequent transmission by the mosquito vector.

Each serotype of DENV consists of 4-5 genotypes (Klungthong et al., 2008). Co-circulation of different serotypes provides a broad spectrum of disease symptoms and host immunity. Genbank data reveal that DEN4V genotype I has been dominating in Thailand for decades. Meanwhile, many strains of DEN4V genotype II had been isolated from both human and *Ae. aegypti* mosquitoes in Thailand since 2013 (Kittichai et al., 2015). Phylogenetic analysis revealed that DEN4V 1036 was categorized as genotype II strain. Ideally, a dengue vaccine candidate should provide sufficient protection against every serotype/genotype of DENV. In *Cynomolgus macaque*, a tetravalent CYD elicited seroneutralizing antibodies against all known and observed DENV serotypes, genotypes, geographic origins and isolated years (Barban et al., 2012).

CHAPTER VI
CONCLUSION

Part A; we have described the molecular epidemiology of dengue infection in Bangkok, Thailand during 2006-2015. The results demonstrated that there were 4 serotypes of DENV circulating in Thailand. DEN1V, DEN2V and DEN4V have only 1 genotype. DEN3V has 2 genotypes (II and III), thereby the genotype III was predominant in recent years. Our study offered genetic information that complements current knowledge on dengue epidemiology, evolution, and transmission dynamics. In further study, we plan to use the data of molecular epidemiology to prevention and control dengue diseases.

Part B; IC-DEN4V-1036-PDK40 shows attenuated characterization including small/pinpoint plaque sizes, temperature sensitivity and low growth efficiency in *Ae. aegypti*. These results indicate safety of IC-DEN4V-1036-PDK40. This infectious clone might be useful for use as vaccine or a backbone for construct chimera vaccine. In further studies, we plan to determine the immunogenicity of IC-DEN4V-1036-PDK40 in vivo and identify the attenuation mutations and to construct a DENV chimera using IC-DEN4V-PDK40 as backbone.

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APPENDIX A

A. RNA extraction

Materials

1. Culture supernatant
2. E.Z.N.A viral RNA extraction kit

No.	Component	Amount
1.	HiBind RNA Mini Columns	50 pieces
2.	2 ml Collection Tubes	150 pieces
3.	QVL Lysis Buffer	30 ml
4.	RNA Wash Buffer II	12 ml
5.	VHB Buffer	15 ml
6.	Carrier RNA (Poly A)	320 μg
7.	DEPC Water	10 ml

Additional material

No.	Component	Volume (ml)
1.	Absolute ethanol Cat No. UN1170, BDH Prolabo, USA	2,500

Methods

1. Add 500 µl of QVL and 5 µl of carrier RNA into a 1.5 ml microcentrifuge tube.
2. Add 150 µl of cell culture supernatant to mixture. Vortex for 30 seconds.
3. Incubate at RT for 10 mins.
4. Centrifuge briefly.
5. Add 350 µl of absolute ETOH. Vortex for 30 seconds to mix thoroughly.
6. Centrifuge briefly.
7. Insert a HiBind RNA Mini Column into a 2 mL Collection Tube.
8. Transfer 750 µl sample to the HiBind RNA Mini Column.
9. Centrifuge at maximum speed (13,000 RPM) for 1 mins.
10. Discard filtrate and reuse the collection tube.
11. Repeat Steps 9-11.
12. Transfer the HiBind RNA Mini Column to a new 2 ml Collection Tube.
13. Add 500 µl of VHB Buffer.
14. Centrifuge at maximum speed (13,000 RPM) for 1 mins.
15. Discard the filtrate and the collection tube.
16. Transfer the HiBind RNA Mini Column to a new 2 ml Collection Tube.
17. Add 500 µl of RNA Wash Buffer II.
18. Centrifuge at maximum speed for 15 seconds.
19. Discard filtrate and reuse the collection tube.
20. Repeat Steps 18-20 for a second RNA Wash Buffer II wash step.
21. Centrifuge at maximum speed (13,000 RPM) for 2 minutes.
22. Transfer the Column to a clean 5 mL microcentrifuge tube.
23. Add 50 µl of DEPC Water directly to the center of column matrix.
24. Centrifuge at maximum speed (13,000 RPM) for 1 minute.
25. Store RNA at -70°C.

B. cDNA synthesis

Materials

1. RNA
2. Maxima H Minus First Strand cDNA Synthesis Kit

Methods

1. Add the 13 µl of RNA, 1 µl of Random primers and 1 µl of 10mM dNTP into a 0.2 ml PCR tube.
2. Mix gently and incubate at 65°C for 5 min. Chill on ice.
3. Add 4 µl of RT buffer and 1 µl of Maxima H minus enzyme mix
4. Mix gently and centrifuge briefly.
5. Incubate for 10 min at 25°C followed by 30 min at 50°C and 5 min at 85°C.
6. Store cDNA at -20°C.

C. Standard PCR

Materials

1. DNA template
2. Primer set
3. KAPA TaqReadyMix kit

Methods

1. Calculate the required volumes of each component based on the following table:

Component	**Volume (20 µl rxn)**	
	1 rxn	**10 rxns**
2× KAPA Taq ReadyMix	10 µl	100 µl
10 mM Forward primer	0.5 µl	5.0 µl
10 mM Reverse primer	0.5 µl	5.0 µl
Nuclease-free water	7.0 µl	70.0 µl
cDNA Template	2.0 µl	20.0 µl

2. Perform PCR with the following cycling protocol:

Step	Temperature	Time	Cycle
Initial denature	95°C	2 min	1
Denature	95°C	30 sec	35
Annealing	55-60°C	30 sec	
Extension	72°C	1kb/min	
Final extension	72°C	2 min	1

D. DENV qRT-PCR

Materials

1. DNA/RNA template
2. qRT-PCR Primer probe set
3. KAPA PROBE FAST Universal One-step qRT-PCR Kit

Methods

1. Calculate the required volumes of each component based on the following table:

Component	Volume (20 µl rxn)		
	1 rxn	**8 rxns**	**10 rxns**
KAPA PROBE FAST qPCR Master Mix (2×)	10 µl	80 µl	100 µl
10 mM D1 primer	0.5 µl	4.0 µl	5.0 µl
10 mM D2 primer	0.5 µl	4.0 µl	5.0 µl
10 mM D3 primer	0.5 µl	4.0 µl	5.0 µl
10 mM D4 primer	0.5 µl	4.0 µl	5.0 µl
10 mM D1 probe	0.2 µl	1.6 µl	2.0 µl
10 mM D2 probe	0.2 µl	1.6 µl	2.0 µl
10 mM D3 probe	0.2 µl	1.6 µl	2.0 µl
10 mM D4 probe	0.2 µl	1.6 µl	2.0 µl
KAPA RT Mix (50×)	0.4 µl	3.2 µl	4.0 µl
Nuclease-free water	4.8 µl	38.4 µl	48.0 µl
RNA Template	2.0 µl	16.0 µl	20.0 µl

2. Perform PCR with the following cycling protocol:

Step	Temperature	Time	Cycle
cDNA synthesis	42°C	5 min	1
Initial denature	95°C	5 min	1
Denature	95°C	3 sec	40
Annealing+Plate read	60°C	30 sec	

E. Long length high-fidelity PCR

Materials

1. DNA template
2. Primer set
3. Phusion flash high-fidelity PCR mastermix kit

Methods

1. Calculate the required volumes of each component based on the following table:

Component	**Volume (20 µl rxn)**	
	1 rxn	**10 rxns**
2× Phusion Flash PCR Master Mix	10 µl	100 µl
10 mM Forward primer	0.5 µl	5.0 µl
10 mM Reverse primer	0.5 µl	5.0 µl
Nuclease-free water	7.0 µl	70.0 µl
cDNA Template	2.0 µl	20.0 µl

2. Perform PCR with the following cycling protocol:

Step	Temperature	Time	Cycle
Initial denature	98°C	10 min	1
Denature	98°C	1 sec	30
Annealing	55-60°C	5 sec	
Extension	72°C	1kb/15 sec	
Final extension	72°C	1 min	1

F. Reagents for cultivation of bacteria for cloning assay

1. Ampicillin 100 mg/ml

Ampicillin	1	g

Add 10 ml sterile MilliQ water. Aliquot 1 ml and store at -20°C until use.

2. Luria-Bertani (LB) medium

2.1 Luria-Bertani plate containing 100 μg/ml ampicillin

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g
Bacto agar	15	g

Adjust the volume to 1 liter with MilliQ water. Autoclave at 121°C under pressure of 15 psi for 15 minutes. After autoclaved, cool medium to approximate 55°C in water bath. Add 1 ml of 100 mg/ml amplicillin and pour the plate. After agar polymerized, 1-2 plates are randomly selected for sterility test at 37°C overnight. Store agar plates at 4°C until use.

2.2 Luria-Bertani broth

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

Adjust the volume to 1 liter with MilliQ water. Autoclave at 121°C under pressure of 15 psi for 15 mins. Store LB broth at 4°C until use.

G. Reagents for PCR and electrophoresis assay

1. TBE solution (0.5X)

TBE powder	8.5	g
Distilled water	900	ml

Add 1 l sterile MilliQ water. Store at room temperature

2. 1% agarose gel containing SYBR safe

Agarose	1	g
TBE solution (0.5X)	100	ml

Disolve by heating in microwave at 800 watt for 1.5 mins. Cool down agarose gel to 55 °C. Add 10 μl of SYBR safe (10,000X). Pour agarose gel containing SYBR safe to chamber.

3. 2% agarose gel containing SYBR safe

Agarose	2	g
TBE working solution (0.5X)	100	ml

Disolve by heating in microwave at 800 watts for 1.5 mins. Cool down agarose gel to 55 ℃. Add 10 µl of SYBR safe (10,000X). Pour agarose gel containing SYBR safe to chamber.

APPENDIX B

Appendix B: Reference DENV from Genbank database

Serotypes	No.	Country of origin	Isolation (years)	Genotypes	Genebank accession #
DEN1V (n= 28)	1	Cambodia	2000	I	GQ868637
	2	China	2004	I	DQ836632
	3	China	2004	I	DQ193572
	4	Sri Lanka	2009	I	HQ891315
	5	Thailand	1981	I	AY732483
	6	Thailand	1982	I	AY732481
	7	Thailand	1991	I	AY732478
	8	Thailand	1991	I	AY732477
	9	Thailand	1994	I	AY732480
	10	Thailand	1994	I	AY732475
	11	Thailand	2001	I	AY732482
	12	Thailand	2001	I	AY732479
	13	Vietnam	2004	I	FJ882569
	14	Indonesia	1988	II	AB074761
	15	Indonesia	2002	II	AB111075
	16	Philippines	1974	II	AF425627
	17	Tahiti	2001	II	AB111070
	18	Thailand	1963	II	AF425629
	19	Thailand	1964	II	AF180817
	20	Malaysia	1972	III	EF457905
	21	Brunei	2006	IV	EU179861
	22	China	1991	IV	FJ196845
	23	USA	2001	IV	DQ672564
	24	Colombia	1996	V	AF425617
	25	Thailand	1980	V	AY732429
	26	Thailand	1980	V	AY732474
	27	USA	1986	V	FJ562106
	28	USA	2000	V	FJ850071

Appendix B: Reference DENV from Genbank database (continued)

Serotypes	No.	Country of origin	Isolation (years)	Genotypes	Genebank accession #
DEN2V (n=32)	1	Columbia	1986	American	AY702040
	2	Tonga	1974	American	AY744147
	3	Venezuela	1987	American	AF100465
	4	Venezuela	1992	American	AF100469
	5	Thailand	1964	Asian I	GQ868591
	6	Thailand	1974	Asian I	DQ181806
	7	Thailand	1979	Asian I	DQ181805
	8	Thailand	1984	Asian I	DQ181804
	9	Thailand	1985	Asian I	DQ181803
	10	Thailand	1988	Asian I	DQ181802
	11	Thailand	1995	Asian I	DQ181800
	12	Thailand	1998	Asian I	DQ181799
	13	Thailand	1999	Asian I	DQ181798
	14	Thailand	2001	Asian I	DQ181797
	15	Thailand	2004	Asian I	JQ993224
	16	Thailand	2004	Asian I	JQ993219
	17	Thailand	2005	Asian I	JQ993226
	18	Thailand	2006	Asian I	JQ993225
	19	Thailand	2006	Asian I	JF812112
	20	Thailand	2007	Asian I	JQ993227
	21	Thailand	2007	Asian I	JQ993208
	22	Thailand	2010	Asian I	JN568274
	23	New Guinea	1944	Asian II	AF038403
	24	Dominican	2001	Asian/American	AB122022
	25	Jamaica	1983	Asian/American	M20558
	26	Martinique	1998	Asian/American	AF208496
	27	Thailand	1990	Asian/American	DQ181801
	28	Australia	1993	Cosmopolitan	AY037116
	29	China	1999	Cosmopolitan	AF359579
	30	Indonesia	1998	Cosmopolitan	AB189122
	31	Indonesia	2004	Cosmopolitan	AY858035
	32	Guinea	1981	Sylvatic	EF105378

Appendix B: Reference DENV from Genbank database (continued)

Serotypes	No.	Country of origin	Isolation (years)	Genotypes	Genebank accession #
DEN3V (n=25)	1	Philippines	1964	I	KU725665
	2	Indonesia	1978	I	AY648961
	3	Tahiti	1989	I	AY744677
	4	Thailand	1987	II	AY676353
	5	Thailand	1987	II	AY676352
	6	Thailand	1993	II	AY676351
	7	Thailand	1993	II	AY676350
	8	Thailand	1998	II	AY676349
	9	Thailand	1998	II	AY676348
	10	Thailand	2004	II	JQ993230
	11	Thailand	2005	II	JQ993229
	12	Thailand	2006	II	JF812104
	13	Thailand	2012	II	KP100251
	14	Thailand	2012	II	KP100252
	15	Thailand	2012	II	KP100253
	16	Thailand	2012	II	KP100254
	17	Thailand	2012	II	KP100255
	18	Thailand	2012	II	KP100256
	19	Martinique	1999	III	AY099337
	20	Sri Lanka	2000	III	AY099336
	21	Thailand	2012	III	KP176707
	22	Thailand	2013	III	KP100257
	23	Thailand	2013	III	KP176708
	24	Puerto Rico	1963	IV	AY146762
	25	Puerto Rico	1977	IV	AY146761

Appendix B: Reference DENV from Genbank database (continued)

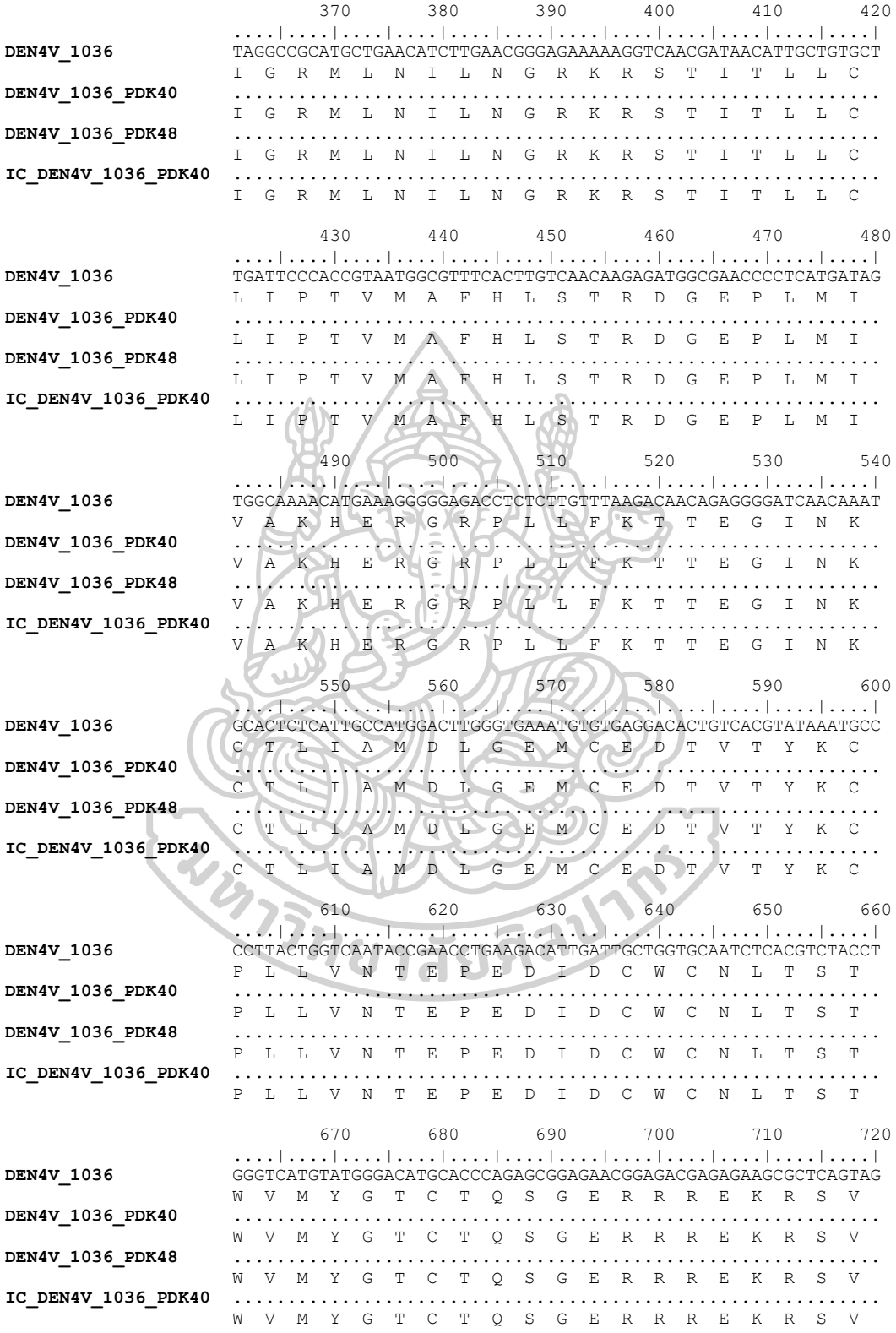
Serotypes	No.	Country of origin	Isolation (years)	Genotypes	Genebank accession #
DEN4V (n=23)	1	China	1990	I	AF289029
	2	India	2007	I	HM237349
	3	Thailand	1977	I	AY618991
	4	Thailand	1991	I	AY618990
	5	Thailand	2000	I	AY618938
	6	Thailand	2001	I	AY618992
	7	Thailand	2001	I	AY618943
	8	Thailand	2002	I	AY618946
	9	Thailand	2003	I	EU448457
	10	Thailand	2004	I	JQ993298
	11	Thailand	2004	I	JQ993292
	12	Thailand	2005	I	JF812108
	13	Thailand	2005	I	JQ993274
	14	Thailand	2005	I	JQ993271
	15	Thailand	2006	I	JF812107
	16	Thailand	2006	I	JF812110
	17	Thailand	2010	I	JN575594
	18	China	2010	II	JN599977
	19	Dominican	1981	II	AF326573
	20	Solomon islands	2007	II	EU448462
	21	Thailand	2000	II	AY618993
	22	Thailand	1997	III	AY618989
	23	Thailand	1997	III	AY618988

APPENDIX C

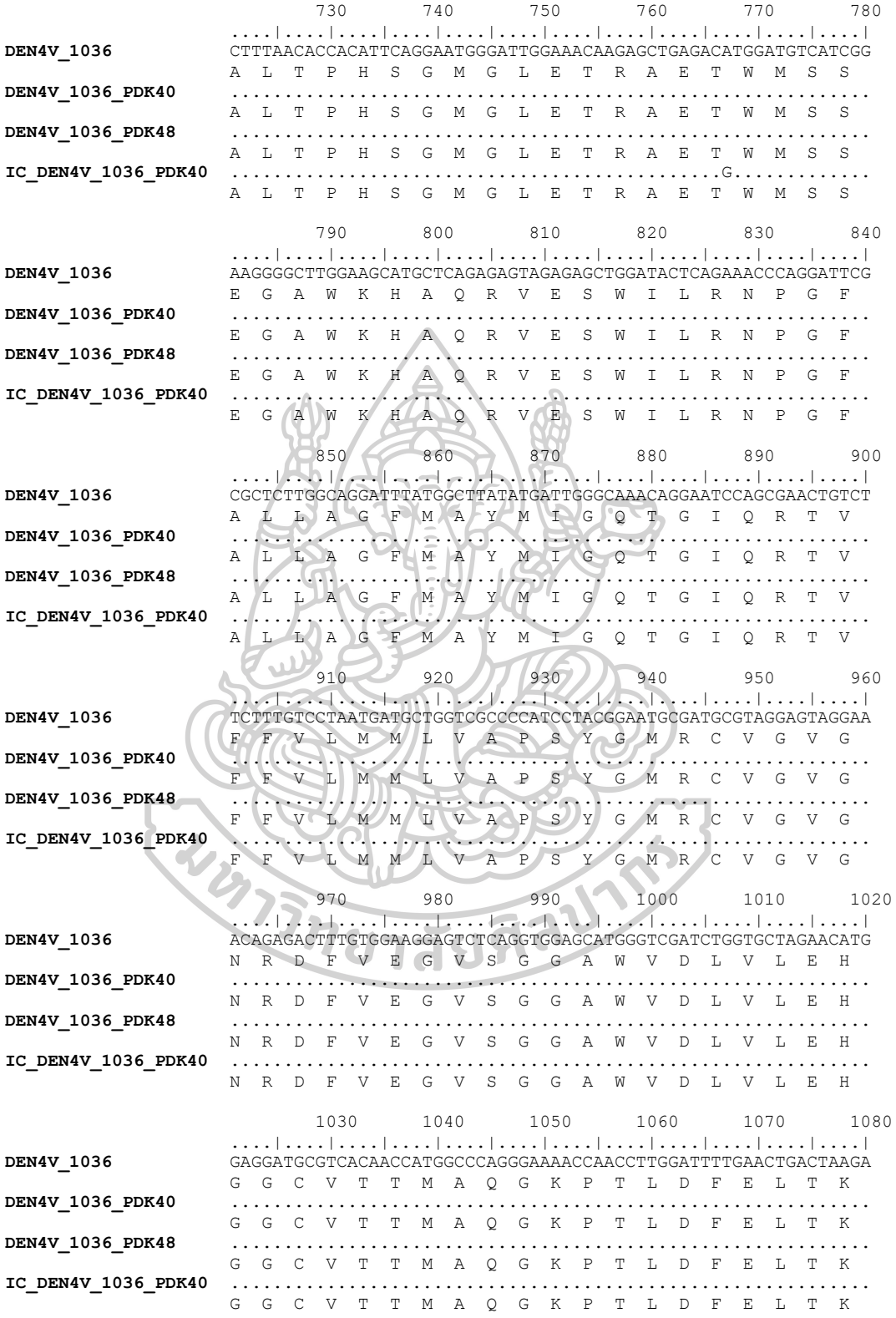
Appendix C: Genome alignment of DEN4V 1036 and its derivatives.

```
                          10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AGTTGTTAGTCTGTGTGGACCGACAAGGACAGTTCCAAATCGGAAGCTTGCTTAACACAG

DEN4V_1036_PDK40    ............................................................

DEN4V_1036_PDK48    ............................................................

IC_DEN4V_1036_PDK40 ............................................................


                                  70        80        90       100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTCTAACAGTTTGTTTGAATAGAGAGCAGATCTCTGGAAAAATGAACCAACGAAAAAAGG
                                                          M  N  Q  R  K  K
DEN4V_1036_PDK40    ............................................................
                                                          M  N  Q  R  K  K
DEN4V_1036_PDK48    ............................................................
                                                          M  N  Q  R  K  K
IC_DEN4V_1036_PDK40 ............................................................
                                                          M  N  Q  R  K  K

                                 130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGGTTAGACCACCTTTCAATATGCTGAAACGCGAGAGAAACCGCGTATCAACCCCTCAAG
                    V  V  R  P  P  F  N  M  L  K  R  E  R  N  R  V  S  T  P  Q
DEN4V_1036_PDK40    ............................................................
                    V  V  R  P  P  F  N  M  L  K  R  E  R  N  R  V  S  T  P  Q
DEN4V_1036_PDK48    ............................................................
                    V  V  R  P  P  F  N  M  L  K  R  E  R  N  R  V  S  T  P  Q
IC_DEN4V_1036_PDK40 ............................................................
                    V  V  R  P  P  F  N  M  L  K  R  E  R  N  R  V  S  T  P  Q

                                 190       200       210       220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GGTTGGTGAAGAGATTCTCAACCGGACTTTTTTCTGGGAAAGGACCCTTACGGATGGTGC
                    G  L  V  K  R  F  S  T  G  L  F  S  G  K  G  P  L  R  M  V
DEN4V_1036_PDK40    ............................................................
                    G  L  V  K  R  F  S  T  G  L  F  S  G  K  G  P  L  R  M  V
DEN4V_1036_PDK48    ............................................................
                    G  L  V  K  R  F  S  T  G  L  F  S  G  K  G  P  L  R  M  V
IC_DEN4V_1036_PDK40 ............................................................
                    G  L  V  K  R  F  S  T  G  L  F  S  G  K  G  P  L  R  M  V

                                 250       260       270       280       290       300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TAGCATTCATCACGTTTTTGCGAGTCCTTTCCATCCCACCAACAGCAGGGATTCTGAAAA
                    L  A  F  I  T  F  L  R  V  L  S  I  P  P  T  A  G  I  L  K
DEN4V_1036_PDK40    ............................................................
                    L  A  F  I  T  F  L  R  V  L  S  I  P  P  T  A  G  I  L  K
DEN4V_1036_PDK48    ............................................................
                    L  A  F  I  T  F  L  R  V  L  S  I  P  P  T  A  G  I  L  K
IC_DEN4V_1036_PDK40 ............................................................
                    L  A  F  I  T  F  L  R  V  L  S  I  P  P  T  A  G  I  L  K

                                 310       320       330       340       350       360
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GATGGGGACAGTTGAAGAAAAATAAGGCCATCAGGATACTGATTGGATTCAGGAAGGAGA
                    R  W  G  Q  L  K  K  N  K  A  I  R  I  L  I  G  F  R  K  E
DEN4V_1036_PDK40    ............................................................
                    R  W  G  Q  L  K  K  N  K  A  I  R  I  L  I  G  F  R  K  E
DEN4V_1036_PDK48    ............................................................
                    R  W  G  Q  L  K  K  N  K  A  I  R  I  L  I  G  F  R  K  E
IC_DEN4V_1036_PDK40 ............................................................
                    R  W  G  Q  L  K  K  N  K  A  I  R  I  L  I  G  F  R  K  E
```

Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



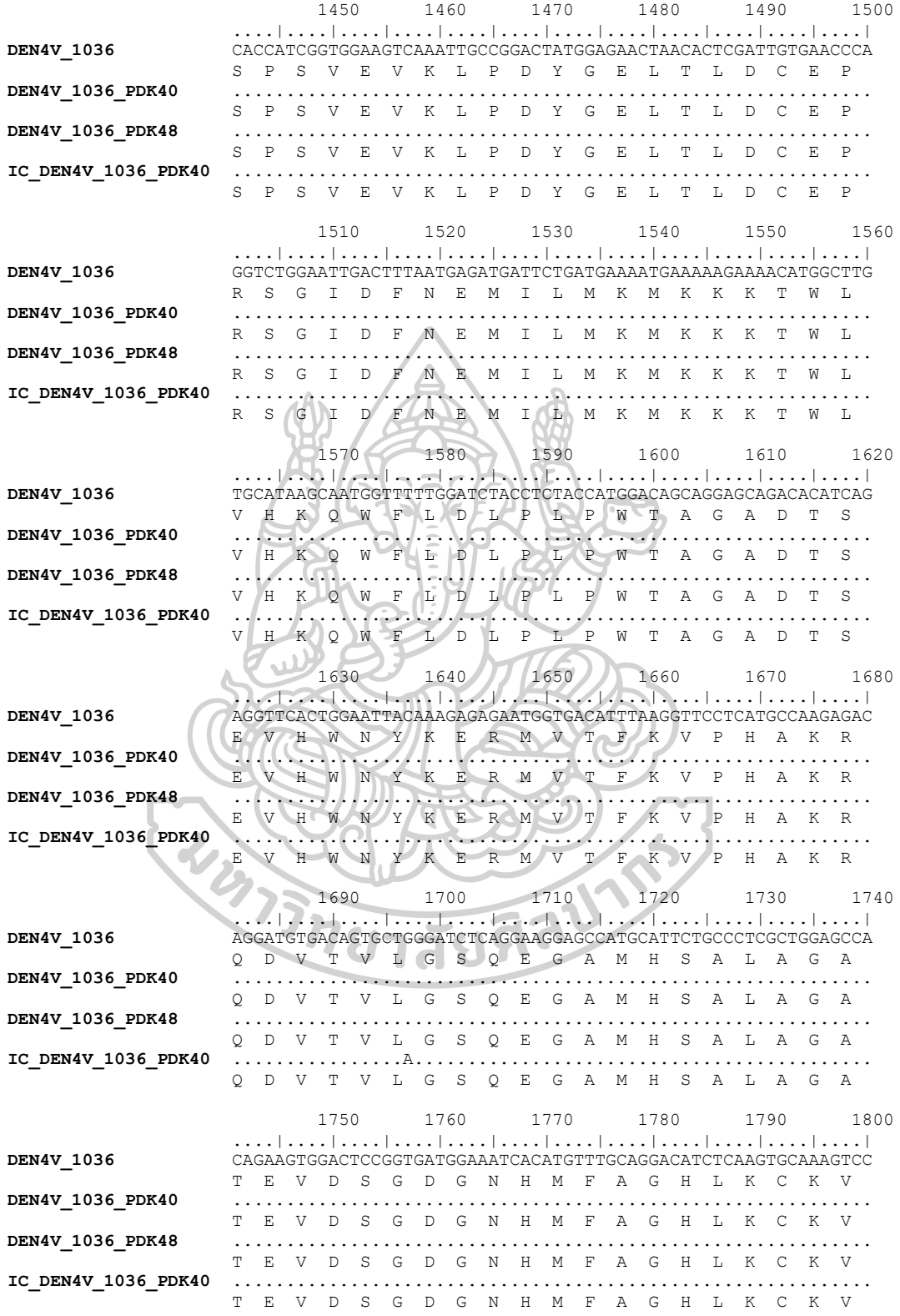
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          1090      1100      1110      1120      1130      1140
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CAACAGCCAAGGAAGTGGCTCTGTTAAGAACCTATTGCATTGAAGCCTCAATATCAAACA
                    T  T  A  K  E  V  A  L  L  R  T  Y  C  I  E  A  S  I  S  N
DEN4V_1036_PDK40    ............................................................
                    T  T  A  K  E  V  A  L  L  R  T  Y  C  I  E  A  S  I  S  N
DEN4V_1036_PDK48    ............................................................
                    T  T  A  K  E  V  A  L  L  R  T  Y  C  I  E  A  S  I  S  N
IC_DEN4V_1036_PDK40 ............................................................
                    T  T  A  K  E  V  A  L  L  R  T  Y  C  I  E  A  S  I  S  N

                          1150      1160      1170      1180      1190      1200
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TAACCACGGCAACAAGATGTCCAACGCAAGGAGAGCCTTATCTAAAAGAGGAACAAGACC
                    I  T  T  A  T  R  C  P  T  Q  G  E  P  Y  L  K  E  E  Q  D
DEN4V_1036_PDK40    ............................................................
                    I  T  T  A  T  R  C  P  T  Q  G  E  P  Y  L  K  E  E  Q  D
DEN4V_1036_PDK48    ............................................................
                    I  T  T  A  T  R  C  P  T  Q  G  E  P  Y  L  K  E  E  Q  D
IC_DEN4V_1036_PDK40 ............................................................
                    I  T  T  A  T  R  C  P  T  Q  G  E  P  Y  L  K  E  E  Q  D

                          1210      1220      1230      1240      1250      1260
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AACAGTACATTTGCCGGAGAGATGTGGTAGACAGAGGGTGGGGCAATGGCTGTGGCTTGT
                    Q  Q  Y  I  C  R  R  D  V  V  D  R  G  W  G  N  G  C  G  L
DEN4V_1036_PDK40    ............................................................
                    Q  Q  Y  I  C  R  R  D  V  V  D  R  G  W  G  N  G  C  G  L
DEN4V_1036_PDK48    .........C..................................................
                    Q  Q  Y  I  C  R  R  D  V  V  D  R  G  W  G  N  G  C  G  L
IC_DEN4V_1036_PDK40 ............................................................
                    Q  Q  Y  I  C  R  R  D  V  V  D  R  G  W  G  N  G  C  G  L

                          1270      1280      1290      1300      1310      1320
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTGGAAAAGGAGGAGTTGTGACATGTGCGAAGTTTTCATGTTCGGGGAAGATAACAGGCA
                    F  G  K  G  G  V  V  T  C  A  K  F  S  C  S  G  K  I  T  G
DEN4V_1036_PDK40    ............................................................
                    F  G  K  G  G  V  V  T  C  A  K  F  S  C  S  G  K  I  T  G
DEN4V_1036_PDK48    ............................................................
                    F  G  K  G  G  V  V  T  C  A  K  F  S  C  S  G  K  I  T  G
IC_DEN4V_1036_PDK40 ............................................................
                    F  G  K  G  G  V  V  T  C  A  K  F  S  C  S  G  K  I  T  G

                          1330      1340      1350      1360      1370      1380
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          ATTTGGTCCAAATTGAGAACCTTGAATACACAGTGGTTGTAACAGTCCACAATGGAGACA
                    N  L  V  Q  I  E  N  L  E  Y  T  V  V  V  T  V  H  N  G  D
DEN4V_1036_PDK40    ............................................................
                    N  L  V  Q  I  E  N  L  E  Y  T  V  V  V  T  V  H  N  G  D
DEN4V_1036_PDK48    ............................................................
                    N  L  V  Q  I  E  N  L  E  Y  T  V  V  V  T  V  H  N  G  D
IC_DEN4V_1036_PDK40 ............................................................
                    N  L  V  Q  I  E  N  L  E  Y  T  V  V  V  T  V  H  N  G  D

                          1390      1400      1410      1420      1430      1440
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CCCATGCAGTAGGAAATGACACATCCAATCATGGAGTTACAGCCACGATAACTCCCAGGT
                    T  H  A  V  G  N  D  T  S  N  H  G  V  T  A  T  I  T  P  R
DEN4V_1036_PDK40    ............................................................
                    T  H  A  V  G  N  D  T  S  N  H  G  V  T  A  T  I  T  P  R
DEN4V_1036_PDK48    ............................................................
                    T  H  A  V  G  N  D  T  S  N  H  G  V  T  A  T  I  T  P  R
IC_DEN4V_1036_PDK40 ............................................................
                    T  H  A  V  G  N  D  T  S  N  H  G  V  T  A  T  I  T  P  R
```

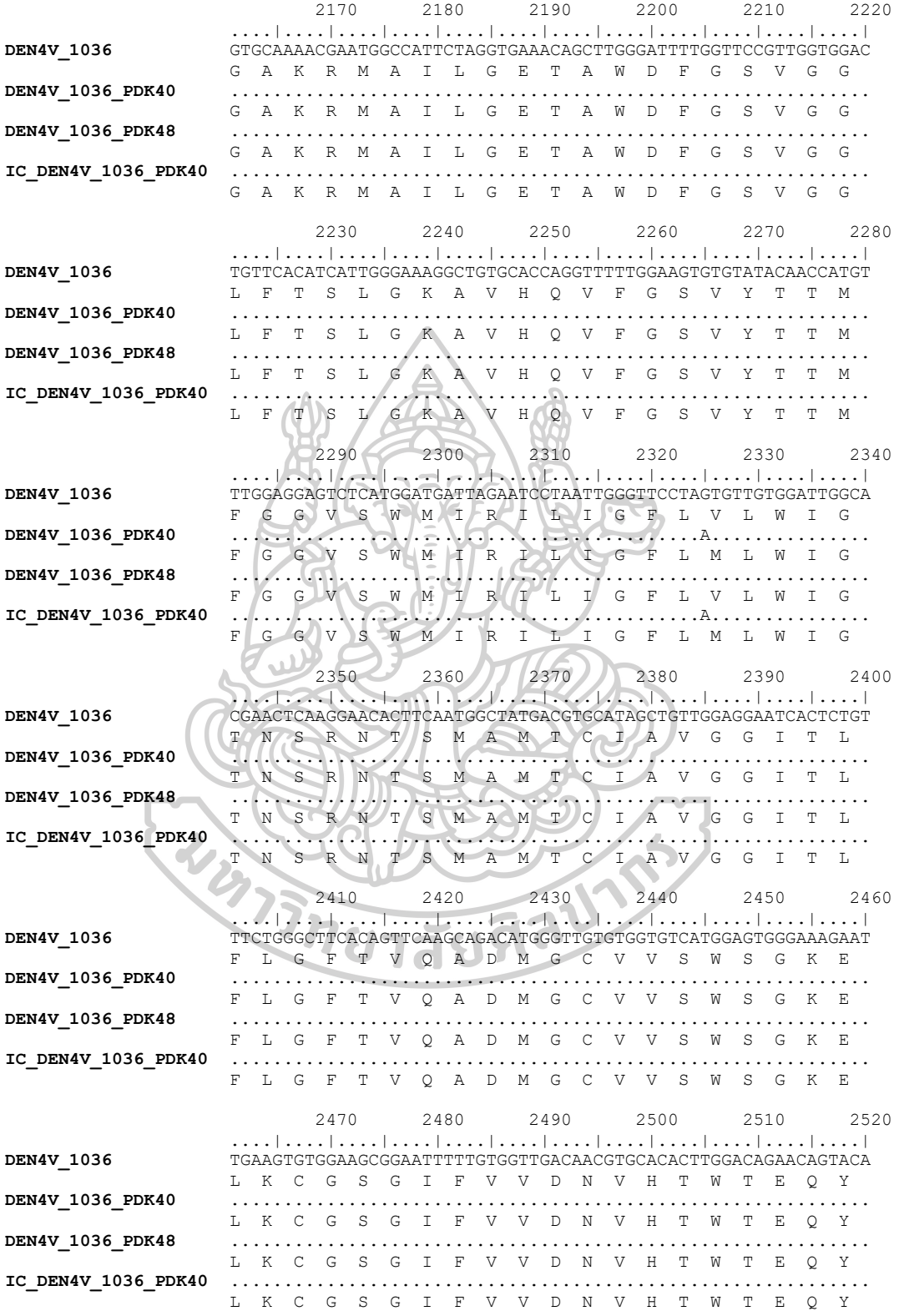
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



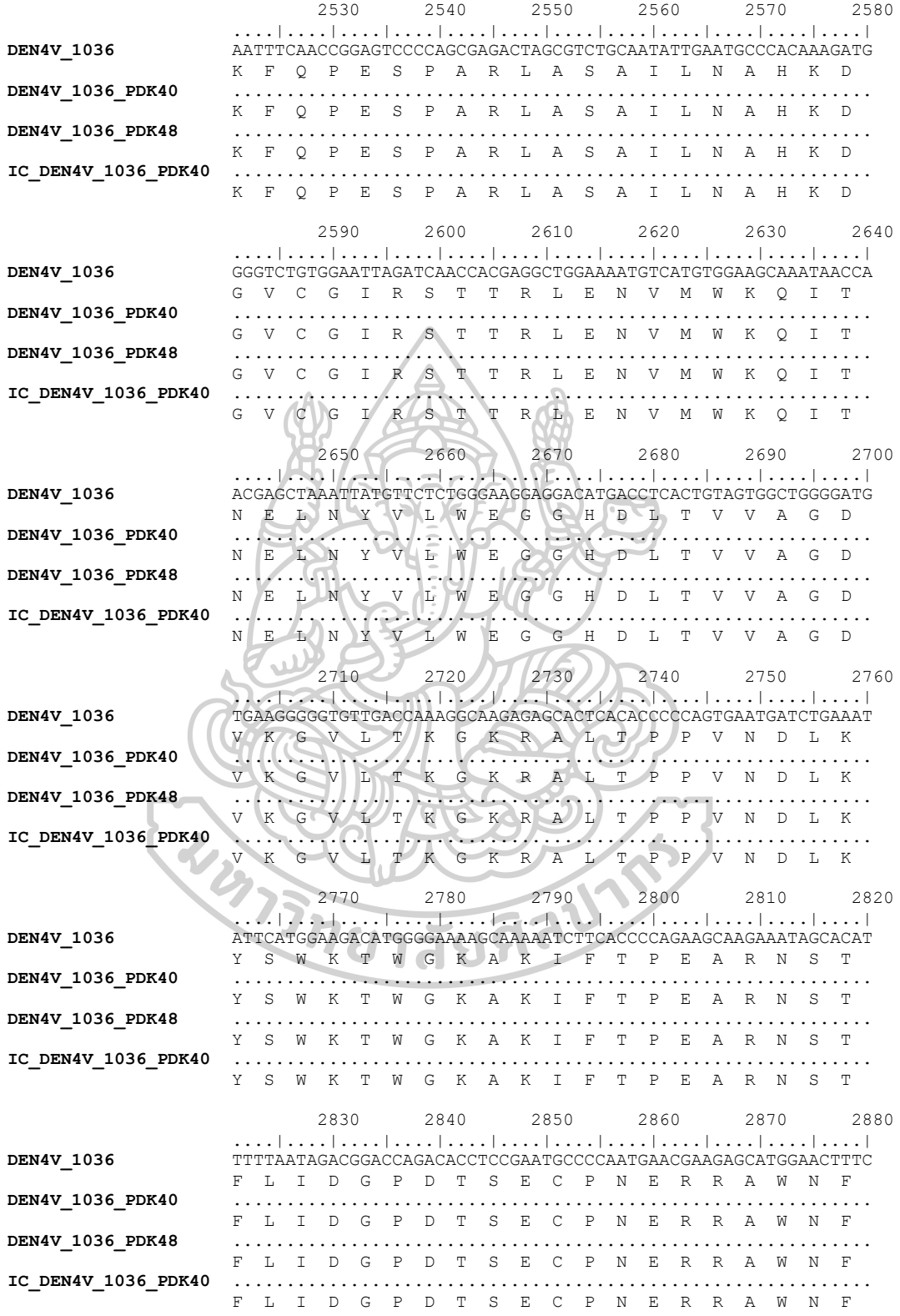
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          1810      1820      1830      1840      1850      1860
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GTATGGAGAAATTGAGAATCAAGGGAATGTCATACACGATGTGTTCAGGAAAGTTCTCAA
                    R  M  E  K  L  R  I  K  G  M  S  Y  T  M  C  S  G  K  F  S
DEN4V_1036_PDK40    ............................................................
                    R  M  E  K  L  R  I  K  G  M  S  Y  T  M  C  S  G  K  F  S
DEN4V_1036_PDK48    ............................................................
                    R  M  E  K  L  R  I  K  G  M  S  Y  T  M  C  S  G  K  F  S
IC_DEN4V_1036_PDK40 ............................................................
                    R  M  E  K  L  R  I  K  G  M  S  Y  T  M  C  S  G  K  F  S

                          1870      1880      1890      1900      1910      1920
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTGACAAAGAGATGGCAGAAACACAGCATGGGACAACAGTGGTGAAAGTCAAGTATGAAG
                    I  D  K  E  M  A  E  T  Q  H  G  T  T  V  V  K  V  K  Y  E
DEN4V_1036_PDK40    ............................................................
                    I  D  K  E  M  A  E  T  Q  H  G  T  T  V  V  K  V  K  Y  E
DEN4V_1036_PDK48    ............................................................
                    I  D  K  E  M  A  E  T  Q  H  G  T  T  V  V  K  V  K  Y  E
IC_DEN4V_1036_PDK40 ............................................................
                    I  D  K  E  M  A  E  T  Q  H  G  T  T  V  V  K  V  K  Y  E

                          1930      1940      1950      1960      1970      1980
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GTGCTGGAGCTCCGTGTAAAGTCCCCATAGAGATAAGAGATGTGAACAAGGAAAAAGTGG
                    G  A  G  A  P  C  K  V  P  I  E  I  R  D  V  N  K  E  K  V
DEN4V_1036_PDK40    ..........................................A.................
                    G  A  G  A  P  C  K  V  P  I  E  I  R  D  V  N  K  K  K  V
DEN4V_1036_PDK48    ..........................................A.................
                    G  A  G  A  P  C  K  V  P  I  E  I  R  D  V  N  K  K  K  V
IC_DEN4V_1036_PDK40 ..........................................A.................
                    G  A  G  A  P  C  K  V  P  I  E  I  R  D  V  N  K  K  K  V

                          1990      2000      2010      2020      2030      2040
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTGGGCGTATCATCTCATCCACCCCTTTGGCTGAGAATACCAACAGTGCAACCAACATAG
                    V  G  R  I  I  S  S  T  P  L  A  E  N  T  N  S  A  T  N  I
DEN4V_1036_PDK40    ............................................................
                    V  G  R  I  I  S  S  T  P  L  A  E  N  T  N  S  A  T  N  I
DEN4V_1036_PDK48    ............................................................
                    V  G  R  I  I  S  S  T  P  L  A  E  N  T  N  S  A  T  N  I
IC_DEN4V_1036_PDK40 ............................................................
                    V  G  R  I  I  S  S  T  P  L  A  E  N  T  N  S  A  T  N  I

                          2050      2060      2070      2080      2090      2100
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AGTTAGAACCCCCCTTTGGGGACAGCTACATAGTGATAGGTGTTGGAAACAGTGCATTAA
                    E  L  E  P  P  F  G  D  S  Y  I  V  I  G  V  G  N  S  A  L
DEN4V_1036_PDK40    ............................................................
                    E  L  E  P  P  F  G  D  S  Y  I  V  I  G  V  G  N  S  A  L
DEN4V_1036_PDK48    ............................................................
                    E  L  E  P  P  F  G  D  S  Y  I  V  I  G  V  G  N  S  A  L
IC_DEN4V_1036_PDK40 ............................................................
                    E  L  E  P  P  F  G  D  S  Y  I  V  I  G  V  G  N  S  A  L

                          2110      2120      2130      2140      2150      2160
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CACTCCATTGGTTCAGGAAAGGGAGTTCCATTGGCAAGATGTTTGAGTCCACATACAGAG
                    T  L  H  W  F  R  K  G  S  S  I  G  K  M  F  E  S  T  Y  R
DEN4V_1036_PDK40    ............................................................
                    T  L  H  W  F  R  K  G  S  S  I  G  K  M  F  E  S  T  Y  R
DEN4V_1036_PDK48    ............................................................
                    T  L  H  W  F  R  K  G  S  S  I  G  K  M  F  E  S  T  Y  R
IC_DEN4V_1036_PDK40 ............................................................
                    T  L  H  W  F  R  K  G  S  S  I  G  K  M  F  E  S  T  Y  R
```

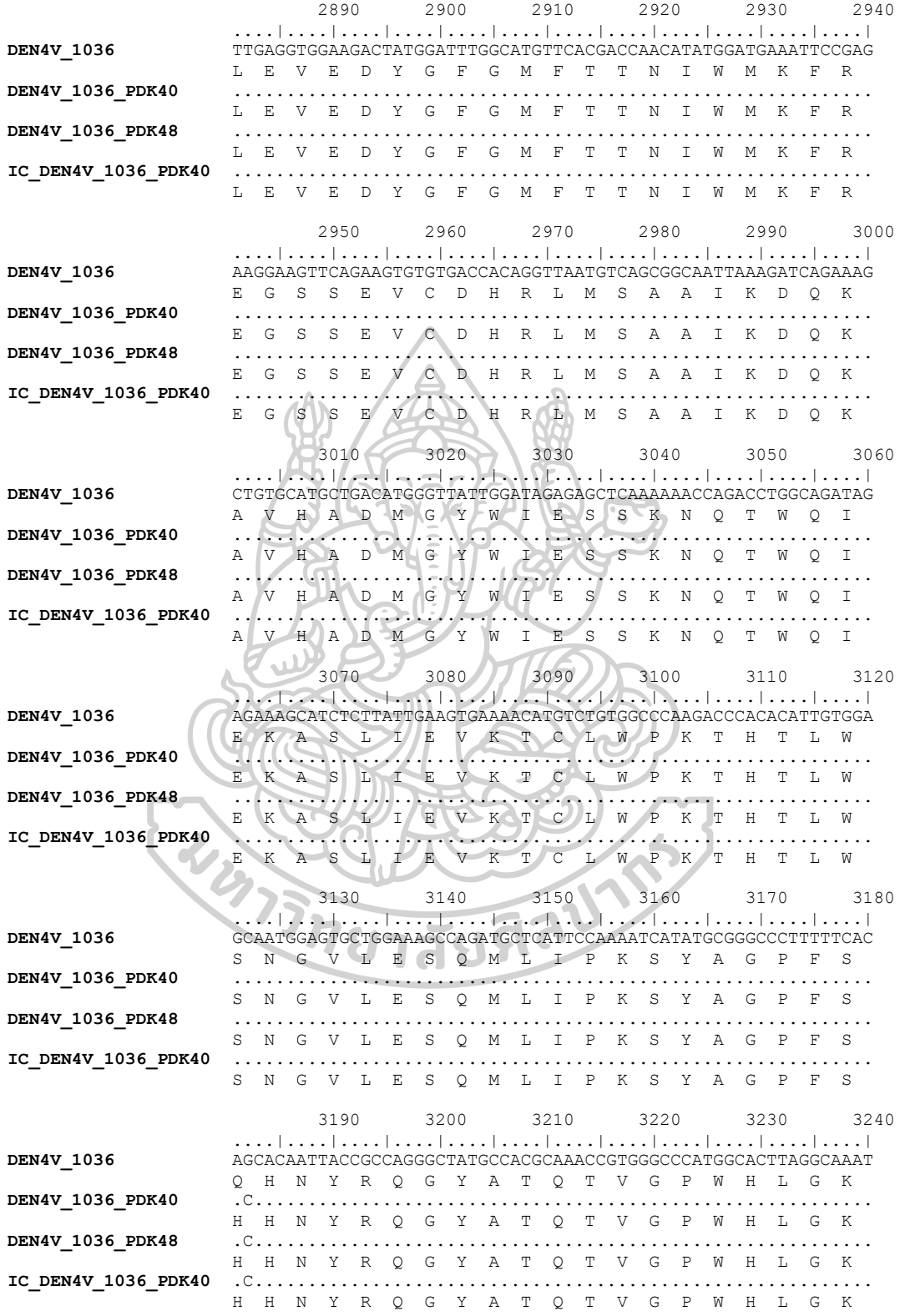
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



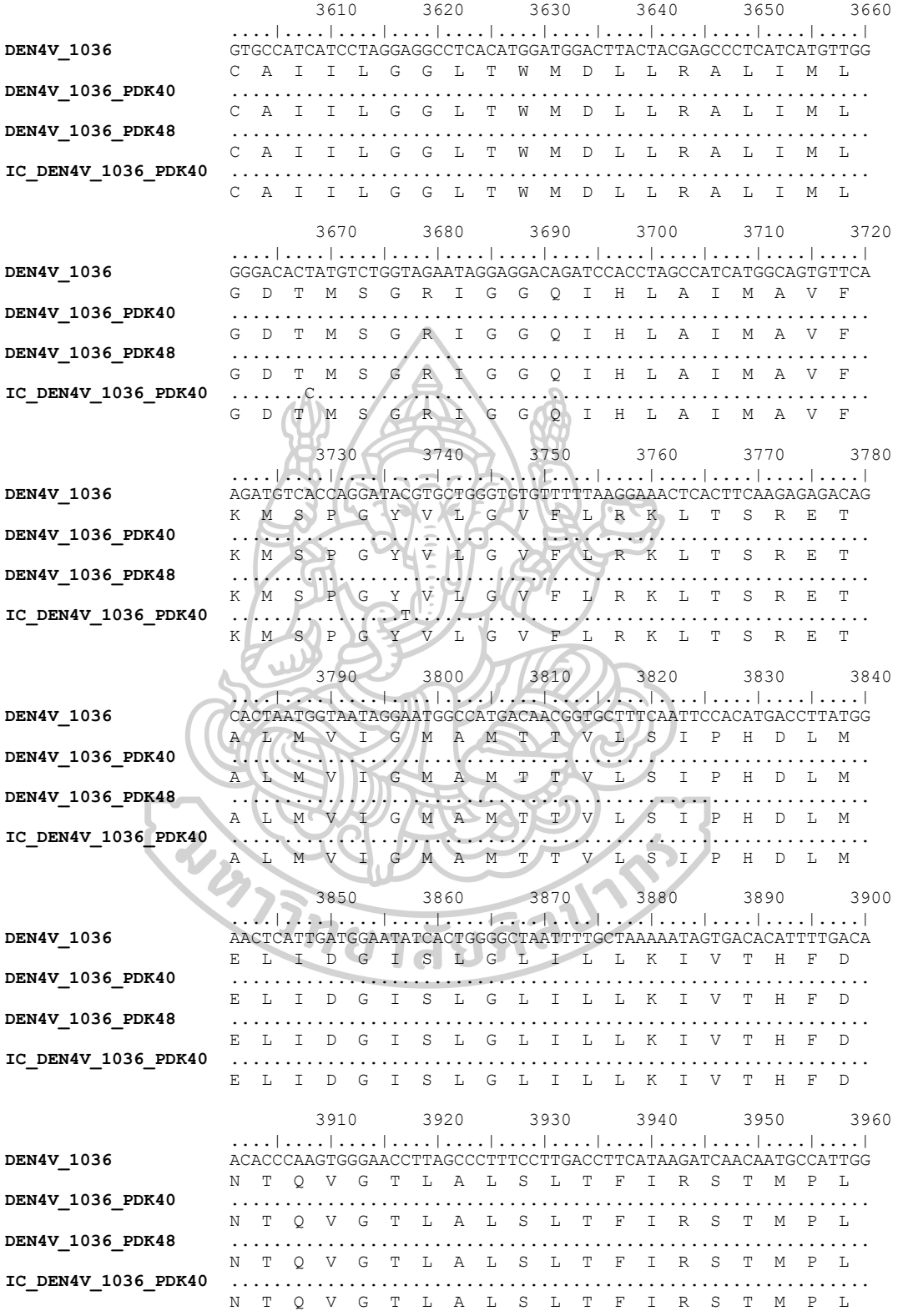
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



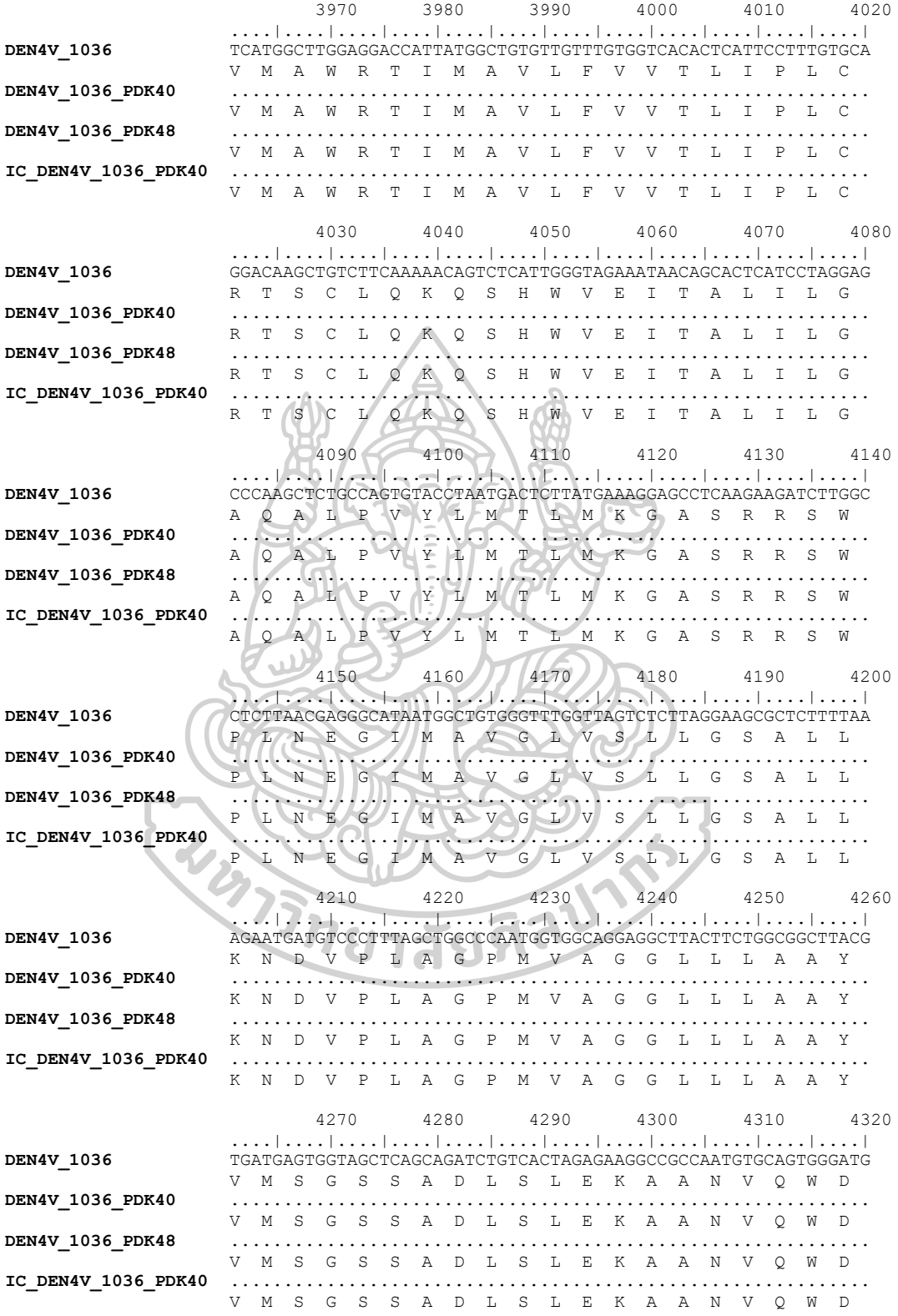
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          3250      3260      3270      3280      3290      3300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TAGAGATAGACTTTGGAGAATGCCCCGGAACAACAGTCGCAATTCAGGAGGATTGTGACC
                    L  E  I  D  F  G  E  C  P  G  T  T  V  A  I  Q  E  D  C  D
DEN4V_1036_PDK40    ............................................................
                    L  E  I  D  F  G  E  C  P  G  T  T  V  A  I  Q  E  D  C  D
DEN4V_1036_PDK48    ............................................................
                    L  E  I  D  F  G  E  C  P  G  T  T  V  A  I  Q  E  D  C  D
IC_DEN4V_1036_PDK40 ............................................................
                    L  E  I  D  F  G  E  C  P  G  T  T  V  A  I  Q  E  D  C  D

                          3310      3320      3330      3340      3350      3360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          ATAGAGGCCCATCTTTGAGGACCACCACTGCATCTGGAAAACTAGTCACGCAATGGTGCT
                    H  R  G  P  S  L  R  T  T  T  A  S  G  K  L  V  T  Q  W  C
DEN4V_1036_PDK40    ............................................................
                    H  R  G  P  S  L  R  T  T  T  A  S  G  K  L  V  T  Q  W  C
DEN4V_1036_PDK48    ............................................................
                    H  R  G  P  S  L  R  T  T  T  A  S  G  K  L  V  T  Q  W  C
IC_DEN4V_1036_PDK40 ............................................................
                    H  R  G  P  S  L  R  T  T  T  A  S  G  K  L  V  T  Q  W  C

                          3370      3380      3390      3400      3410      3420
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GCCGCTCCTGCACGATGCCTCCCTTAAGGTTCTTGGGAGAAGATGGGTGCTGGTATGGGA
                    C  R  S  C  T  M  P  P  L  R  F  L  G  E  D  G  C  W  Y  G
DEN4V_1036_PDK40    ............................................................
                    C  R  S  C  T  M  P  P  L  R  F  L  G  E  D  G  C  W  Y  G
DEN4V_1036_PDK48    ............................................................
                    C  R  S  C  T  M  P  P  L  R  F  L  G  E  D  G  C  W  Y  G
IC_DEN4V_1036_PDK40 ............................................................
                    C  R  S  C  T  M  P  P  L  R  F  L  G  E  D  G  C  W  Y  G

                          3430      3440      3450      3460      3470      3480
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGGAGATTAGGCCCTTGAGTGAAAAAGAAGAGAACATGGTCAAATCACAGGTAACGGCCG
                    M  E  I  R  P  L  S  E  K  E  E  N  M  V  K  S  Q  V  T  A
DEN4V_1036_PDK40    ............................................................
                    M  E  I  R  P  L  S  E  K  E  E  N  M  V  K  S  Q  V  T  A
DEN4V_1036_PDK48    ............................................................
                    M  E  I  R  P  L  S  E  K  E  E  N  M  V  K  S  Q  V  T  A
IC_DEN4V_1036_PDK40 ............................................................
                    M  E  I  R  P  L  S  E  K  E  E  N  M  V  K  S  Q  V  T  A

                          3490      3500      3510      3520      3530      3540
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GACAGGGCACATCAGAAACTTTTTCTATGGGTCTGTTGTGCCTGACCTTGTTTGTGGAAG
                    G  Q  G  T  S  E  T  F  S  M  G  L  L  C  L  T  L  F  V  E
DEN4V_1036_PDK40    ............................................................
                    G  Q  G  T  S  E  T  F  S  M  G  L  L  C  L  T  L  F  V  E
DEN4V_1036_PDK48    ............................................................
                    G  Q  G  T  S  E  T  F  S  M  G  L  L  C  L  T  L  F  V  E
IC_DEN4V_1036_PDK40 ............................................................
                    G  Q  G  T  S  E  T  F  S  M  G  L  L  C  L  T  L  F  V  E

                          3550      3560      3570      3580      3590      3600
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AATGCTTGAGGAGAAGAGTCACTAGGAAACACATGATATTGGTTGTGGTGATCACTCTTT
                    E  C  L  R  R  R  V  T  R  K  H  M  I  L  V  V  V  I  T  L
DEN4V_1036_PDK40    ............................................................
                    E  C  L  R  R  R  V  T  R  K  H  M  I  L  V  V  V  I  T  L
DEN4V_1036_PDK48    ............................................................
                    E  C  L  R  R  R  V  T  R  K  H  M  I  L  V  V  V  I  T  L
IC_DEN4V_1036_PDK40 ............................................................
                    E  C  L  R  R  R  V  T  R  K  H  M  I  L  V  V  V  I  T  L
```

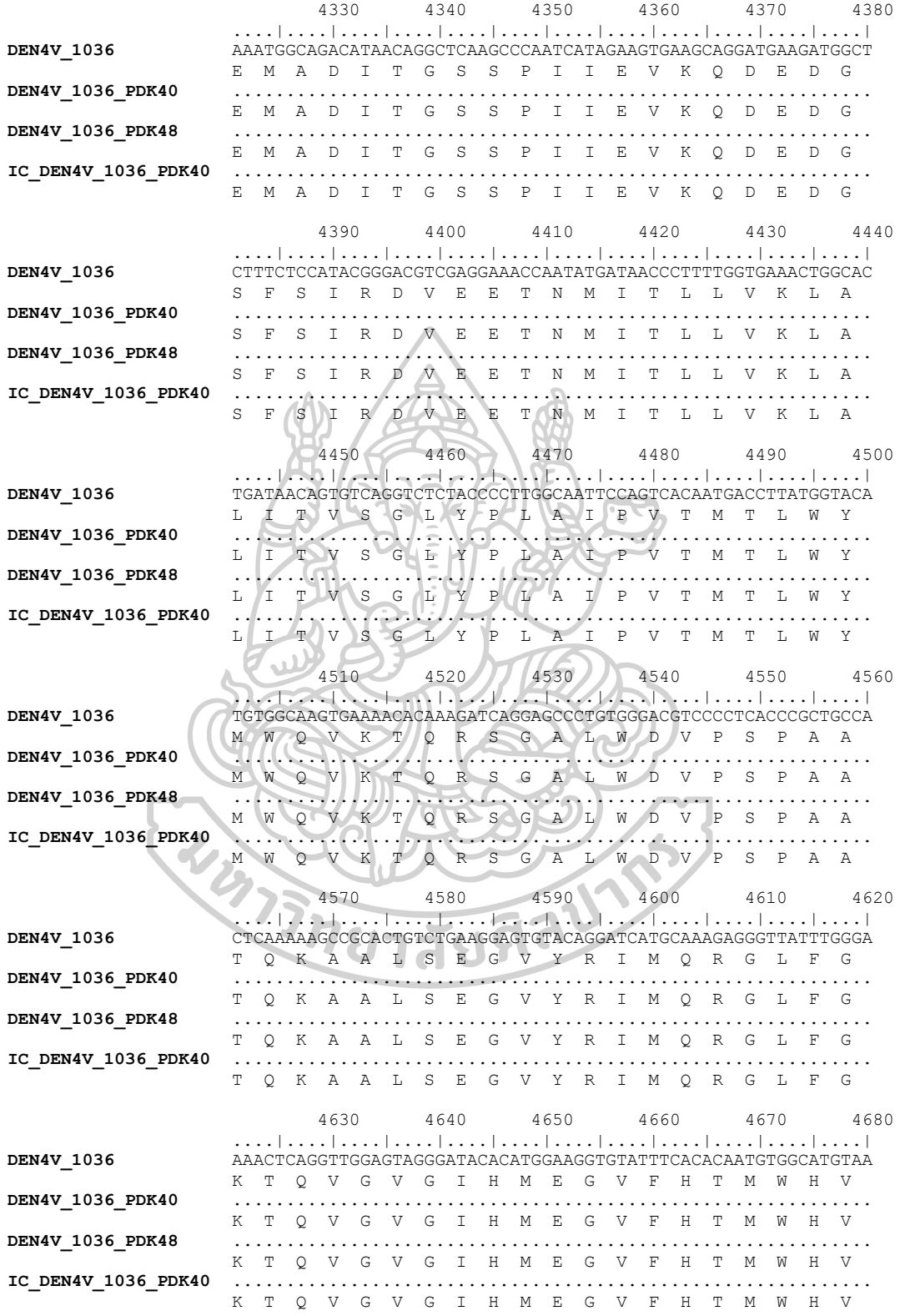
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



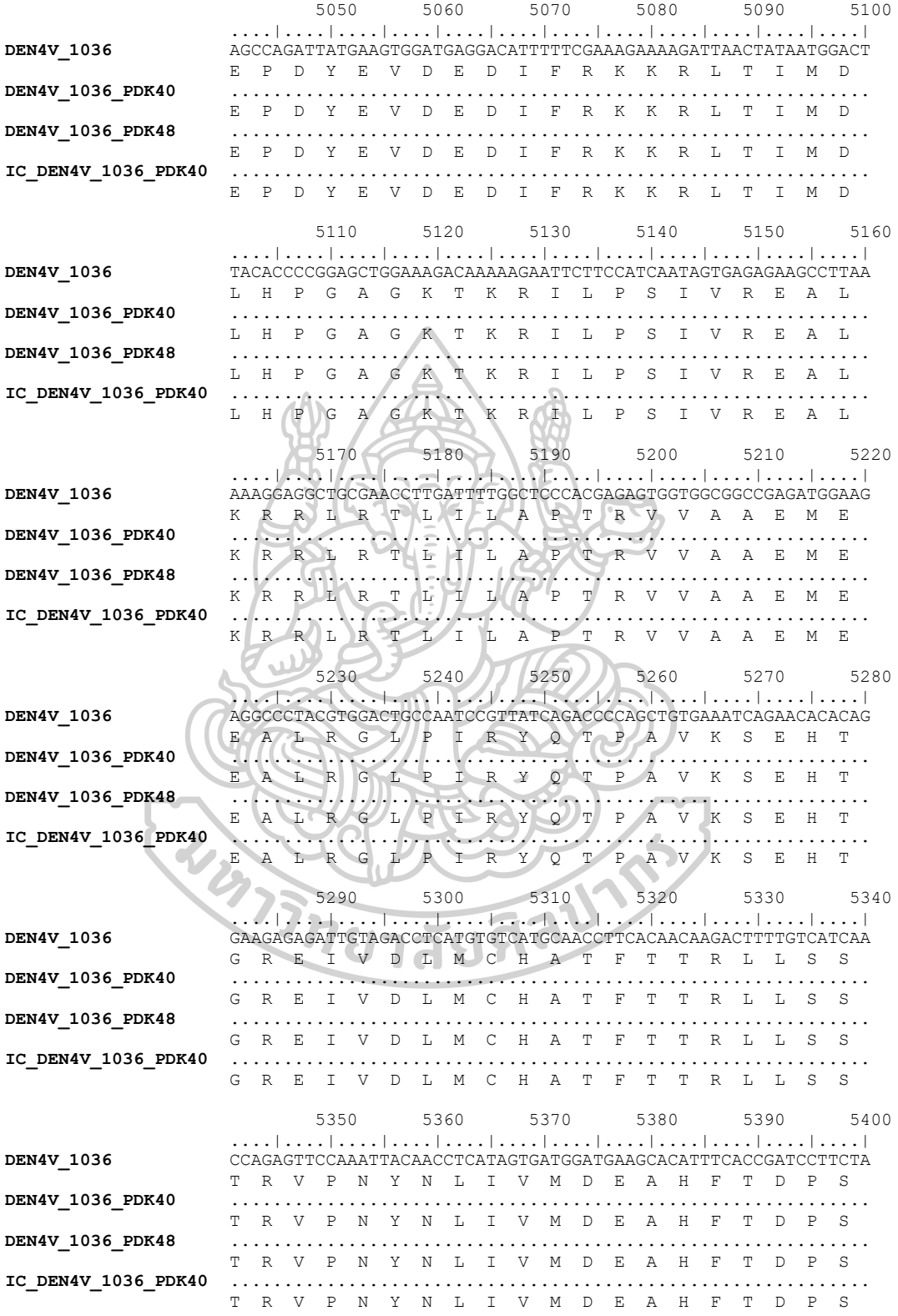
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



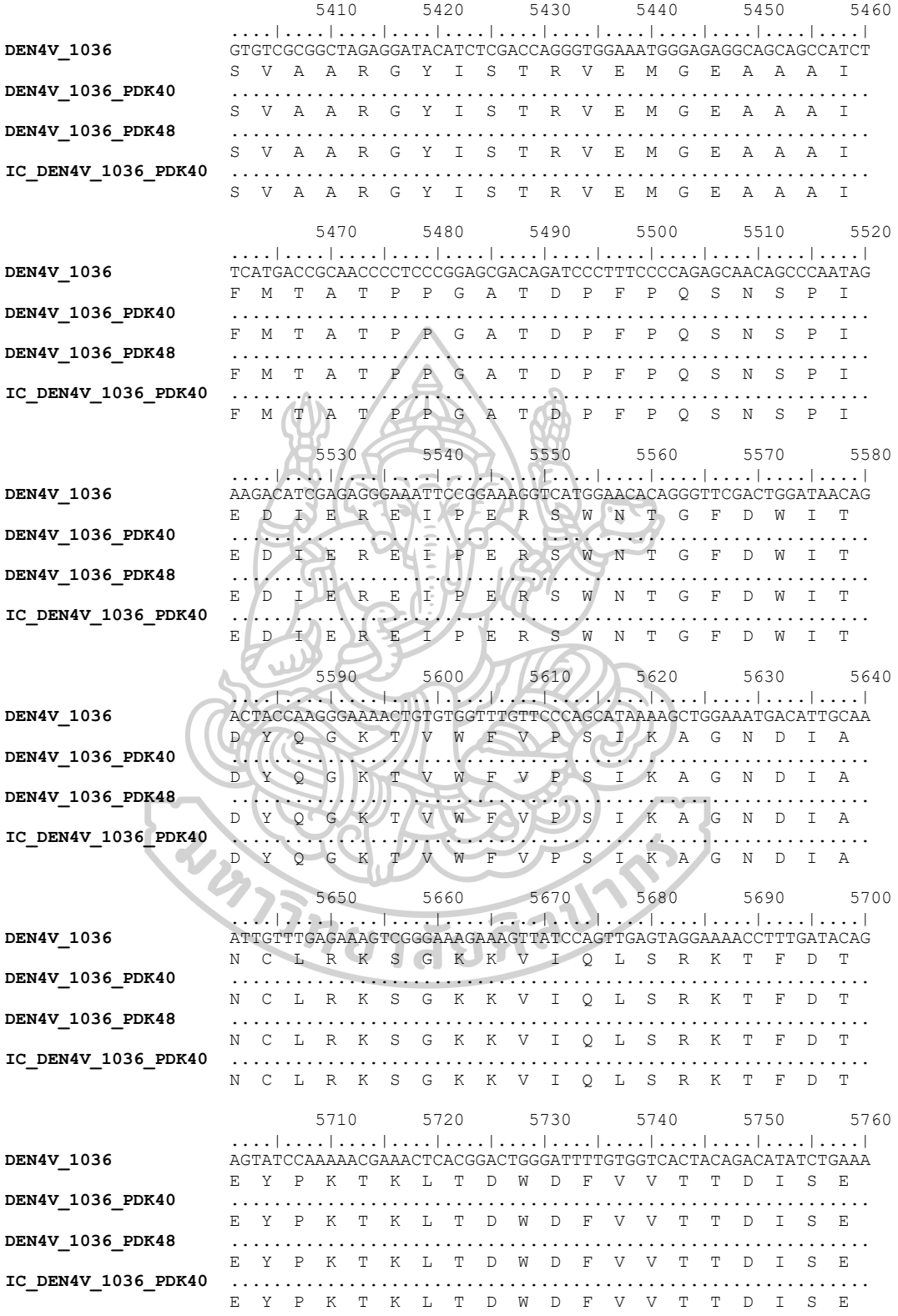
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          4690      4700      4710      4720      4730      4740
                     ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036           CAAGAGGATCAGTGATCTGCCATGAGACTGGGAGATTGGAGCCATCTTGGGCTGACGTCA
                     T  R  G  S  V  I  C  H  E  T  G  R  L  E  P  S  W  A  D  V
DEN4V_1036_PDK40     ............................................................
                     T  R  G  S  V  I  C  H  E  T  G  R  L  E  P  S  W  A  D  V
DEN4V_1036_PDK48     ............................................................
                     T  R  G  S  V  I  C  H  E  T  G  R  L  E  P  S  W  A  D  V
IC_DEN4V_1036_PDK40  ............................................................
                     T  R  G  S  V  I  C  H  E  T  G  R  L  E  P  S  W  A  D  V

                          4750      4760      4770      4780      4790      4800
                     ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036           GGAATGACATGATATCATACGGTGGGGGATGGAGACTTGGAGACAAATGGGACAAAGAAG
                     R  N  D  M  I  S  Y  G  G  G  W  R  L  G  D  K  W  D  K  E
DEN4V_1036_PDK40     ............................................................
                     R  N  D  M  I  S  Y  G  G  G  W  R  L  G  D  K  W  D  K  E
DEN4V_1036_PDK48     ............................................................
                     R  N  D  M  I  S  Y  G  G  G  W  R  L  G  D  K  W  D  K  E
IC_DEN4V_1036_PDK40  ............................................................
                     R  N  D  M  I  S  Y  G  G  G  W  R  L  G  D  K  W  D  K  E

                          4810      4820      4830      4840      4850      4860
                     ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036           AAGATGTTCAGGTCCTCGCCATAGAACCAGGAAAAAATCCTAAACATGTCCAAACGAAAC
                     E  D  V  Q  V  L  A  I  E  P  G  K  N  P  K  H  V  Q  T  K
DEN4V_1036_PDK40     ............................................................
                     E  D  V  Q  V  L  A  I  E  P  G  K  N  P  K  H  V  Q  T  K
DEN4V_1036_PDK48     ............................................................
                     E  D  V  Q  V  L  A  I  E  P  G  K  N  P  K  H  V  Q  T  K
IC_DEN4V_1036_PDK40  ............................................................
                     E  D  V  Q  V  L  A  I  E  P  G  K  N  P  K  H  V  Q  T  K

                          4870      4880      4890      4900      4910      4920
                     ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036           CCGGCCTTTTCAAGACCCTAACTGGAGAAATTGGAGCAGTAACATTAGATTTCAAACCCG
                     P  G  L  F  K  T  L  T  G  E  I  G  A  V  T  L  D  F  K  P
DEN4V_1036_PDK40     ............................................................
                     P  G  L  F  K  T  L  T  G  E  I  G  A  V  T  L  D  F  K  P
DEN4V_1036_PDK48     ............................................................
                     P  G  L  F  K  T  L  T  G  E  I  G  A  V  T  L  D  F  K  P
IC_DEN4V_1036_PDK40  ............................................................
                     P  G  L  F  K  T  L  T  G  E  I  G  A  V  T  L  D  F  K  P

                          4930      4940      4950      4960      4970      4980
                     ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036           GAACGTCTGGTTCTCCCATCATCAACAGGAAAGGAAAAGTCATCGGACTCTATGGAAATG
                     G  T  S  G  S  P  I  I  N  R  K  G  K  V  I  G  L  Y  G  N
DEN4V_1036_PDK40     ............................................................
                     G  T  S  G  S  P  I  I  N  R  K  G  K  V  I  G  L  Y  G  N
DEN4V_1036_PDK48     ............................................................
                     G  T  S  G  S  P  I  I  N  R  K  G  K  V  I  G  L  Y  G  N
IC_DEN4V_1036_PDK40  ............................................................
                     G  T  S  G  S  P  I  I  N  R  K  G  K  V  I  G  L  Y  G  N

                          4990      5000      5010      5020      5030      5040
                     ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036           GAGTAGTTACCAAATCAGGTGATTACGTCAGTGCCATAACGCAAGCCGAAAGAATTGGAG
                     G  V  V  T  K  S  G  D  Y  V  S  A  I  T  Q  A  E  R  I  G
DEN4V_1036_PDK40     ............................................................
                     G  V  V  T  K  S  G  D  Y  V  S  A  I  T  Q  A  E  R  I  G
DEN4V_1036_PDK48     ............................................................
                     G  V  V  T  K  S  G  D  Y  V  S  A  I  T  Q  A  E  R  I  G
IC_DEN4V_1036_PDK40  ............................................................
                     G  V  V  T  K  S  G  D  Y  V  S  A  I  T  Q  A  E  R  I  G
```

Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



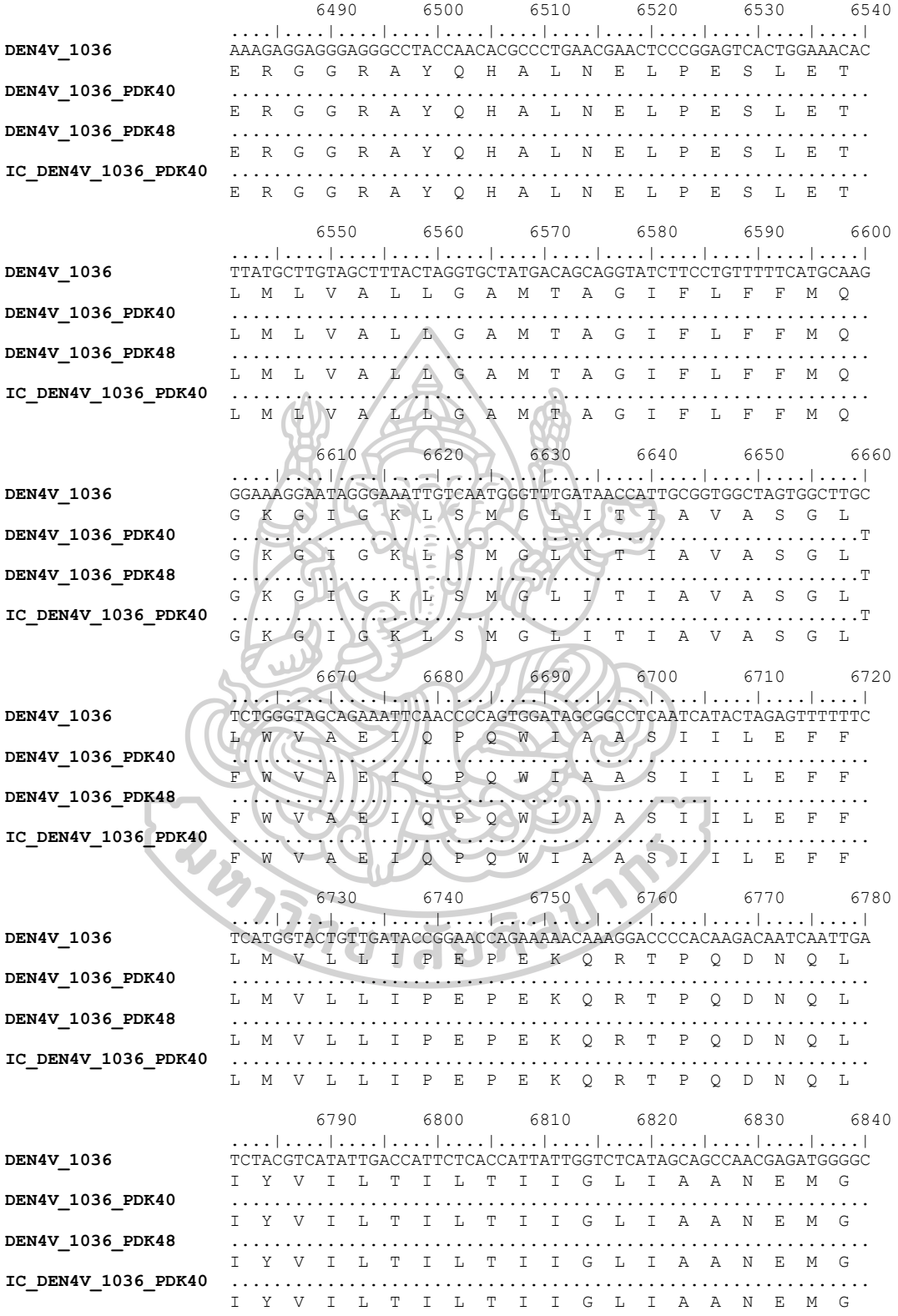
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          5770      5780      5790      5800      5810      5820
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGGGGGCCAATTTTAGAGCTGGGAGAGTGATAGACCCTAGGAGATGCCTCAAGCCAGTTA
                    M  G  A  N  F  R  A  G  R  V  I  D  P  R  R  C  L  K  P  V
DEN4V_1036_PDK40    ............................................................
                    M  G  A  N  F  R  A  G  R  V  I  D  P  R  R  C  L  K  P  V
DEN4V_1036_PDK48    ............................................................
                    M  G  A  N  F  R  A  G  R  V  I  D  P  R  R  C  L  K  P  V
IC_DEN4V_1036_PDK40 ............................................................
                    M  G  A  N  F  R  A  G  R  V  I  D  P  R  R  C  L  K  P  V

                          5830      5840      5850      5860      5870      5880
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TCCTAACAGATGGGCCAGAGAGAGTCATTTTAGCAGGTCCTATTCCAGTGACTCCAGCAA
                    I  L  T  D  G  P  E  R  V  I  L  A  G  P  I  P  V  T  P  A
DEN4V_1036_PDK40    ............................................................
                    I  L  T  D  G  P  E  R  V  I  L  A  G  P  I  P  V  T  P  A
DEN4V_1036_PDK48    ............................................................
                    I  L  T  D  G  P  E  R  V  I  L  A  G  P  I  P  V  T  P  A
IC_DEN4V_1036_PDK40 ............................................................
                    I  L  T  D  G  P  E  R  V  I  L  A  G  P  I  P  V  T  P  A

                          5890      5900      5910      5920      5930      5940
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GCGCTGCTCAGAGAAGAGGGCGAATAGGAAGGAACCCAGCACAAGAAGACGACCAATACG
                    S  A  A  Q  R  R  G  R  I  G  R  N  P  A  Q  E  D  D  Q  Y
DEN4V_1036_PDK40    ............................................................
                    S  A  A  Q  R  R  G  R  I  G  R  N  P  A  Q  E  D  D  Q  Y
DEN4V_1036_PDK48    ............................................................
                    S  A  A  Q  R  R  G  R  I  G  R  N  P  A  Q  E  D  D  Q  Y
IC_DEN4V_1036_PDK40 ............................................................
                    S  A  A  Q  R  R  G  R  I  G  R  N  P  A  Q  E  D  D  Q  Y

                          5950      5960      5970      5980      5990      6000
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTTTCTCCGGAGACCCACTAAAAAATGATGAAGATCATGCCCACTGGACAGAAGCAAAGA
                    V  F  S  G  D  P  L  K  N  D  E  D  H  A  H  W  T  E  A  K
DEN4V_1036_PDK40    ............................................................
                    V  F  S  G  D  P  L  K  N  D  E  D  H  A  H  W  T  E  A  K
DEN4V_1036_PDK48    ............................................................
                    V  F  S  G  D  P  L  K  N  D  E  D  H  A  H  W  T  E  A  K
IC_DEN4V_1036_PDK40 ..........................C.............................
                    V  F  S  G  D  P  L  K  N  D  E  D  H  A  H  W  T  E  A  K

                          6010      6020      6030      6040      6050      6060
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGCTGCTTGACAATATCTACACCCCAGAAGGGATCATTCCAACATTGTTTGGTCCGGAAA
                    M  L  L  D  N  I  Y  T  P  E  G  I  I  P  T  L  F  G  P  E
DEN4V_1036_PDK40    ............................................................
                    M  L  L  D  N  I  Y  T  P  E  G  I  I  P  T  L  F  G  P  E
DEN4V_1036_PDK48    ............................................................
                    M  L  L  D  N  I  Y  T  P  E  G  I  I  P  T  L  F  G  P  E
IC_DEN4V_1036_PDK40 ............................................................
                    M  L  L  D  N  I  Y  T  P  E  G  I  I  P  T  L  F  G  P  E

                          6070      6080      6090      6100      6110      6120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GGGAAAAAACCCAAGCCATTGATGGAGAGTTTCGCCTCAGAGGGGAACAAAGGAAGACTT
                    R  E  K  T  Q  A  I  D  G  E  F  R  L  R  G  E  Q  R  K  T
DEN4V_1036_PDK40    ............................................................
                    R  E  K  T  Q  A  I  D  G  E  F  R  L  R  G  E  Q  R  K  T
DEN4V_1036_PDK48    ............................................................
                    R  E  K  T  Q  A  I  D  G  E  F  R  L  R  G  E  Q  R  K  T
IC_DEN4V_1036_PDK40 ............................................................
                    R  E  K  T  Q  A  I  D  G  E  F  R  L  R  G  E  Q  R  K  T
```

Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          6130      6140      6150      6160      6170      6180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTGTGGAATTAATGAGGAGAGGAGACCTTCCGGTGTGGCTGAGCTATAAGGTAGCTTCTG
                    F  V  E  L  M  R  R  G  D  L  P  V  W  L  S  Y  K  V  A  S
DEN4V_1036_PDK40    ............................................................
                    F  V  E  L  M  R  R  G  D  L  P  V  W  L  S  Y  K  V  A  S
DEN4V_1036_PDK48    ............................................................
                    F  V  E  L  M  R  R  G  D  L  P  V  W  L  S  Y  K  V  A  S
IC_DEN4V_1036_PDK40 ............................................................
                    F  V  E  L  M  R  R  G  D  L  P  V  W  L  S  Y  K  V  A  S

                          6190      6200      6210      6220      6230      6240
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CTGGCATTTCTTACAAAGATCGGGAATGGTGCTTCACAGGGGAAAGGAATAACCAAATTT
                    A  G  I  S  Y  K  D  R  E  W  C  F  T  G  E  R  N  N  Q  I
DEN4V_1036_PDK40    ............................................................
                    A  G  I  S  Y  K  D  R  E  W  C  F  T  G  E  R  N  N  Q  I
DEN4V_1036_PDK48    ............................................................
                    A  G  I  S  Y  K  D  R  E  W  C  F  T  G  E  R  N  N  Q  I
IC_DEN4V_1036_PDK40 ............................................................
                    A  G  I  S  Y  K  D  R  E  W  C  F  T  G  E  R  N  N  Q  I

                          6250      6260      6270      6280      6290      6300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TAGAAGAAAACATGGAGGTTGAAATTTGGACTAGAGAGGGAGAAAAGAAAAAGCTAAGGC
                    L  E  E  N  M  E  V  E  I  W  T  R  E  G  E  K  K  K  L  R
DEN4V_1036_PDK40    ............................................................
                    L  E  E  N  M  E  V  E  I  W  T  R  E  G  E  K  K  K  L  R
DEN4V_1036_PDK48    ............................................................
                    L  E  E  N  M  E  V  E  I  W  T  R  E  G  E  K  K  K  L  R
IC_DEN4V_1036_PDK40 ............................................................
                    L  E  E  N  M  E  V  E  I  W  T  R  E  G  E  K  K  K  L  R

                          6310      6320      6330      6340      6350      6360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CAAGATGGTTAGATGCACGTGTATACGCTGACCCCATGGCTTTGAAGGATTTTAAGGAGT
                    P  R  W  L  D  A  R  V  Y  A  D  P  M  A  L  K  D  F  K  E
DEN4V_1036_PDK40    ............................................................
                    P  R  W  L  D  A  R  V  Y  A  D  P  M  A  L  K  D  F  K  E
DEN4V_1036_PDK48    ............................................................
                    P  R  W  L  D  A  R  V  Y  A  D  P  M  A  L  K  D  F  K  E
IC_DEN4V_1036_PDK40 ............................................................
                    P  R  W  L  D  A  R  V  Y  A  D  P  M  A  L  K  D  F  K  E

                          6370      6380      6390      6400      6410      6420
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTGCTAGTGGAAGGAAGAGCATAACTCTCGACATCCTAACAGAGATTGCCAGTTTGCCAA
                    F  A  S  G  R  K  S  I  T  L  D  I  L  T  E  I  A  S  L  P
DEN4V_1036_PDK40    ............................................................
                    F  A  S  G  R  K  S  I  T  L  D  I  L  T  E  I  A  S  L  P
DEN4V_1036_PDK48    ............................................................
                    F  A  S  G  R  K  S  I  T  L  D  I  L  T  E  I  A  S  L  P
IC_DEN4V_1036_PDK40 ............................................................
                    F  A  S  G  R  K  S  I  T  L  D  I  L  T  E  I  A  S  L  P

                          6430      6440      6450      6460      6470      6480
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CTTACCTTTCCTCTAGGGCCAAGCTCGCCCTTGATAACATAGTCATGCTCCACACAACAG
                    T  Y  L  S  S  R  A  K  L  A  L  D  N  I  V  M  L  H  T  T
DEN4V_1036_PDK40    ............................................................
                    T  Y  L  S  S  R  A  K  L  A  L  D  N  I  V  M  L  H  T  T
DEN4V_1036_PDK48    ............................................................
                    T  Y  L  S  S  R  A  K  L  A  L  D  N  I  V  M  L  H  T  T
IC_DEN4V_1036_PDK40 ............................................................
                    T  Y  L  S  S  R  A  K  L  A  L  D  N  I  V  M  L  H  T  T
```

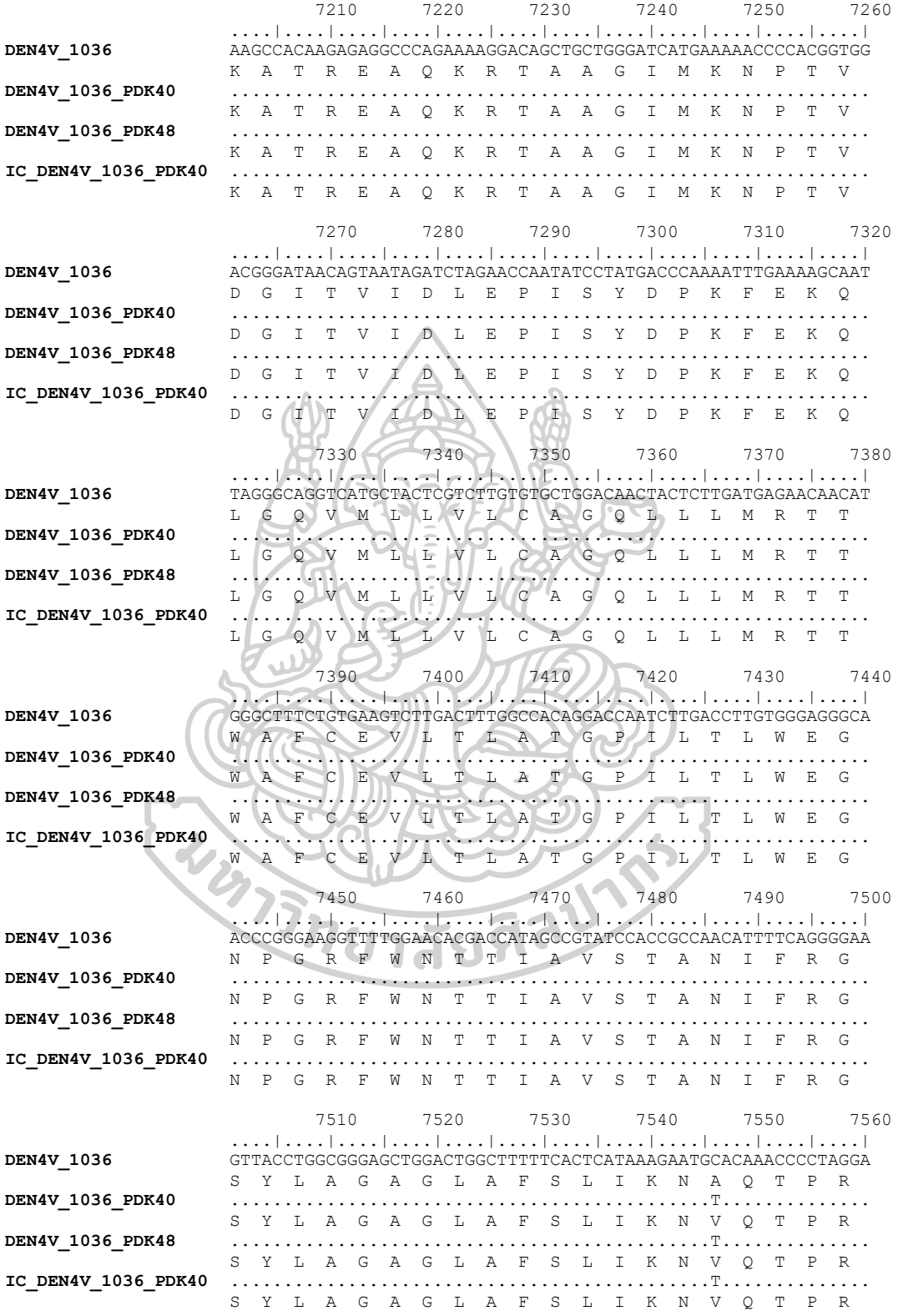
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          6850      6860      6870      6880      6890      6900
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGATTGAAAAAACAAAAACGGATTTTGGGTTTTACCAGGTAAAAACAGAAACCACCATCC
                    L  I  E  K  T  K  T  D  F  G  F  Y  Q  V  K  T  E  T  T  I
DEN4V_1036_PDK40    ............................................................
                    L  I  E  K  T  K  T  D  F  G  F  Y  Q  V  K  T  E  T  T  I
DEN4V_1036_PDK48    ............................................................
                    L  I  E  K  T  K  T  D  F  G  F  Y  Q  V  K  T  E  T  T  I
IC_DEN4V_1036_PDK40 ............................................................
                    L  I  E  K  T  K  T  D  F  G  F  Y  Q  V  K  T  E  T  T  I

                          6910      6920      6930      6940      6950      6960
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TCGATGTGGACTTGAGACCAGCTTCAGCATGGACGCTCTATGCAGTAGCCACCACAATTC
                    L  D  V  D  L  R  P  A  S  A  W  T  L  Y  A  V  A  T  T  I
DEN4V_1036_PDK40    ............................................................T...
                    L  D  V  D  L  R  P  A  S  A  W  T  L  Y  A  V  A  T  T  F
DEN4V_1036_PDK48    ............................................................T...
                    L  D  V  D  L  R  P  A  S  A  W  T  L  Y  A  V  A  T  T  F
IC_DEN4V_1036_PDK40 ............................................................T...
                    L  D  V  D  L  R  P  A  S  A  W  T  L  Y  A  V  A  T  T  F

                          6970      6980      6990      7000      7010      7020
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGACTCCCATGCTGAGACACACCATAGAAAACACGTCGGCCAACCTATCTCTAGCAGCCA
                    L  T  P  M  L  R  H  T  I  E  N  T  S  A  N  L  S  L  A  A
DEN4V_1036_PDK40    ............................................................
                    L  T  P  M  L  R  H  T  I  E  N  T  S  A  N  L  S  L  A  A
DEN4V_1036_PDK48    ............................................................
                    L  T  P  M  L  R  H  T  I  E  N  T  S  A  N  L  S  L  A  A
IC_DEN4V_1036_PDK40 ............................................................
                    L  T  P  M  L  R  H  T  I  E  N  T  S  A  N  L  S  L  A  A

                          7030      7040      7050      7060      7070      7080
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTGCCAACCAGGCGGCCGTCCTAATGGGGCTTGGAAAAGGATGGCCGCTCCACAGAATGG
                    I  A  N  Q  A  A  V  L  M  G  L  G  K  G  W  P  L  H  R  M
DEN4V_1036_PDK40    ............................................................
                    I  A  N  Q  A  A  V  L  M  G  L  G  K  G  W  P  L  H  R  M
DEN4V_1036_PDK48    ............................................................
                    I  A  N  Q  A  A  V  L  M  G  L  G  K  G  W  P  L  H  R  M
IC_DEN4V_1036_PDK40 ............................................................
                    I  A  N  Q  A  A  V  L  M  G  L  G  K  G  W  P  L  H  R  M

                          7090      7100      7110      7120      7130      7140
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          ACCTCGGTGTGCCGCTGTTAGCAATGGGATGCTATTCTCAAGTGAACCCAACAACTTTGA
                    D  L  G  V  P  L  L  A  M  G  C  Y  S  Q  V  N  P  T  T  L
DEN4V_1036_PDK40    ............................................................
                    D  L  G  V  P  L  L  A  M  G  C  Y  S  Q  V  N  P  T  T  L
DEN4V_1036_PDK48    ............................................................
                    D  L  G  V  P  L  L  A  M  G  C  Y  S  Q  V  N  P  T  T  L
IC_DEN4V_1036_PDK40 ............................................................
                    D  L  G  V  P  L  L  A  M  G  C  Y  S  Q  V  N  P  T  T  L

                          7150      7160      7170      7180      7190      7200
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CAGCATCCTTAGTCATGCTTTTAGTCCATTATGCAATAATAGGTCCAGGATTGCAGGCAA
                    T  A  S  L  V  M  L  L  V  H  Y  A  I  I  G  P  G  L  Q  A
DEN4V_1036_PDK40    ......................C.....................................
                    T  A  S  L  V  M  L  S  V  H  Y  A  I  I  G  P  G  L  Q  A
DEN4V_1036_PDK48    ......................C.....................................
                    T  A  S  L  V  M  L  S  V  H  Y  A  I  I  G  P  G  L  Q  A
IC_DEN4V_1036_PDK40 ......................C.....................................
                    T  A  S  L  V  M  L  S  V  H  Y  A  I  I  G  P  G  L  Q  A
```

Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



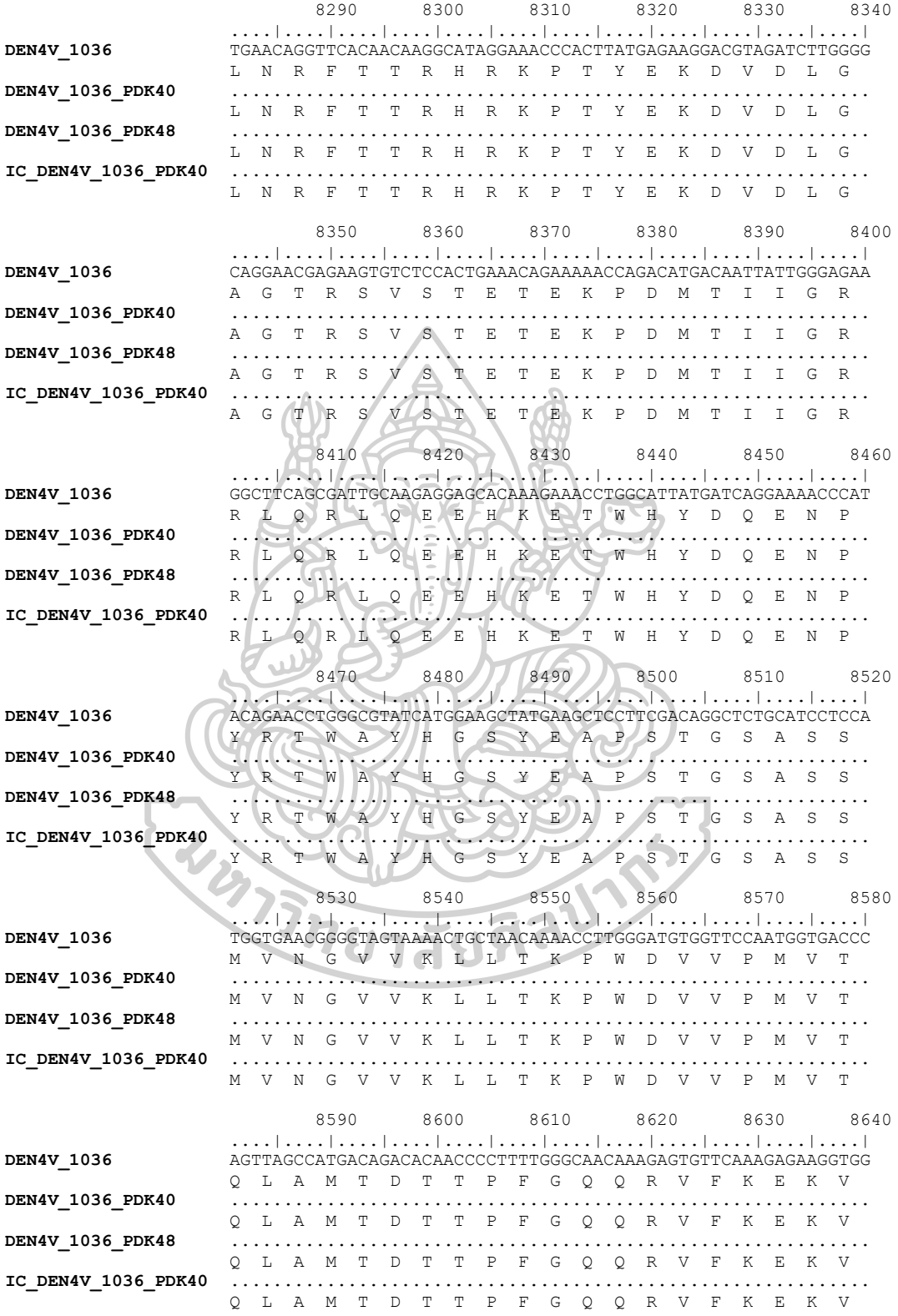
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          7570      7580      7590      7600      7610      7620
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GGGGAACTGGGACCACAGGAGAGACACTGGGAGAGAAGTGGAAGAGACAGCTAAACTCAT
                    R  G  T  G  T  T  G  E  T  L  G  E  K  W  K  R  Q  L  N  S
DEN4V_1036_PDK40    ............................................................
                    R  G  T  G  T  T  G  E  T  L  G  E  K  W  K  R  Q  L  N  S
DEN4V_1036_PDK48    ............................................................
                    R  G  T  G  T  T  G  E  T  L  G  E  K  W  K  R  Q  L  N  S
IC_DEN4V_1036_PDK40 ............................................................
                    R  G  T  G  T  T  G  E  T  L  G  E  K  W  K  R  Q  L  N  S

                          7630      7640      7650      7660      7670      7680
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TAGACAGAAAAGAGTTTGAAGAGTATAAAAGAAGTGGAATACTAGAAGTGGACAGGACTG
                    L  D  R  K  E  F  E  E  Y  K  R  S  G  I  L  E  V  D  R  T
DEN4V_1036_PDK40    ............................................................
                    L  D  R  K  E  F  E  E  Y  K  R  S  G  I  L  E  V  D  R  T
DEN4V_1036_PDK48    ..T.........................................................
                    L  Y  R  K  E  F  E  E  Y  K  R  S  G  I  L  E  V  D  R  T
IC_DEN4V_1036_PDK40 ............................................................
                    L  D  R  K  E  F  E  E  Y  K  R  S  G  I  L  E  V  D  R  T

                          7690      7700      7710      7720      7730      7740
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AAGCCAAGTCTGCCCTGAAAGATGGGTCTAAAATCAAGCATGCAGTATCTAGAGGGTCCA
                    E  A  K  S  A  L  K  D  G  S  K  I  K  H  A  V  S  R  G  S
DEN4V_1036_PDK40    ............................................................
                    E  A  K  S  A  L  K  D  G  S  K  I  K  H  A  V  S  R  G  S
DEN4V_1036_PDK48    ............................................................
                    E  A  K  S  A  L  K  D  G  S  K  I  K  H  A  V  S  R  G  S
IC_DEN4V_1036_PDK40 ............................................................
                    E  A  K  S  A  L  K  D  G  S  K  I  K  H  A  V  S  R  G  S

                          7750      7760      7770      7780      7790      7800
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GTAAGATTAGATGGATTGTTGAGAGAGGGATGGTAAAGCCAAAAGGGAAAGTTGTAGATC
                    S  K  I  R  W  I  V  E  R  G  M  V  K  P  K  G  K  V  V  D
DEN4V_1036_PDK40    ............................................................
                    S  K  I  R  W  I  V  E  R  G  M  V  K  P  K  G  K  V  V  D
DEN4V_1036_PDK48    ............................................................
                    S  K  I  R  W  I  V  E  R  G  M  V  K  P  K  G  K  V  V  D
IC_DEN4V_1036_PDK40 ............................................................
                    S  K  I  R  W  I  V  E  R  G  M  V  K  P  K  G  K  V  V  D

                          7810      7820      7830      7840      7850      7860
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TTGGCTGTGGGAGAGGAGGATGGTCTTATTACATGGCGACGCTCAAGAACGTGACTGAAG
                    L  G  C  G  R  G  G  W  S  Y  Y  M  A  T  L  K  N  V  T  E
DEN4V_1036_PDK40    ............................................................
                    L  G  C  G  R  G  G  W  S  Y  Y  M  A  T  L  K  N  V  T  E
DEN4V_1036_PDK48    ............................................................
                    L  G  C  G  R  G  G  W  S  Y  Y  M  A  T  L  K  N  V  T  E
IC_DEN4V_1036_PDK40 ............................................................
                    L  G  C  G  R  G  G  W  S  Y  Y  M  A  T  L  K  N  V  T  E

                          7870      7880      7890      7900      7910      7920
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGAAAGGGTATACAAAAGGAGGTCCAGGACATGAAGAACCGATTCCCATGGCTACTTATG
                    V  K  G  Y  T  K  G  G  P  G  H  E  E  P  I  P  M  A  T  Y
DEN4V_1036_PDK40    ............................................................
                    V  K  G  Y  T  K  G  G  P  G  H  E  E  P  I  P  M  A  T  Y
DEN4V_1036_PDK48    ............................................................
                    V  K  G  Y  T  K  G  G  P  G  H  E  E  P  I  P  M  A  T  Y
IC_DEN4V_1036_PDK40 ............................................................
                    V  K  G  Y  T  K  G  G  P  G  H  E  E  P  I  P  M  A  T  Y
```

Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          7930      7940      7950      7960      7970      7980
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GCTGGAATTTGGTCAAACTCCATTCAGGGGTTGACGTGTTCTACAAACCCACAGAGCAAG
                    G  W  N  L  V  K  L  H  S  G  V  D  V  F  Y  K  P  T  E  Q
DEN4V_1036_PDK40    ............................................................
                    G  W  N  L  V  K  L  H  S  G  V  D  V  F  Y  K  P  T  E  Q
DEN4V_1036_PDK48    ............................................................
                    G  W  N  L  V  K  L  H  S  G  V  D  V  F  Y  K  P  T  E  Q
IC_DEN4V_1036_PDK40 ..........................................T.................
                    G  W  N  L  V  K  L  H  S  G  V  D  V  F  Y  K  P  T  E  Q

                          7990      8000      8010      8020      8030      8040
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGGACACCCTGCTCTGTGATATTGGGGAGTCATCTTCTAATCCAACAATAGAGGAAGGAA
                    V  D  T  L  L  C  D  I  G  E  S  S  S  N  P  T  I  E  E  G
DEN4V_1036_PDK40    ............................................................
                    V  D  T  L  L  C  D  I  G  E  S  S  S  N  P  T  I  E  E  G
DEN4V_1036_PDK48    ............................................................
                    V  D  T  L  L  C  D  I  G  E  S  S  S  N  P  T  I  E  E  G
IC_DEN4V_1036_PDK40 ............................................................
                    V  D  T  L  L  C  D  I  G  E  S  S  S  N  P  T  I  E  E  G

                          8050      8060      8070      8080      8090      8100
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GAACATTAAGAGTTTTGAAGATGGTGGAGCCATGGCTCTCTTCAAAACCTGAATTCTGCA
                    R  T  L  R  V  L  K  M  V  E  P  W  L  S  S  K  P  E  F  C
DEN4V_1036_PDK40    ............................................................
                    R  T  L  R  V  L  K  M  V  E  P  W  L  S  S  K  P  E  F  C
DEN4V_1036_PDK48    ............................................................
                    R  T  L  R  V  L  K  M  V  E  P  W  L  S  S  K  P  E  F  C
IC_DEN4V_1036_PDK40 ............................................................
                    R  T  L  R  V  L  K  M  V  E  P  W  L  S  S  K  P  E  F  C

                          8110      8120      8130      8140      8150      8160
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TCAAAGTCCTTAACCCCTACATGCCAACAGTCATAGAAGAGCTGGAGAAACTGCAGAGAA
                    I  K  V  L  N  P  Y  M  P  T  V  I  E  E  L  E  K  L  Q  R
DEN4V_1036_PDK40    ............................................................
                    I  K  V  L  N  P  Y  M  P  T  V  I  E  E  L  E  K  L  Q  R
DEN4V_1036_PDK48    ............................................................
                    I  K  V  L  N  P  Y  M  P  T  V  I  E  E  L  E  K  L  Q  R
IC_DEN4V_1036_PDK40 ............................................................
                    I  K  V  L  N  P  Y  M  P  T  V  I  E  E  L  E  K  L  Q  R

                          8170      8180      8190      8200      8210      8220
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AACATGGTGGGAACCTTGTCAGATGCCCGCTGTCCAGGAACTCCACCCATGAGATGTATT
                    K  H  G  G  N  L  V  R  C  P  L  S  R  N  S  T  H  E  M  Y
DEN4V_1036_PDK40    ............................................................
                    K  H  G  G  N  L  V  R  C  P  L  S  R  N  S  T  H  E  M  Y
DEN4V_1036_PDK48    ............................................................
                    K  H  G  G  N  L  V  R  C  P  L  S  R  N  S  T  H  E  M  Y
IC_DEN4V_1036_PDK40 ............................................................
                    K  H  G  G  N  L  V  R  C  P  L  S  R  N  S  T  H  E  M  Y

                          8230      8240      8250      8260      8270      8280
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GGGTGTCAGGAGCGTCGGGAAACATTGTGAGCTCTGTGAACACAACATCAAAGATGTTGT
                    W  V  S  G  A  S  G  N  I  V  S  S  V  N  T  T  S  K  M  L
DEN4V_1036_PDK40    ............................................................
                    W  V  S  G  A  S  G  N  I  V  S  S  V  N  T  T  S  K  M  L
DEN4V_1036_PDK48    ............................................................
                    W  V  S  G  A  S  G  N  I  V  S  S  V  N  T  T  S  K  M  L
IC_DEN4V_1036_PDK40 ............................................................
                    W  V  S  G  A  S  G  N  I  V  S  S  V  N  T  T  S  K  M  L
```

Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



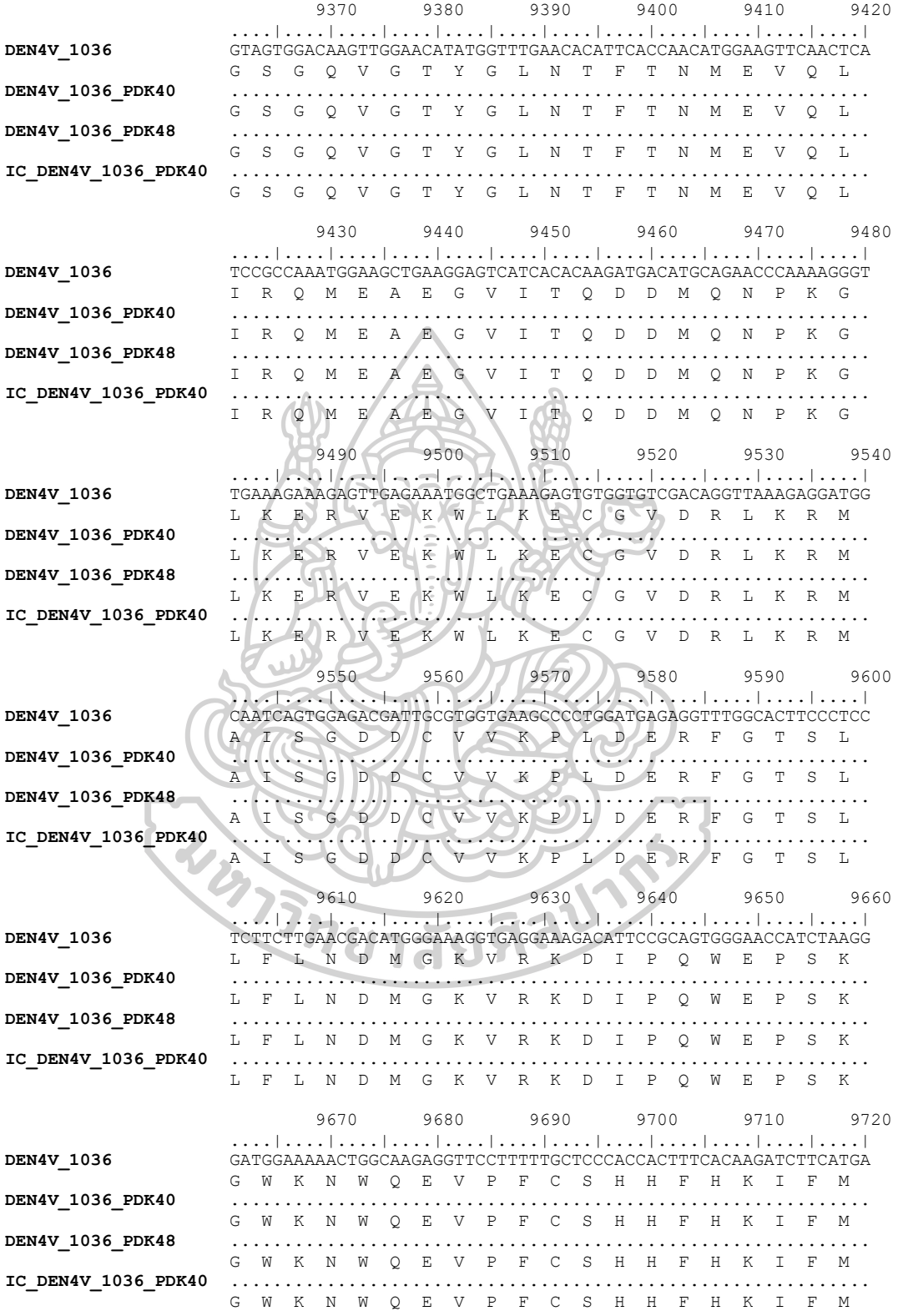
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          8650      8660      8670      8680      8690      8700
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          ATACCAGAACACCACAACCAAAACCCGGTACACGAATGGTTATGACCACGACAGCCAATT
                    D  T  R  T  P  Q  P  K  P  G  T  R  M  V  M  T  T  T  A  N
DEN4V_1036_PDK40    ............................................................
                    D  T  R  T  P  Q  P  K  P  G  T  R  M  V  M  T  T  T  A  N
DEN4V_1036_PDK48    ............................................................
                    D  T  R  T  P  Q  P  K  P  G  T  R  M  V  M  T  T  T  A  N
IC_DEN4V_1036_PDK40 ............................................................
                    D  T  R  T  P  Q  P  K  P  G  T  R  M  V  M  T  T  T  A  N

                          8710      8720      8730      8740      8750      8760
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GGCTGTGGGCCCTCCTTGGGAAGAAGAAAAATCCCAGACTGTGCACAAGGGAAGAGTTCA
                    W  L  W  A  L  L  G  K  K  K  N  P  R  L  C  T  R  E  E  F
DEN4V_1036_PDK40    ............................................................
                    W  L  W  A  L  L  G  K  K  K  N  P  R  L  C  T  R  E  E  F
DEN4V_1036_PDK48    ............................................................
                    W  L  W  A  L  L  G  K  K  K  N  P  R  L  C  T  R  E  E  F
IC_DEN4V_1036_PDK40 ............................................................
                    W  L  W  A  L  L  G  K  K  K  N  P  R  L  C  T  R  E  E  F

                          8770      8780      8790      8800      8810      8820
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TCTCAAAAGTTAGATCAAACGCAGCCATAGGCGCAGTCTTTCAGGAAGAACAGGGATGGA
                    I  S  K  V  R  S  N  A  A  I  G  A  V  F  Q  E  E  Q  G  W
DEN4V_1036_PDK40    ............................................................
                    I  S  K  V  R  S  N  A  A  I  G  A  V  F  Q  E  E  Q  G  W
DEN4V_1036_PDK48    ............................................................
                    I  S  K  V  R  S  N  A  A  I  G  A  V  F  Q  E  E  Q  G  W
IC_DEN4V_1036_PDK40 ............................................................
                    I  S  K  V  R  S  N  A  A  I  G  A  V  F  Q  E  E  Q  G  W

                          8830      8840      8850      8860      8870      8880
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CATCAGCCAGTGAAGCTGTGAATGACAGCCGGTTTTGGGAACTGGTTGACAAAGAAAGGG
                    T  S  A  S  E  A  V  N  D  S  R  F  W  E  L  V  D  K  E  R
DEN4V_1036_PDK40    ............................................................
                    T  S  A  S  E  A  V  N  D  S  R  F  W  E  L  V  D  K  E  R
DEN4V_1036_PDK48    ............................................................
                    T  S  A  S  E  A  V  N  D  S  R  F  W  E  L  V  D  K  E  R
IC_DEN4V_1036_PDK40 ............................................................
                    T  S  A  S  E  A  V  N  D  S  R  F  W  E  L  V  D  K  E  R

                          8890      8900      8910      8920      8930      8940
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CCCTACACCAGGAAGGGAAATGTGAATCGTGTGTCTACAACATGATGGGAAAACGTGAGA
                    A  L  H  Q  E  G  K  C  E  S  C  V  Y  N  M  M  G  K  R  E
DEN4V_1036_PDK40    ............................................................
                    A  L  H  Q  E  G  K  C  E  S  C  V  Y  N  M  M  G  K  R  E
DEN4V_1036_PDK48    ............................................................
                    A  L  H  Q  E  G  K  C  E  S  C  V  Y  N  M  M  G  K  R  E
IC_DEN4V_1036_PDK40 ............................................................
                    A  L  H  Q  E  G  K  C  E  S  C  V  Y  N  M  M  G  K  R  E

                          8950      8960      8970      8980      8990      9000
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AAAAGTTAGGAGAGTTTGGCAGAGCCAAGGGAAGCCGAGCAATCTGGTACATGTGGCTGG
                    K  K  L  G  E  F  G  R  A  K  G  S  R  A  I  W  Y  M  W  L
DEN4V_1036_PDK40    ............................................................
                    K  K  L  G  E  F  G  R  A  K  G  S  R  A  I  W  Y  M  W  L
DEN4V_1036_PDK48    ............................................................
                    K  K  L  G  E  F  G  R  A  K  G  S  R  A  I  W  Y  M  W  L
IC_DEN4V_1036_PDK40 ............................................................
                    K  K  L  G  E  F  G  R  A  K  G  S  R  A  I  W  Y  M  W  L
```

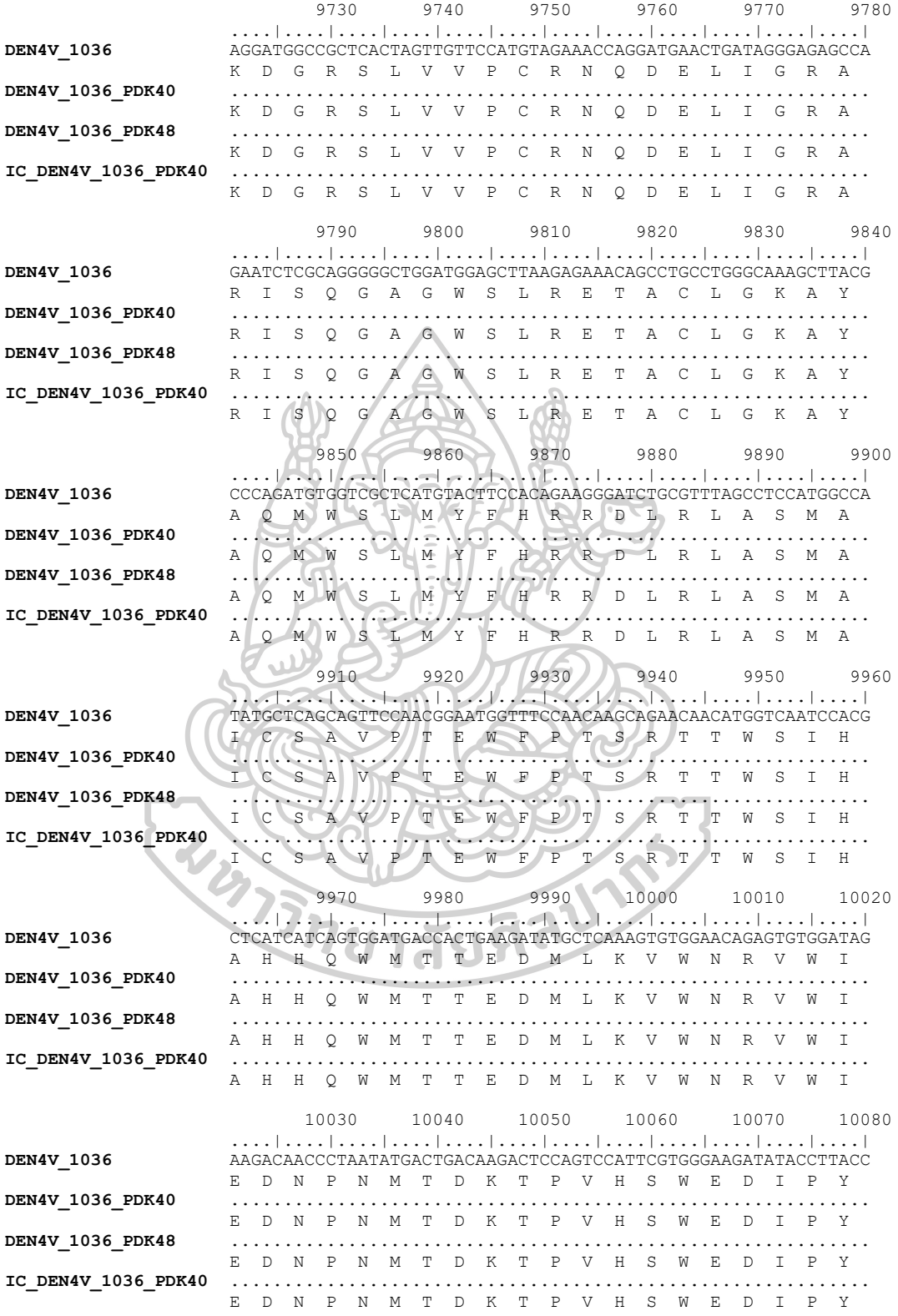
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          9010      9020      9030      9040      9050      9060
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GAGCGCGGTTTCTGGAATTTGAAGCCCTGGGTTTTTTTGAATGAAGATCACTGGTTTGGCA
                    G  A  R  F  L  E  F  E  A  L  G  F  L  N  E  D  H  W  F  G
DEN4V_1036_PDK40    ............................................................
                    G  A  R  F  L  E  F  E  A  L  G  F  L  N  E  D  H  W  F  G
DEN4V_1036_PDK48    ............................................................
                    G  A  R  F  L  E  F  E  A  L  G  F  L  N  E  D  H  W  F  G
IC_DEN4V_1036_PDK40 ............................................................
                    G  A  R  F  L  E  F  E  A  L  G  F  L  N  E  D  H  W  F  G

                          9070      9080      9090      9100      9110      9120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          GAGAAAATTCATGGAGTGGAGTGGAAGGGGAAGGTCTGCACAGATTGGGATATATCCTGG
                    R  E  N  S  W  S  G  V  E  G  E  G  L  H  R  L  G  Y  I  L
DEN4V_1036_PDK40    ............................................................
                    R  E  N  S  W  S  G  V  E  G  E  G  L  H  R  L  G  Y  I  L
DEN4V_1036_PDK48    ............................................................
                    R  E  N  S  W  S  G  V  E  G  E  G  L  H  R  L  G  Y  I  L
IC_DEN4V_1036_PDK40 ............................................................
                    R  E  N  S  W  S  G  V  E  G  E  G  L  H  R  L  G  Y  I  L

                          9130      9140      9150      9160      9170      9180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AGGAGATAGACAAGAAGGATGGAGACCTAATGTATGCTGATGACACAGCAGGCTGGGACA
                    E  E  I  D  K  K  D  G  D  L  M  Y  A  D  D  T  A  G  W  D
DEN4V_1036_PDK40    ............................................................
                    E  E  I  D  K  K  D  G  D  L  M  Y  A  D  D  T  A  G  W  D
DEN4V_1036_PDK48    ............................................................
                    E  E  I  D  K  K  D  G  D  L  M  Y  A  D  D  T  A  G  W  D
IC_DEN4V_1036_PDK40 ............................................................
                    E  E  I  D  K  K  D  G  D  L  M  Y  A  D  D  T  A  G  W  D

                          9190      9200      9210      9220      9230      9240
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CAAGAATCACTGAGGATGACCTTCAAAATGAAGAACTGATCACGGAACAGATGGCCCCCC
                    T  R  I  T  E  D  D  L  Q  N  E  E  L  I  T  E  Q  M  A  P
DEN4V_1036_PDK40    ............................................................
                    T  R  I  T  E  D  D  L  Q  N  E  E  L  I  T  E  Q  M  A  P
DEN4V_1036_PDK48    ............................................................
                    T  R  I  T  E  D  D  L  Q  N  E  E  L  I  T  E  Q  M  A  P
IC_DEN4V_1036_PDK40 ............................................................
                    T  R  I  T  E  D  D  L  Q  N  E  E  L  I  T  E  Q  M  A  P

                          9250      9260      9270      9280      9290      9300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          ACCACAAGATCCTAGCCAAAGCCATTTTCAAACTAACCTATCAAAACAAAGTGGTGAAAG
                    H  H  K  I  L  A  K  A  I  F  K  L  T  Y  Q  N  K  V  V  K
DEN4V_1036_PDK40    ............................................................
                    H  H  K  I  L  A  K  A  I  F  K  L  T  Y  Q  N  K  V  V  K
DEN4V_1036_PDK48    ............................................................
                    H  H  K  I  L  A  K  A  I  F  K  L  T  Y  Q  N  K  V  V  K
IC_DEN4V_1036_PDK40 ............................................................
                    H  H  K  I  L  A  K  A  I  F  K  L  T  Y  Q  N  K  V  V  K

                          9310      9320      9330      9340      9350      9360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TCCTCAGACCCACACCGAGAGGAGCGGTGATGGATATCATATCCAGGAAAGACCAAAGAG
                    V  L  R  P  T  P  R  G  A  V  M  D  I  I  S  R  K  D  Q  R
DEN4V_1036_PDK40    ............................................................
                    V  L  R  P  T  P  R  G  A  V  M  D  I  I  S  R  K  D  Q  R
DEN4V_1036_PDK48    ............................................................
                    V  L  R  P  T  P  R  G  A  V  M  D  I  I  S  R  K  D  Q  R
IC_DEN4V_1036_PDK40 ............................................................
                    V  L  R  P  T  P  R  G  A  V  M  D  I  I  S  R  K  D  Q  R
```

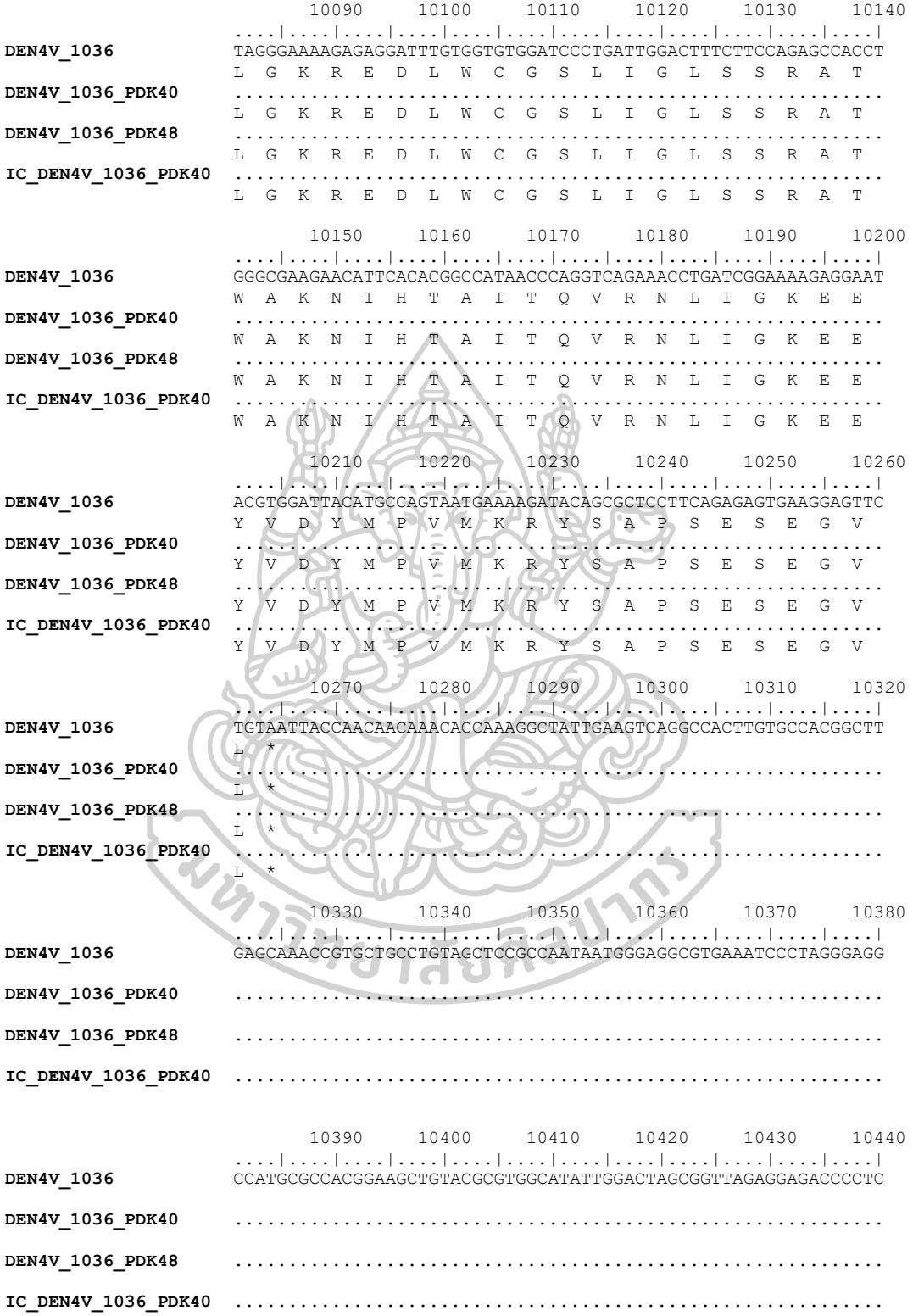
Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).



Appendix C: Genome alignment of DEN4V 1036 and its derivatives (continued).

```
                          10450     10460     10470     10480     10490     10500
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          CCATCACTGACAAAACGCAGCAAAAGGGGGCCCGAAGCCAGGAGGAAGCTGTACTCCTGG

DEN4V_1036_PDK40    ............................................................

DEN4V_1036_PDK48    ............................................................

IC_DEN4V_1036_PDK40 ............................................................


                          10510     10520     10530     10540     10550     10560
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          TGGAAGGACTAGAGGTTAGAGGAGACCCCCCCAACACAAAAACAGCATATTGACGCTGGG

DEN4V_1036_PDK40    ............................................................

DEN4V_1036_PDK48    ............................................................

IC_DEN4V_1036_PDK40 ............................................................


                          10570     10580     10590     10600     10610     10620
                    ....|....|....|....|....|....|....|....|....|....|....|....|
DEN4V_1036          AAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGAAGCAAGATG

DEN4V_1036_PDK40    ..........................................................CC........

DEN4V_1036_PDK48    ............................................................

IC_DEN4V_1036_PDK40 ..........................................................CC........


                          10630     10640
                    ....|....|....|....|....|...
DEN4V_1036          GATTGGTGTTGTTGATCCAACAGGTTCT

DEN4V_1036_PDK40    ............................

DEN4V_1036_PDK48    ............................

IC_DEN4V_1036_PDK40 ............................
```

VITA

NAME Kumchol Chaiyo

DATE OF BIRTH 19 February 1987

PLACE OF BIRTH Ratchaburi

INSTITUTIONS ATTENDED
Prince of Songkla University, 2004 – 2008
Bachelor Degree of Science (Microbiology)
Mahidol University, 2008 – 2011
Master Degree of Science (Microbiology)
Silpakorn University, 2013 – 2016
Doctor of Philosophy (Biology)

HOME ADDRESS 23/75 Soi 1, Kaosand Rd., Kaorubchang, Meung, Songkhla 90000

PUBLICATION
1. Nitatpattana, N., Chaiyo, K., Rajakam, S., Poolam, K., Chansiprasert, K., Pesirikan, N., Buree, S., Rodpai, E., Yoksan, S. (2018). Complete Genome Sequence of a Zika Virus Strain Isolated from the Serum of an Infected Patient in Thailand in 2006. Genome Announc 6(10)
2. Nitatpattana, N., Moné, Y., Gouilh, M., Chaiyo, K., Joyjinda, Y., Ratchakum, S., Wacharapluesadee, S., Yoksan, S., Hemachudha, T., Veas, F., Vincent, T., Gonzalez, J. P. (2018). Genetic Diversity of Dengue-4 Virus Strains Isolated from Patients During a Single Outbreak of Dengue Fever, Thailand (2011). J Fever 2(1): 1009
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