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APPENDICES

APPENDIX A

Chemical and reagent preparations

0.5 M EDTA (pH 8.0)

Dissolve EDTA.2H₂O 186.1 g in distilled water 800 ml. Then mix gently and adjust pH to 8.0 with NaOH. Autoclave the solution at 121 °C, 15 lb/inch² for 15 minutes.

5X Tris borate (TBE)

Tris base	54	g
Boric acid	27.5	g
0.5 M EDTA (pH 8.0)	20	ml

Adjust the volume to 1000 ml by distilled water. Keep the solution at room temperature.

Phosphate buffer saline (PBS) pH 7.4

NaCl	8	g
KCl	0.2	g
Na ₂ HPO ₄	1.44	g
KH ₂ PO ₄	0.24	g

Dissolve in distilled water, adjust volume to 1000 ml and pH to 7.4. Incubate 37°C overnight and Autoclave the solution at 121 °C, 15 lb/inch² for 15 minutes.

5M NaOH

NaOH	200	g
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Dissolve in distilled water and adjust volume to 1000 ml. Keep the solution at room temperature.

5M NaCl

NaCl	292.2 g
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Dissolve in distilled water and adjust volume to 1000 ml. Keep the solution at room temperature.

1M Tris-HCl (pH 8.0)

Tris base	121.1 g
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Dissolve in distilled water and adjust pH to 8.0 with HCl. Then adjust volume to 1000 ml. Autoclave the solution at 121 °C, 15 lb/inch² for 15 minutes.

0.5 µg/ml ethidium bromide

Stock ethidium bromide 10 mg/ml	12.5 µl
Distilled water	237.5 ml

Mix gently and keep the solution in the dark chamber (light protection).

1.5% and 2% agarose gel electrophoresis

Agarose	1.5 g (for 1.5%) and 2 g (for 2%)
1X TBE	100 ml

Mix gently and heat with a microwave machine. Wait the gel solution until it cools down. Then pour the gel into gel chamber.

100 mg/ml spectinomycin

Spectinomycin	1 g
DNase and Rnase free water	10 ml

Mix gently and filter the solution through 0.2 µm pore size paper. Divide the solution in small volume and keep it at -20°C.

SOB medium*

Tryptone	20 g
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Yeast extract	5	g
NaCl	5	g

Add distilled to a final of 1000 ml and autoclave the solution at 121 °C, 15 lb/inch² for 15 minutes. Add 10 ml of filter-sterilized 1M MgCl₂ and 10 ml of filtered sterilized 1M MgSO₄ before use.

SOC medium*

Prepare immediately before use by adding 1 ml of filter-sterilized 2M glucose and adjust to final volume of 100 ml with SOC medium.

LB (Luria-Bertani) broth with 100 µg/ml spectinomycin

Tryptone	2.5	g
Yeast extract	1.25	g
NaCl	2.5	g

Dissolve in distilled water, adjust pH 7.0 and final volume of 250 ml. Autoclave the solution at 121 °C, 15 lb/inch² for 15 minutes. Wait the medium becomes warm. Then add 250µl of 10 mg/ml spectinomycin to prepare LB broth with 100 µg/ml.

LB (Luria-Bertani) agar with 100 µg/ml spectinomycin

Tryptone	2	g
Yeast extract	1	g
NaCl	2	g
Agar	4	g

Dissolve in distilled water, adjust pH 7.0 and final volume of 200 ml. Autoclave the solution at 121 °C, 15 lb/inch² for 15 minutes. Wait the medium becomes warm. Then add 200µl of 10 mg/ml spectinomycin to prepare LB agar with 100 µg/ml. Pour the medium into petri dishes (~25 ml/100 mm plate).

* The protocols are taken from StrataCloneTMSoloPack®Competent Cells data sheets (USA).

APPENDIX B

Summary data of specimen collections in dengue and non-dengue infected patients

Table 34: Summary data of specimen collections from both dengue and
non dengue-infected patients

Code	DOF	Acute period (Febrile period)				Early convalescent period (After fever – day 25 of illness)				Late convalescent period (day 26 – day 90 of illness)			
		PI	PB	S	U	PI	PB	S	U	PI	PB	S	U
N2	4	+	+	+	+	+	+	+	+	+	+	+	+
		(3)	(3)	(3)	(3)	(21)	(21)	(21)	(21)	(90)	(90)	(90)	(90)
N3	7	+	+	+	+	+	+	+	+	+	+	+	+
		(7)	(7)	(7)	(7)	(22)	(22)	(22)	(22)	(75)	(75)	(75)	(75)
N4	5	+	+	+	+	+	+	+	+	ND	ND	ND	ND
		(5)	(5)	(5)	(5)	(21)	(21)	(21)	(21)				
N5	7	+	+	+	+	+	+	+	+	ND	ND	ND	ND
		(6)	(6)	(6)	(6)	(23)	(23)	(23)	(23)				
N6	6	+	+	+	+	+	+	+	+	+	+	+	+
		(4)	(4)	(4)	(4)	(23)	(23)	(23)	(23)	(80)	(80)	(80)	(80)
N8 ^v	5	ND	ND	ND	ND	+	+	+	+	ND	ND	ND	ND
						(7)	(7)	(7)	(7)				
						(25)	(25)	(25)	(25)				
N9 ^o	9	+	+	+	+	+	+	+	+	+	+	+	+
		(9)	(9)	(9)	(9)	(15)	(15)	(15)	(15)	(30)	(30)	(30)	(30)
										+	+	+	+
										(90)	(90)	(90)	(90)
N10 ^o	5	+	+	+	+	ND	ND	ND	ND	+	+	+	+
		(4)	(4)	(4)	(4)					(27)	(27)	(27)	(27)
										(90)	(90)	(90)	(90)
N12 ^o	6	+	+	+	+	+	+	+	+	+	+	+	+
		(4)	(4)	(4)	(4)	(12)	(12)	(12)	(12)	(26)	(26)	(26)	(26)
										(75)	(75)	(75)	(75)
N13 ^{v o}	5	ND	ND	ND	ND	+	+	+	+	+	+	+	+
						(8)	(8)	(8)	(8)	(29)	(29)	(29)	(29)
						(15)	(15)	(15)	(15)	(64)	(64)	(64)	(64)
N17	7	+	+	+	+	+	+	+	+	+	+	+	+
		(7)	(7)	(7)	(7)	(13)	(13)	(13)	(13)	(33)	(33)	(33)	(33)
N20	8	ND	ND	+	+	ND	ND	ND	ND	+	+	+	+
				(6)	(6)					(30)	(30)	(30)	(30)
N21 ^v	7	+	+	+	+	+	+	+	+	ND	ND	ND	ND
		(7)	(7)	(7)	(7)	(14)	(14)	(14)	(14)				

Code	DOF	Acute period (Febrile period)				Early convalescent period (After fever – day 25 of illness)				Late convalescent period (day 26 – day 90 of illness)			
		PI	PB	S	U	PI	PB	S	U	PI	PB	S	U
N21 [†]	7					(24)	(24)	(24)	(24)				
N22	7	+	+	+	+	+	+	+	+	ND	ND	ND	ND
		(4)	(4)	(4)	(4)	(24)	(24)	(24)	(24)				
N23	8	+	+	+	+	+	+	+	+	+	+	+	+
		(7)	(7)	(7)	(7)	(17)	(17)	(17)	(17)	(45)	(45)	(45)	(45)
N24 [°]	6	+	+	+	+	+	+	+	+	+	+	+	+
		(3)	(3)	(3)	(3)	(13)	(13)	(13)	(13)	(32)	(32)	(32)	(32)
										(49)	(49)	(49)	(49)
N28 [°]	8	+	+	+	+	+	+	+	+	+	+	+	+
		(6)	(6)	(6)	(6)	(14)	(14)	(14)	(14)	(46)	(46)	(46)	(46)
										(90)	(90)	(90)	(90)
N29	8	+	+	+	+	+	+	+	+	+	+	+	+
		(5)	(5)	(5)	(5)	(19)	(19)	(19)	(19)	(33)	(33)	(33)	(33)
N30	9	+	+	+	+	+	+	+	+	+	+	+	+
		(7)	(7)	(7)	(7)	(20)	(20)	(20)	(20)	(38)	(38)	(38)	(38)
N33 [†]	6	ND	ND	ND	ND	+	+	+	+	+	+	+	+
						(7)	(7)	(7)	(7)	(31)	(31)	(31)	(31)
						(18)	(18)	(18)	(18)				
N34 [†]	6	ND	ND	ND	ND	+	+	+	+	+	+	+	+
						(8)	(8)	(8)	(8)	(28)	(28)	(28)	(28)
						(14)	(14)	(14)	(14)				
N35 [†]	5	ND	ND	ND	ND	+	+	+	+	+	+	+	+
						(7)	(7)	(7)	(7)	(27)	(27)	(27)	(27)
						(13)	(13)	(13)	(13)				
N40	4	+	+	+	+	+	+	+	+	+	+	+	+
		(4)	(4)	(4)	(4)	(21)	(21)	(21)	(21)	(71)	(71)	(71)	(71)
N16 ^{†°}	7	+	+	+	+	+	+	+	+	+	+	+	+
		(6)	(6)	(6)	(6)	(13)	(13)	(13)	(13)	(34)	(34)	(34)	(34)
										(64)	(64)	(64)	(64)
N27 [†]	6	+	+	+	+	+	+	+	+	+	+	+	+
		(5)	(5)	(5)	(5)	(14)	(14)	(14)	(14)	(54)	(54)	(54)	(54)
N37 [†]	5	+	+	+	+	ND	ND	ND	ND	+	+	+	+
		(4)	(4)	(4)	(4)					(28)	(28)	(28)	(28)
N39 [†]	9	+	+	+	+	+	+	+	+	+	+	+	+
		(1)	(1)	(1)	(1)	(15)	(15)	(15)	(15)	(33)	(33)	(33)	(33)
N43 [†]	15	+	+	+	+	+	+	+	+	+	+	+	+
		(5)	(5)	(5)	(5)	(23)	(23)	(23)	(23)	(33)	(33)	(33)	(33)

+ = have specimen collection. ND = not have specimen collection. DOF = duration of fever.

[†] = have double specimen collections during early convalescent period. [°] = have double specimen collections during late convalescent period. * = non dengue-infected patients. PI = plasma, PB = PBMCs, S = saliva, U = urine. The number in "()" presents the day of specimen collection.

APPENDIX C

The ELISA results of all DENV and non-DENV infected patients

Table 35: The ELISA results of 23 DENV-infected and 5 non-DENV infected patients

Code	DOF (day)	Clinical diagnosis	Day of specimen collection	IgM (Units)	IgG (Units)	IgM:IgG ratio	Interpretation
N2	4	DHF II	3	53	107	< 1.8	Secondary
			21	37	128		
			90	1	61		
N3	7	DHF II	7	55	25	< 1.8	Secondary
			22	58	66		
			75	38	38		
N4	5	DHF II	5	6	134	< 1.8	Secondary
			21	0	134		
N5	7	DHF II	6	25	126	< 1.8	Secondary
			23	62	129		
N6	6	DHF I	4	112	126	< 1.8	Secondary
			23	96	112		
			80	32	61		
N8	5	DHF I	7	64	86	< 1.8	Secondary
			25	41	93		
N9	9	DHF II	9	66	122	< 1.8	Secondary
			15	51	116		
			30	30	103		
			90	0	44		
N10	5	DHF II	4	4	10	< 1.8	Secondary
			27	66	129		
			90	48	103		
N12	6	DHF II	4	56	74	< 1.8	Secondary
			12	73	113		
			26	65	119		
			75	32	76		
N13	5	DHF I	8	101	101	< 1.8	Secondary
			15	97	95		

Code	DOF (day)	Clinical diagnosis	Day of specimen collection	IgM (Units)	IgG (Units)	IgM:IgG ratio	Interpretation
N13	5	DHF I	29	85	85		
			64	33	33		
N17	7	DHF I	7	84	123	< 1.8	Secondary
			13	85	136		
			33	72	137		
N20	8	DHF I	6	ND	ND	< 1.8	Secondary
			30	75	112		
N21	7	DF	7	24	97	< 1.8	Secondary
			14	37	129		
			24	32	115		
N22	7	DHF II	4	66	93	< 1.8	Secondary
			24	59	94		
N23	8	DHF I	7	44	101	< 1.8	Secondary
			17	64	130		
			45	19	102		
N24	6	DSS	3	0	8	< 1.8	Secondary
			13	28	136		
			32	10	108		
			49	0	44		
N28	8	DHF II	6	49	0	< 1.8	Secondary
			14	87	66		
			46	78	53		
			90	62	38		
N29	8	DHF II	5	1	54	< 1.8	Secondary
			19	126	128		
			33	117	122		
N30	9	DHF III	7	9	36	< 1.8	Secondary
			20	32	122		
			38	23	110		
N33	6	DHF III	7	26	93	< 1.8	Secondary
			18	49	113		
			31	55	106		
N34	6	DHF II	8	98	9	>1.8	Primary
			14	101	27		

Code	DOF (day)	Clinical diagnosis	Day of specimen collection	IgM (Units)	IgG (Units)	IgM:IgG ratio	Interpretation
N34	6	DHF II	28	75	17		
N35	5	DHF II	7	24	120	< 1.8	Secondary
			13	27	123		
			27	17	105		
N40	4	DHF II	4	3	13	<1.8	Secondary
			21	59	117		
			71	6	82		
Negative control patient results (non-DENV-infected patients)							
N16	7	Graves' disease	6	9	13	ND	ND
			13	9	10		
			34	7	4		
			64	8	8		
N27	6	Unspecified viral infection	5	1	19	ND	ND
			14	3	13		
			54	0	18		
N37	5	Influenza virus infection	4	4	13	ND	ND
			28	0	10		
N39	9	Unspecified viral infection	7	1	8	ND	ND
			15	0	3		
			33	0	2		
N43	15	Influenza virus infection	4	2	15	ND	ND
			23	2	13		
			33	1	10		

DOF= duration of fever. ND = not determined.

The IgM \geq 40 units and IgG \geq 100 units are considered as positive. IgM: IgG \geq 1.8 is interpreted as primary DENV infection whereas < 1.8 is secondary infection.

Non-dengue diagnosis means the patients diagnosed other febrile illness.

The controls of this assay give the acceptable results (OD of NC = 0.076, < 0.1 is acceptable and OD of weak positive control (WPC) of mixed 4 serotypes = 0.288 (IgM) and 0.290 (IgG), acceptable OD is 0.25-0.60 for mixed 4 serotypes IgM and IgG).

Definitions: acute period (duration of fever), early convalescent period (first day of fever recovery until day 25 of illness) and late convalescent period (day 26 – day 90 of illness).

APPENDIX D

Comparison of the longest time of DENV detection

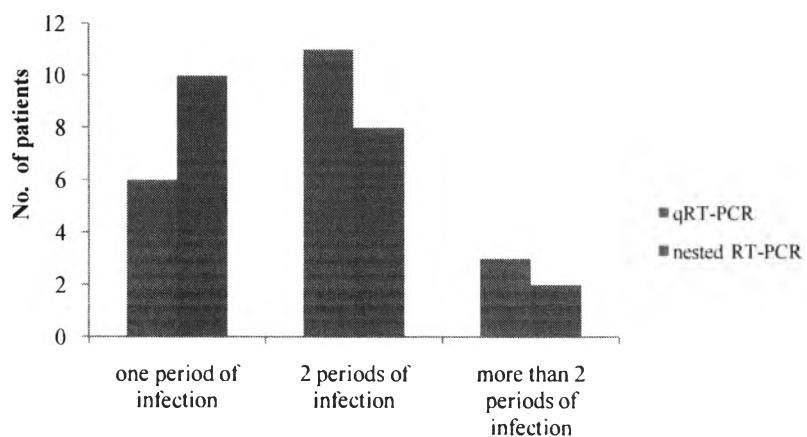


Figure 46: The comparison of the latest time of DENV detection in dengue-infected patients when using real time RT-PCR (qRT-PCR) and nested RT-PCR.

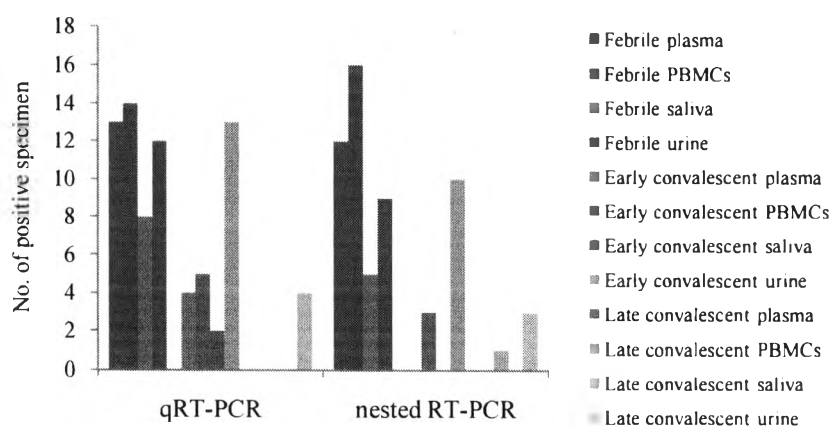


Figure 47: The comparison of DENV detection in different specimens and time points of dengue-infected patients when using real time RT-PCR (qRT-PCR) and nested RT-PCR.

APPENDIX E

Molecular techniques for negative strand detection (Tagged RT-PCR)

During viral replication of positive sense single strand RNA viruses, negative strand is synthesized as a template for new positive strand synthesis. The presence of negative strand may point out the evidence of viral replication. RT-PCR is applied to detect viral negative strand by using forward primer to generated cDNA and following by PCR assay. Strand specific RT-PCR known as tagged RT-PCR has been developed to detect negative strand detection.

Tagged RT-PCR is based on the use of modified forward primer with short oligonucleotide sequence at 5'end (Tag-F primer). Added oligonucleotide sequences are known as 'tag' primers not randomly bind with the target gene of interest. After cDNA synthesis, the reaction mixture is purified to reduce trace primer and non binding RNA with tag-F primer. PCR is done by using tag primers (without forward primer) as forward primer and reverse gene specific primer. Tagged RT-PCR can also be applied in real time RT-PCR. However, gel electrophoresis to confirm the result after doing real time RT-PCR is necessary because the result of real time PCR may be inconclusive especially when using SYBR Green I detection system. The diagram of tagged RT-PCR is presented in Figure 48.

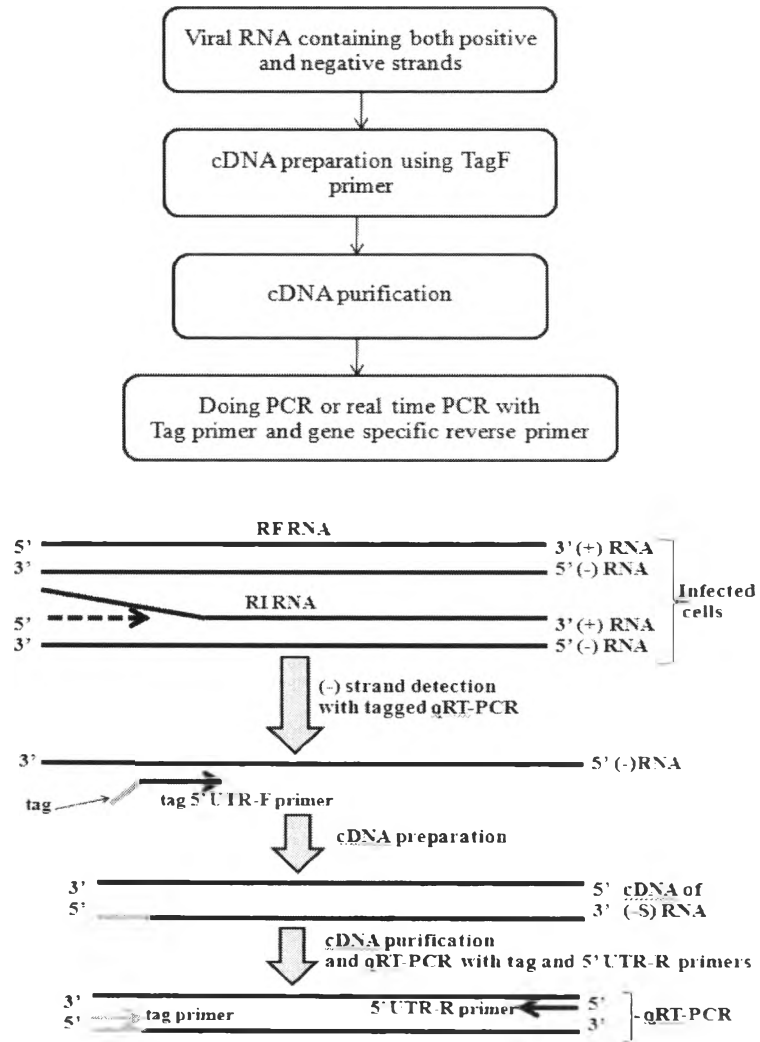


Figure 48: The strategy of tagged RT-PCR assay (Adapted from Peyrefitte *et al.* [82])

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2. **Oral Presentation:** “Prolonged and differential shedding of dengue virus serotype 2 (DEN2) in plasma, PBMCs, saliva and urine of adult patients during acute infection” 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) London, United Kingdom, 31 March – 4 April 2012.
3. **Poster Presentation:** “Presence of multiple dengue serotypes in various body compartments in different time points of single clinical episodes” 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) London, United Kingdom, 31 March – 4 April 2012.
4. **Poster Presentation:** “Presence of heterogeneous population of dengue virus serotype 2 (DENV2) in different body compartments and time points in adults” IDWEEKTM2012, San Diego (CA), USA, 17-21 October 2012.

PUBLICATIONS: In preparation for 2 international publications