APPENDICES

Appendix A

Titration of K204 dengue mutant virus set

Day after transfection	K204A virus (ffu/mL)	K204D virus (ffu/mL)	K204H virus (ffu/mL)	K204R virus (ffu/mL)	K204S virus (ffu/mL)	Dengue 16681 wt (ffu/mL)
4	ND	ND	ND	0	ND	7.00×10^{1}
7	0	0	0	0	0	2.00×10^{4}
11	ND	ND	ND	2.73×10^{4}	ND	1.73×10^{7}
14	ND	ND	ND	2.64×10^{7}	ND	2.45×10^{7}
21	ND	ND	ND	4.18×10^{6}	ND	5.09×10^{6}
28	0	0	0	1.00×10^{7}	0	4.50×10^{6}
35	ND	ND	ND	9.09×10^{6}	ND	5.23×10^{6}
42	ND	ND	ND	1.35×10^{6}	ND	3.55×10^{6}
49	ND	ND	ND	2.00×10^{6}	ND	6.21×10^{5}
56	0	0	0	1.10×10^{6}	0	3.33×10^{5}

ND, not done.

Appendix B

Titration of R205 dengue mutant virus set

Day after transfection	R205A virus (ffu/mL)	R205D virus (ffu/mL)	R205H virus (ffu/mL)	R205K virus exp.I (ffu/mL)	R205K virus exp.II (ffu/mL)	R205D virus (ffu/mL)
4	ND	ND	ND	0	0	ND
7	0	0	0	0	0	0
11	ND	ND	ND	0	0	ND
14	ND	ND	ND	4.00×10^{1}	1.00×10^{1}	ND
21	ND	ND	ND	1.82×10^{1}	2.82×10^{4}	ND
28	0	0	0	1.36×10^{2}	1.45×10^{4}	0
35	ND	ND	ND	2.18×10^{4}	2.09×10^{4}	ND
42	ND	ND	ND	8.00×10^{5}	1.27×10^{4}	ND
49	ND	ND	ND	6.00×10^{6}	1.82×10^{4}	ND
56	0	0	0	1.27×10^{7}	4.82×10^{4}	0

ND, not done.

Appendix C

Burst size of the K204R dengue mutant virus

Day[1] / Foci no.	Day 11	Day 14	Day 56
1	417	230	161
2	255	176	83
3	118	155	45
4		337	170
5		272	335
6		265	140
7		180	117
8		169	265
9		252	118
10		138	18
11		109	24
12		38	110
13		45	88
14		50	124
15		33	317
16		351	123
17		344	45
18		230	106
19		106	87
20		332	205

Appendix C (Continue)

Day[A] / Foci no.	Day 11	Day 14	Day 56
21		63	91
22		157	198
23		40	94
24		211	157
25		167	145
26		108	
27		51	
28		341	
Total average	790/3 =263.3	4950/28 =176.8	3420/25 = 136.8
	5740/31=185.2		3420/25 = 136.8
	9160/56=163.6		

A, day after transfection

Appendix D

Burst size of the R205K dengue mutant virus (transfection I)

Day[A] / Foci no.	Day 14	Day 21	Day 28	Day 56
1	5	5	7	202
2	3	10	7	110
3	19		2	201
4	4		6	495
5	2		7	111
6	3		8	428
7			9	159
8			5	118
9			4	121
10			8	109
11			5	159
12				208
13				210
14				76
15				384
16				212
17				563
Total average	36/6 = 6 cells/focus	15/2 = 7.5 cells/focus	68/11 = 6.2 cells/focus	3866/17 =227.4cells/focus
	119/19 = 6.3 cells/focus			3866/17 = 227.4 cells/focus

A, day after transfection

Appendix E

Percent of small and large foci of K204R mutant virus

Day after transfection	Foci size (cell/focus)			Percent of foci size	
	Small[A]	Large[B]	Total	Small[A]	Large[B]
4	0	0	0	0	0
7	0	0	0	0	0
11	0	3	3	0	100
14	0	29	29	0	100
21	3	43	46	6.52	93.48
28	0	11	11	0	100
35	1	9	10	10.00	90.00
42	0	15	15	0	100
49	0	22	22	0	100
56	2	9	11	18.18	81.82

A, small foci are the focus have 1-20 cells in a focus.

B, large foci are the focus that larger than 20 cells in a focus.

Appendix F

Percent of small and large foci R205K mutant virus (transfection I)

Day after transfection	Foci size (cell/focus)			Percent of foci size	
	Small[A]	Large[B]	Total	Small[A]	Large[B]
4	0	0	0	0	0
7	0	0	0	0	0
11	0	0	0	0	0
14	4	0	4	100	0
21	2	0	2	100	0
28	14	1	15	93.3	6.7
35	6	18	24	25.0	75.0
42	2	6	8	25.0	75.0
49	0	6	6	0	100
56	2	12	14	14.3	85.7

A, small foci are the focus have 1-20 cells in a focus.

B, large foci are the focus that larger than 20 cells in a focus.

Appendix G

Percent of small and large foci of R205K mutant virus (transfection II)

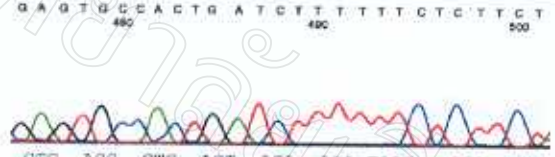
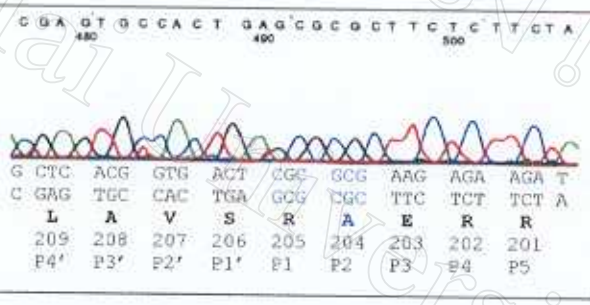
Day after transfection	Foci size (cell/focus)			Percent of foci size	
	Small[A]	Large[B]	Total	Small[A]	Large[B]
4	0	0	0	0	0
7	0	0	0	0	0
11	0	0	0	0	0
14	1	0	1	100	0
21	31	0	31	100	0
28	10	6	16	62.5	37.5
35	13	10	23	56.5	43.5
42	13	1	14	7.1	92.9
49	8	12	20	40.0	60.0
56	24	29	53	45.3	54.7

A, small foci are the focus have 1-20 cells in a focus.

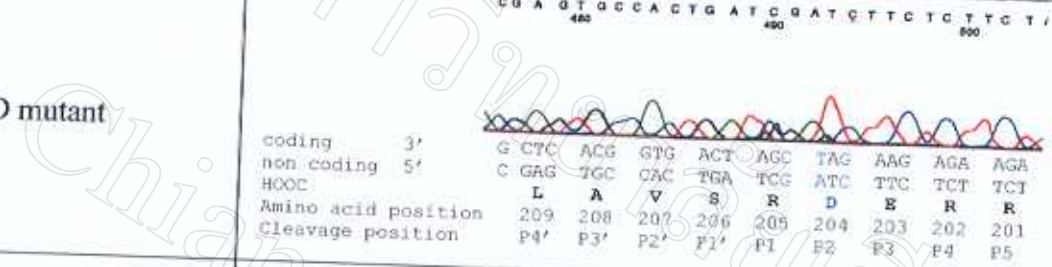
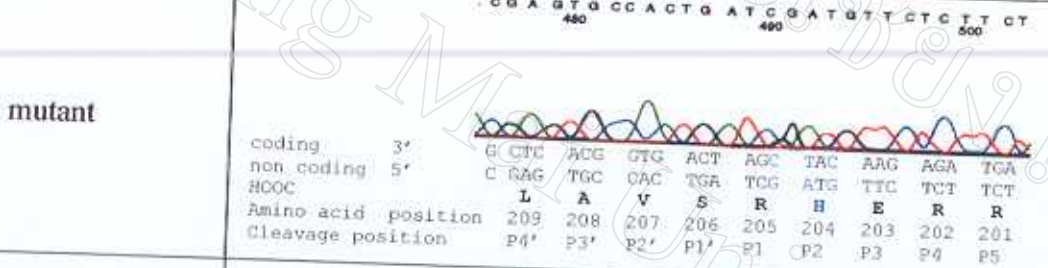
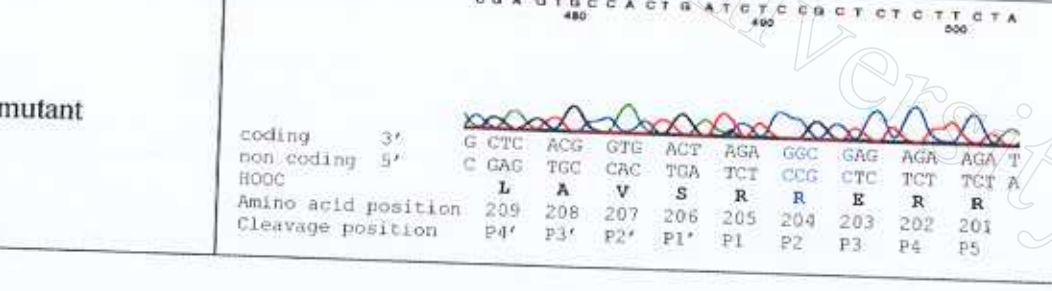
B, large foci are the focus that larger than 20 cells in a focus.

Appendix H

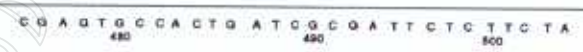
Nucleotide sequence analysis at prM-M junction of dengue mutant clones by using primer C1204

cDNA	Sequence at prM/M junction[A]
Dengue serotype 2 strain 16681 wild type	
K204A mutant	

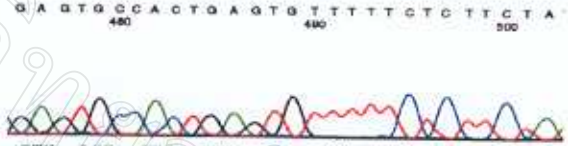
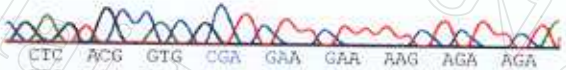
Appendix H (continue)

cDNA	Sequence at prM/M junction[A]
K204D mutant	
K204H mutant	
K204R mutant	

Appendix H (continue)

cDNA	Sequence at prM/M junction[A]
K204S mutant	 coding 3′ G CTC ACG GTG ACT AGC GCT AAG AGA AGA T 5′ non coding 5′ C GAG TGC CAC TGA TCG CGA TTC TCT TCT A 3′ HOOC L A V S R S E R R NH_2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5
R205A mutant	 coding 3′ CTC ACG GTG ACT TCG AAA AAG AGA AGA T 5′ non coding 5′ GAG TGC CAC TGA AGC TTT TTC TCT TCT A 3′ HOOC L A V S A K E R R NH_2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5
R205D mutant	 coding 3′ CTC ACG CTG ACT CAG AAA AAG AGA AGA 5′ non coding 5′ GAG TGC GAC TGA GTC TTT TTC TCT TCT 3′ HOOC L A V S D K E R R NH_2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5

Appendix H (continue)

cDNA	Sequence at prM/M junction[A]
R205H mutant	 coding 3′ CTC ACG GTG ACT CAC AAA AAG AGA AGA T 5′ non coding 5′ GAG TGC CAC TGA GTG TTT TTC TCT TCT A 3′ HOOC L A V S H K E R R NH_2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5
R205K mutant	 coding 3′ CTC ACG GTG CGA GAA GAA AAG AGA AGA 5′ non coding 5′ GAG TGC CAC GCT CTT CTT TTC TCT TCT 3′ HOOC L A V S K K E R R NH_2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5
R205S mutant	 coding 3′ CTC ACG GTG ACT CGA GAA AAG AGA AGA 5′ non coding 5′ GAG TGC CAC TGA GCT CTT TTC TCT TCT 3′ HOOC L A V S S K E R R NH_2 Amino acid position 209 208 207 206 205 204 203 202 201 Cleavage position P4′ P3′ P2′ P1′ P1 P2 P3 P4 P5

A, blue letters indicate intended mutation

Appendix I

Nucleotide and amino acid sequences around the prM-M cleavage-site of K204R and R205K viruses compare with dengue serotype 2 strain 16681.

1. The nucleotide sequence at the cleavage site of the prM gene of dengue serotype 2 strain 16681, K204R (day11) and R205K (day 14, 21 35, 42) by primer C859. Dots represent nucleotides identical of dengue serotype 2 strain 16681 and nucleotide 710 is boxed to highlight a nucleotide changed.

Nucleotide sequence

```
DEN-2(16681)      501 AAGTCTTCTG TTTAAAACAG AGGATGGCGT GAACATGTGT ACCCTCATGG 550
K204R day11           ..... ..... ..... ..... .....
R205K day14           ..... ..... ..... ..... .....
R205K day21           ..... ..... ..... ..... .....
R205K day35           ..... ..... ..... ..... .....
R205K day42           ..... ..... ..... ..... .....

DEN-2(16681)      551 CCATGGACCT TGGTGAATTG TGTGAAGACA CAATCACGTA CAAGTGTCCC 600
K204R day11           ..... ..... ..... ..... .....
R205K day14           ..... ..... ..... ..... .....
R205K day21           ..... ..... ..... ..... .....
R205K day35           ..... ..... ..... ..... .....
R205K day42           ..... ..... ..... ..... .....

DEN-2(16681)      601 CTTCTCAGGC AGAATGAGCC AGAAGACATA GACTGTTGGT GCAACTCTAC 650
K204R day11           ..... ..... ..... ..... .....
R205K day14           ..... ..... ..... ..... .....
R205K day21           ..... ..... ..... ..... .....
R205K day35           ..... ..... ..... ..... .....
R205K day42           ..... ..... ..... ..... .....

DEN-2(16681)      651 GTCCACGTGG GTAACTTATG GGACGTGTAC CACCATGGGA GAACATAGAA 700
K204R day11           ..... ..... ..... ..... .....
R205K day14           ..... ..... ..... ..... .....
R205K day21           ..... ..... ..... ..... .....
R205K day35           ..... ..... ..... ..... .....
R205K day42           ..... ..... ..... ..... .....

DEN-2(16681)      701 GAGAAAAAAG ATCAGTGGCA CTCGTTCCAC ATGTGGGAAT GGGACTGGAG 750
K204R day11           ....GCGG.. ..... ..... ..... .....
R205K day14           .......G.A GAGC.. ..... ..... ..... .....
R205K day21           .......G.A GAGC.. ..... ..... ..... .....
R205K day35           .......G.A/G GAGC.. ..... ..... ..... .....
R205K day42           .......G.G GAGC.. ..... ..... ..... .....
```

```
DEN-2(16681)      751 ACACGAACTG AAACATGGAT GTCATCAGAA GGGGCCTGGA AACATGTCCA 800
K204R day11           .......... .......... .......... .......... ..........
R205K day14           .......... .......... .......... .......... ..........
R205K day21           .......... .......... .......... .......... ..........
R205K day35           .......... .......... .......... .......... ..........
R205K day42           .......... .......... .......... .......... ..........
```

2. Amino acid sequence at the cleavage site of the prM of dengue serotype 2 strain 16681, K204R (day11) and R205K (day 14, 21 35, 42). Dots represent base identical of dengue serotype 2 strain 16681. The cleavage site of prM-M is indicated. The amino acid residue 205 of R205K (day42) mutant was completely reverting.

```
Amino acid sequence

DEN-2(16681)      141 TEDGVNMCTL MAMDLGELCE DTITYKCPLL RQNEPEDIDC WCNSTSTWVT 190
K204R day11           .......... .......... .......... .......... ..........

R205K day14           .......... .......... .......... .......... ..........
R205K day21           .......... .......... .......... .......... ..........
R205K day35           .......... .......... .......... .......... ..........
R205K day42           .......... .......... .......... .......... ..........


                                          ┌─►M
DEN-2(16681)      191 YGTCTTMGEH RREKRSVALV PHVGMGLETR                      230
K204R day11           .......... ...R...... ..........

R205K day14           .......... ....K..... ..........
R205K day21           .......... ....K..... ..........
R205K day35           .......... ....K..... ..........
R205K day42           .......... .....R.... ..........
```

Appendix J

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	Residues 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
Isoluecine	Ile	I	–	113.17
Luecine	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 codon (mRNA) uses.

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 K

Reagents

1. Medium for Bacterial growth

1.1 LB Medium (per Liter)

Tryptone	10.0	g
Yeast extracts	5.0	g
NaCl	10.0	g

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 LB Agar (per Liter)

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

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/100-mm plate).

1.3 LB–Ampicillin Agar (per Liter)

A liter of LB agar was prepared, autoclaved, cooled to 55 °C, added 250 µL of 100.0 mg/mL sterilized ampicillin. Then poured into 100 mm petri-dishes (~25 mL/100-mm plate).

1.4 SOB Medium (per Liter)

Tryptone	20.0	g
Yeast extracts	5.0	g
NaCl	0.5	g

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)

20% (w/v) glucose	2.0	mL

The 2.0 mL of sterile glucose solution were added to 98.0 mL of sterile SOB medium to make final volume of 100.0 mL. Solution was stored at 4°C.

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

2.1 STET buffer

8.0% (w/v) sucrose

0.5% (v/v) Triton X-100

50.0 mM Tris (pH 8.0)

50.0 mM EDTA

All components could be prepared and 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 10.0 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 (pH8.0)

The 1.21 g of Tris-base and 0.37 g of EDTA·$2H_2O$ 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 g of Tris-base were dissolve in 800 mL of 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.

3. Solution for plasmid DNA midi-preparation

3.1 Resuspension buffer

Tris-base	6.06	g
EDTA·$2H_2O$	3.72	g

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. The RNase A was added to100.0 μg/mL before use.

3.2 Lysis buffer

0.2 N NaOH

1% (w/v) SDS

NaOH pellets (8.0 g) were dissolved in distilled water and 50.0 mL of 20% SDS solution was added. The final volume was adjusted to1.0 litter.

3.3 Neutralization buffer

The 294.5 g 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

NaCl	43.83 g
Acid free MOPS	10.46 g
Isopropanol	150.0 mL
10% Triton X-100 solution	15.0 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 by distilled water.

3.5 Wash buffer

NaCl	58.44 g
Acid free MOPS	10.46 g
Isopropanol	150.0 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

NaCl	73.05 g
Tris-base	6.06 g
Isopropanol	150.0 mL

NaCl and Tris-base were dissolved in 800 mL of distilled water. The pH was adjusted to 8.5. Isopropanol 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	g
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 added to 1 liter. Solution was sterilized by autoclaving.

4.2 0.7% Agarose gel

1X TAE buffer	100.0	mL
Agarose gel	0.7	g

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

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

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

The Diethylpyrocarbonate was added to deionized water and shake vigorously to mix. Solution was autoclaved to inactivate the DEPC and stored at room temperature.

5.2 Transcription optimized 5X buffer

200 mM Tris-HCl (pH 7.9)

30 mM $MgCl_2$

10 mM spermidine

50 mM NaCl

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 congentstion

50 μg/mL Ethidium bromide

Recommended 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 Formaldehyde-0.7% agarose gel

Agarose	0.35	g
5X MOPS-EDTA-sodium acetate buffer	10.0	mL
Sterile water	32.0	mL
37% formaldehyde	8.0	mL

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

6.3 MOPS-EDTA-sodium acetate buffer

0.2 M MOPS (pH 7.0)

80 .0 mM sodium acetate

10.0 mM EDTA (pH 8.0)

7. Reagent for focus immunoassay

7.1 Phosphate-buffer 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

The 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 were dissolved in 800 mL of deionizing water. The pH was adjusted

to 7.4 by HCl and then deionizing 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

7.3 3.7% Formaldehyde in sterile 1X PBS

37% Formaldehyde	2.0	mL
Sterile 1X PBS	18.0	mL

7.4 PBS-Tween-fetal Bovine Serum (PBS-TF)

Sterile 1X PBS	25.0	mL
Tween 20	12.5	µL
Fetal Bovine Serum	0.5	mL

7.5 Peroxidase substrate

6%(v/v) H_2O_2	60.0	µL
3, 3 diaminobenzidine (in 1 mL methanol)	0.003	g
Sterile 1X PBS	5.0	mL

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 L

Instruments

1. DNA Thermal cycle model 480 (Perkin Elmer, Forster 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 photography FCR-10 (Fotodyne Incorperated, USA).
6. Genetic Analyzer ABI PRISM 310 (Perkin Elmer, Forester, California, USA).
7. Gene pulser (Bio-Rad™, USA).
8. High speed refrigerated microcentrifuge model 4239R (ALC, Milano, Italy)
9. Incubator (Forma Scientific Inc, USA).
10. Laminar Flow Cabinet
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. pH/Millivolt meter model 661 (Orion Research Incorporated Laboratory product group, USA).
15. Spectrophotometer (Spectronic Genesysz, U.K.).
16. Ultra Violet fluorescent table (Viber Lourmat, France).
17. Waterbath

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