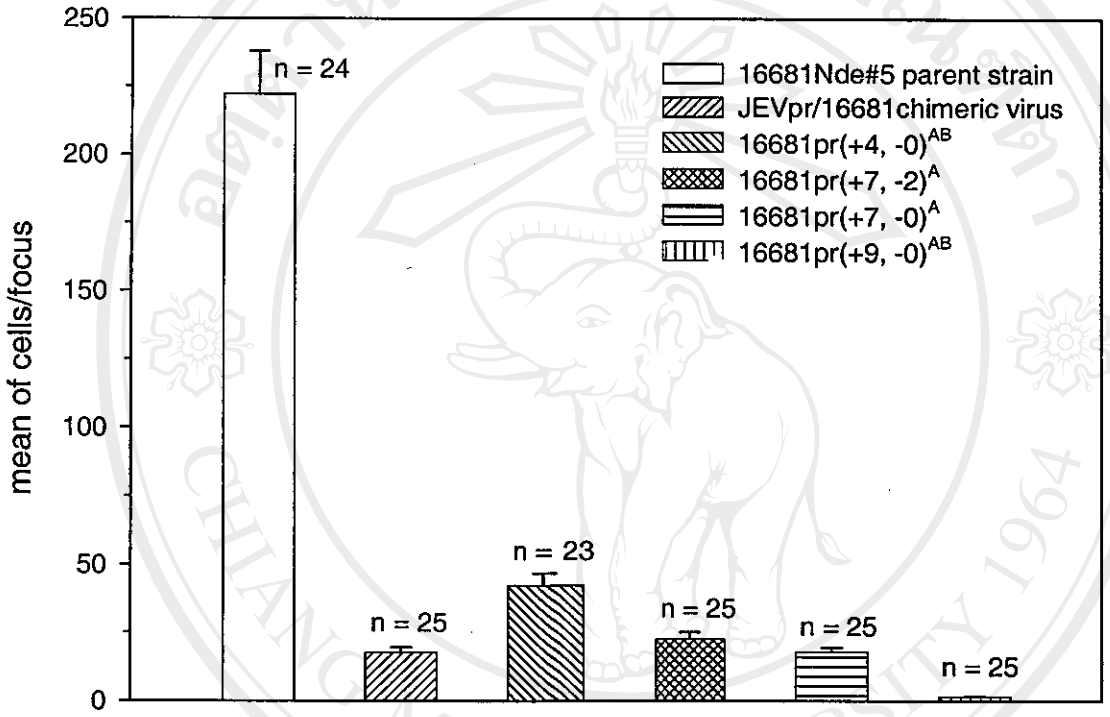
APPENDICES

Appendix A:

Comparison of focus size of four dengue prM mutant viruses by using specific monoclonal antibody to dengue serotype 2 envelope protein and peroxidase anti-peroxidase (PAP) focus immunoassay.



[A] Signification of focus size reduction of dengue mutants compares with 16681Nde#5 derived dengue serotype 2 parent strain ($P < 0.001$). [B] The alteration of focus size of four mutants present to significant, $P < 0.001$, when compared with JEVpr/16681 chimeric virus control (Keelapang et al., 2004).

Appendix B: Properties of amino acids

1. Abbreviation, pKa and molecular weight for the amino acids.

Name of amino acid	Three-letter code	One-letter code	pKa of ionizing side chain	Residue mass (Daltons)
Alanine	Ala	A	–	71.08
Arginine	Arg	R	12.5	156.20
Asparagine	Asn	N	–	114.11
Aspartic acid	Asp	D	3.9	115.09
Cysteine	Cys	C	8.3	103.14
Glutamine	Gln	Q	–	128.14
Glutamic acid	Glu	E	4.2	129.12
Glycine	Gly	G	–	57.06
Histidine	His	H	6.0	137.15
Isoleucine	Ile	I	–	113.17
Leucine	Leu	L	–	113.17
Lysine	Lys	K	10.0	128.18
Methionine	Met	M	–	131.21
Phenylalanine	Phe	F	–	147.18
Proline	Pro	P	–	97.12
Serine	Ser	S	–	87.08
Threonine	Thr	T	–	101.11
Tryptophan	Trp	W	–	186.21
Tyrosine	Tyr	Y	10.1	163.18
Valine	Val	V	–	99.14

2. The genetic code.

Second position

First position	U		C		A		G		Third position
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	C
	UUA	Leu	UCA	Ser	UAA	stop	UGA	stop	A
	UUG	Leu	UCG	Ser	UAG	stop	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G
A	AUU	Ile	ACU	Thr	AUU	Asn	AGU	Ser	U
	AUC	Ile	ACC	Thr	AUC	Asn	AGC	Ser	C
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
	AUG	Met/start	ACG	Thr	AAG	Lys	AGG	Arg	G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G

The codons read in the 5'→3' direction.

Appendix C: Reagents

1. Medium for bacterial growth

1.1. Luria Broth (LB) Medium (per liter)

Tryptone	10.0	gm
Yeast extracts	5.0	gm
NaCl	10.0	gm

Tryptone, yeast extract and NaCl were dissolved in 1,000 ml of deionized H_2O. The medium was adjusted to pH 7.5 with 1 N NaOH, sterilized by autoclaving and stored at 4°C.

1.2. Luria Broth (LB) Agar (per liter)

NaCl	10.0	gm
Tryptone	10.0	gm
Yeast extracts	5.0	gm
Bacto-Agar	20.0	gm

The deionized H_2O was added to a final volume of 1.0 liter and adjusted with 5N NaOH to pH 7.0. The agar was sterilized by autoclaving. Cooled down to 55°C and poured into 100 mm Petri dishes (~25 ml/plate).

1.3. LB-Ampicillin Agar (per liter)

A liter of Luria broth agar was prepared, autoclaved, cooled to 55°C and added 250 μl of 100.0 mg/ml sterilized ampicillin. Then poured into 100 mm Petri dishes (~25 ml/plate).

1.4. SOB Medium (per liter)

Tryptone	20.0	gm
Yeast extracts	5.0	gm
NaCl	0.5	gm

Distilled water was added to 1.0 liter in final volume and sterilized by autoclaving. Ten milliliters of 1.0 M $MgCl_2$ were added. Then 10.0 ml of 1 M $MgSO_4$ were added prior to use, and the solution was sterilized by filter.

1.5. SOC Medium (per 100 ml)

Tryptone or peptone	2.0	gm
Yeast extract	0.5	gm
NaCl	0.05	gm
KCl	0.0186	gm
20% (w/v) glucose	2.0	ml

The components were dissolved by sterile deionized water 100.0 ml. Adjust pH to 7.0 and sterile by autoclaving and then allowed cooling at room temperature. Add 1 M $MgCl_2$ 1 ml (final concentration 0.01 M) and 1 M of glucose 2 ml (final concentration 0.02 M) before use. The solution was stored at 4°C.

2. Solution for plasmid DNA mini-preparation: boiling method

2.1. STET Buffer (per 100 ml)

Sucrose [final conc. 8.0% (w/v)]	8.0	gm
Triton X-100 [final conc. 0.5% (v/v)]	0.5	ml
1 M Tris-HCl (pH 8.0) [final conc. 50 mM]	5.0	ml
0.5 M EDTA [final conc. 50.0 mM]	10.0	ml

The components were mixed gently and sterile by autoclaving. The solution mixture was stored indefinitely at 4°C.

2.2. Lysozyme (10 mg/ml) Stock

Solid lysozyme was dissolved at a concentration of 10.0 mg/ml in 10 mM Tris-HCl (pH 8.0). Stored at -20°C.

2.3. Tris EDTA (TE) Buffer

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

The 1.21 gm of Tris-base and 0.37 gm of $EDTA{\cdot}2H_2O$ in 800 ml of distilled water were disolved. The pH was adjusted to 8.0 by 0.1 M HCl, and volume was adjusted to 1.0 liter by distilled water.

2.4. 10 mM Tris-HCl (pH 8.0)

The 1.21 gm of Tris-base were dissolved in 800 ml of 0.1 % diethylpyrocabonate (DEPC)-treated water and adjusted pH to 8.0 by 0.1 M HCl. The volume was adjusted to 1.0 liter by DEPC-treated water.

2.5. 0.1 M Calcium Chloride (per 100 ml)

$CaCl_2$-$2H_2O$	7.38	gm
Sterile distilled water	100.0	ml

Mix the solution using magnetic stirrer until it completely dissolved. Sterilized by autoclaving and stored at 4°C.

3. Solution for plasmid DNA midi-preparation

3.1. Resuspension Buffer (P1 Buffer)

Tris-base	6.06	gm
EDTA·$2H_2O$	3.72	gm

Tris-base and EDTA·$2H_2O$ were dissolved in 800 ml of distilled H_2O and adjusted the pH was adjusted to 8.0 with HCl. The volume was adjusted to 1.0 liter by distilled water and sterilized by autoclaving. The RNase A was added to 100.0 μg/ml before use.

3.2. Lysis Buffer (P2 Buffer)

0.2 N NaOH

1% (w/v) SDS

NaOH pellets (8.0 gm) were dissolved in distilled water and 50.0 ml of 20% SDS solution was added. The final volume was adjusted to 1.0 litter.

3.3. Neutralization Buffer (P3 Buffer)

The 294.5 gm of potassium acetate were dissolved in 500 ml of distilled water. The pH was adjusted to 5.5 by glacial acetic acid (~110 ml). The final volume was adjusted to 1.0 liter by distilled water. Sterilized by autoclaving.

3.4. Equilibration Buffer (QBT Buffer)

NaCl	43.83	gm
Acid free MOPS	10.46	gm
Isopropanol	150.00	ml
10% Triton X-100 solution	15.00	ml

NaCl and MOPS were dissolved in 800 ml of distilled water and adjusted pH to 7.0. Isopropanol and 10% Triton X-100 solution were added and adjusted final volume to 1.0 liter with distilled water. Sterilized by filtration through a 0.2 μm membrane.

3.5. Wash Buffer (QC Buffer)

NaCl	58.44	gm
Acid free MOPS	10.46	gm
Isopropanol	150.00	ml

NaCl and MOPS were dissolved in 800 ml of distilled H_2O and adjusted pH to 7.0. Isopropanol was added and final volume adjusted to 1.0 liter with distilled water.

3.6. Elution Buffer (QF Buffer)

NaCl	73.05	gm
Tris-base	6.06	gm
Isopropanol	150.00	ml

NaCl and Tris-base were dissolved in 800 ml of distilled water. The pH was adjusted to 8.5. Isopropanol was added and final volume adjusted to 1.0 liter with distilled water.

4. Solution for DNA agarose gel electrophoresis

4.1. 50X TAE Buffer (Stock solution/liter)

Tris-HCl	242.0	gm
Acetic acid	57.1	ml
0.5 M EDTA (pH 8.0)	100.0	ml

Tris-base was dissolved in 800 ml of distilled water. Added acetic acid and 0.5 M EDTA. The pH was adjusted to 8.0 and distilled water was added to 1.0 liter. Solution was sterilized by autoclaving.

4.2. 0.7% Agarose Gel

1X TAE Buffer	100.0	ml
Agarose gel powder (GIBCO BRL)	0.7	gm

Mixture was heated in microwave oven until agarose dissolved completely and then poured into a gel block or tray. The combs were set up to gel-block.

4.3. 6X Gel-loading Buffers

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

30% (v/v) glycerol in H_2O

The 2.5 gm of bromophenol and 2.5 gm of xylene cyanol blue were dissolved in sterile distilled water 350 μl by mixing inversion and then 450 μl of glycerol were added into solution. Adjust volume to 1 ml by sterile distilled water and mix gently. The loading dye was stored at -20°C.

4.4. Stock of Ethidium bromide

Ethidium bromide	100.0	mg
Distilled water	10.0	ml

Ethidium bromide in distilled water was mixed thoroughly. Solution was stored at room temperature in a dark bottle.

5. Solution for *In vitro* transcription

5.1. Diethylpyrocarbonate (DEPC) -treated Water

Diethylpyrocarbonate	0.2	ml
Deionized water	100.0	ml

Diethylpyrocarbonate was added to deionized water and stirred vigorously to mix. After stranding the treated water for overnight, the solution was autoclaved to inactivate the DEPC and stored at room temperature.

6. Reagents for RNA agarose gel electrophoresis

6.1. RNA Sample Loading Buffer

62.5% (v/v) Deionized formamide

1.14 M Formaldehyde

200 μg/ml Bromophenol blue

200 μg/ml Xylene cyanole

MOPS-EDTA-sodium acetate buffer at 1.25X working concentration

Usage: add sample to loading buffer in ratio 1:2 to 1:5. Just before loading, heat to 65°C for 10 min, then chill on ice.

6.2. 6.0 % Formaldehyde-0.7 % Agarose Gel

Agarose	0.35	gm
5X MOPS-EDTA-sodium acetate buffer	10.00	ml
Sterile water	32.00	ml
37% formaldehyde	8.00	ml

Agarose was heated to dissolve in MOPS-EDTA-sodium acetate buffer and water. Wait until it warm and 37% formaldehyde was added, mixed to homogeneous, and poured to gel-block.

6.3. 10X MOPS-EDTA-Sodium acetate (MESA) Buffer [0.2 M MOPS (pH 7.0), 80.0 mM sodium acetate, 10.0 mM EDTA (pH 8.0)]

MOPS	20.92	gm
Sodium acetate	3.28	gm
Sterile DEPC-treated water	450.00	ml

The pH was adjusted to7.0 with 1N NaOH. Add 0.5 M EDTA (pH 8.0) 10 ml and diethylpyrocarbonate 0.5 ml. After standing at room temperature for overnight, this solution was sterilized by filter and stored at room temperature.

7. Reagent for focus immunoassay

7.1. 10 X Phosphate-buffered Saline (PBS) in 1.0 liter (137.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4)

NaCl	80.0	gm
KCl	2.0	gm
Na_2HPO_4	11.5	gm
KH_2PO_4	2.0	gm

The 80.0 gm of NaCl, 2.0 gm of KCl, 11.5 gm of Na_2HPO_4, and 2.0 gm of KH_2PO_4 were dissolved in 800 ml of deionised water. The pH was adjusted with HCl and then deionising water was added to 1.0 litter. The solution was dispensed into aliquots and sterilized by autoclaving for 20 minutes at 1.05 kg/cm^2 on liquid cycle. The buffer was stored at room temperature.

7.2. 2% Triton-X 100

Triton-X 100	0.2	ml
Sterile 1X PBS	10.0	ml

Add 0.2 ml Triton-X 100 into 10 ml of PBS buffer and mix thoroughly.

7.3. 3.7% Formaldehyde in Sterile 1X PBS

37% Formaldehyde	2.0	ml
Sterile 1X PBS	18.0	ml

Mix solution gently before use.

7.4. 1X PBS-0.05% (v/v) Tween-2% (v/v) Fetal Bovine Serum (PBS-TF)

Sterile 1X PBS	9.8	ml
Tween 20	0.5	μl
Fetal bovine serum	0.2	ml

Prepare before use and mix gently.

7.5. Peroxidase Substrate

6%(v/v) H_2O_2	200.0	μl
3, 3 diaminobenzidine (in 1 ml methanol)	50.0	mg

Sterile 1X PBS	9.0	ml

Adjusted final volume to 10.0 ml with sterile 1X PBS. Prepare fresh and mix thoroughly. Prevented from light activation by keeping in the dark.

7.6. Alkaline Phosphatase Buffer (AP Buffer) (per 10 ml)

1 M Tris (pH 9.5)	1.0	ml
5 M NaCl	0.2	ml
1 M MgCl2	0.05	ml
Sterile water up to	9.0	ml

Adjusted final volume to 10.0 ml with sterile water. Prepare fresh and mix thoroughly.

8. Reagents for cell culture

8.1. Leibovitz's L-15 Medium (1X)

The Leibovitz's L-15 Medium is buffered by phosphates and free-base amino acids. The components of Leibovitz's L-15 Medium was: Inorganic salts: 1.26 mM Calcium chloride ($CaCl_2$), 5.30 mM Potassium chloride (KCl), 0.441 mM Potassium phosphate monobasic (KH_2PO_4), 0.986 mM Magnesium chloride ($MgCl_2$), 0.814 mM Magnesium sulfate ($MgSO_4$), 138.00 mM Sodium chloride (NaCl), 1.34 mM Sodium phosphate, dibasic (Na_2HPO_4).

Other compounds: 5.00 mM *D*-Galactose, 5.00 mM Sodium pyruvate, 0.025 mM Phenol Red. Amino acids: 2.52 mM *L*-Alanine, 2.87 mM *L*-Arginine, 1.89 mM *L*-Asparagine, 0.992 mM *L*-Cysteine, 2.055 mM *L*-Glutamine, 2.670 mM Glycine, 1.61 mM *L*-Histidine, 1.910 mM *L*-Isoleucine, 0.954 mM *L*-Leucine, 0.503 mM *L*-Lysine, 1.01 mM *L*-Methionine, 0.76 mM *L*-Phenylalanine, 1.90 mM *L*-Serine, 2.52 mM *L*-Threonine, 0.098 mM *L*-Tryptophan, 1.66 mM *L*-Tyrosine, 0.85 mM *L*-Valine

Vitamins: 0.002 mM *DL*-Calcium pantothenate, 0.0071 mM Choline chloride, 0.0022 mM Folic acid, 0.011 mM *i*-Inositol, 0.0081 mM Niacinamide, 0.0048 mM Pyridoxine hydrochloride, 0.000209 mM Riboflavin 5'-phosphate, Na, 0.00226 mM Thiamine monophosphate.

Appendix D: Instruments

1. DNA thermal cycler, model 480 (Perkin Elmer, Foster City, California, USA).
2. Flip-flop shaker (model FF 120 S, J.S.C. instrument).
3. Freezer, -20°C (Sanyo, Japan).
4. Freezer, -75°C (Forma Scientific Inc, USA).
5. Gel documentation system, model gel doc 1000 (Bio-Rad Laboratories, Inc., RSA).
6. Genetic analyzer ABI PRISM 310 (Perkin Elmer, Foster City, California, USA).
7. High speed refrigerated microcentrifuge, model 4239R (ALC, Milano, Italy).
8. Incubator (Forma Scientific Inc, USA).
9. Incubator, model 1565 (Shellab, USA).
10. Laminar flow cabinet, model NU-425-400 (NuAire, USA).
11. Microcentrifuge (ALC, Milano, Italy).
12. Microwave oven NN-6208 (Matsushita Electric Industrial Co., Ltd., Japan).
13. Orbital shaker bath Model 360 (Precision Scientific, USA).
14. Orbital shaker model Gyromax 737R (Amerex Instruments, Inc., USA).
15. pH meter model 661 (Orion Research Incorporated Laboratory product group, USA).
16. Superspeed refrigerated centrifuge, model Sorvall RC-5 (E.I. Dupont Denemours & Co., USA).
17. Spectrophotometer (Spectronic Genesysz, U.K.).
18. Ultraviolet transilluminator (Vilber Lourmat, France).

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PUBLICATION:

Keelapang P., R. Sriburi, S. Supasa, N. Panyadee, **A. Songjaeng**, A. Jairungsri, C. Puttikhunt, W. Kasinrerk, P. Malasit, and N. Sittisombut. 2004. Alterations of pr-M cleavage and virus export in pr-M junction chimeric dengue viruses. J.Virol. 78: 2367-81