CHAPTER IV

RESULTS

Patient recruitment and specimen collections

Twenty-three diagnosed DENV-infected adult patients and five non-DENV-infected patients from our previous project "Survival of dengue virus in blood, urine, saliva and buccal mucosa in complete-recovery dengue patients" were included in this study. All patients were admitted to King Chulalongkorn Memorial Hospital during 29 April 2007 to 30 November 2007. For the former group, there were 12 males (age: 19-53 years old) and 11 females (age: 18-56 years old). For the latter group, there were 2 males (age: 23-35 years old) and 3 females (age: 33-65 years old). The day of illness in DENV-infected patients were 5-9 days in male and 4-9 days in female while in non-DENV-infected patients were 7-9 days in male and 6-15 days in female. Their clinical diagnoses were based on ELISA test and WHO criteria (2009) [40]. They were classified as DF (1), DHF grade I (6), DHF grade II (13), DHF grade III (2) and DSS (DHF grade IV) (1). According to 23 DENV-infected patients, there were 22 patients diagnosed as secondary DENV infections and 1 patient diagnosed as primary DENV infection according to the ELISA results (Appendix C) of the previous project by Laosakul *et al.* [30]. The summary of enrolled patients is presented in Table 20.

Table 20: The summary of both DENV and non-DENV infected patients in this study

No.	Code	Sex	Age (years old)	DOF	Clinical diagnosis	ELISA interpretation (type of infection)
				DEN	IV-infected patients	
1	N2	F	25	4	DHF II	secondary infection
2	N3	М	20	7	DHF II	secondary infection
3	N4	F	37	5	DHF II	secondary infection
4	N5	М	19	7	DHF II	secondary infection
5	N6	F	55	6	DHF I	secondary infection

No.	Code	Sex	Age	DOF	Clinical diagnosis	ELISA interpretation
No.	Code	Sex	(years old)		Clinical diagnosis	(type of infection)
6	N8	М	23	5	DHF I	secondary infection
7	N9	F	22	9	DHF II	secondary infection
8	N10	М	30	5	DHF II	secondary infection
9	N12	F	35	6	DHF II	secondary infection
10	N13	F	55	5	DHF I	secondary infection
11	NI7	F	56	7	DHF I	secondary infection
12	N20	М	26	8	DHF I	secondary infection
13	N21	М	22	7	DF	secondary infection
14	N22	М	24	7	DHF II	secondary infection
15	N23	М	19	8	DHF I	secondary infection
16	N24	F	18	6	DSS	secondary infection
17	N28	М	26	8	DHF II	secondary infection
18	N29	F	48	8	DHF II	secondary infection
19	N30	М	53	9	DHF III	secondary infection
29	N33	М	28	6	DHF III	secondary infection
21	N34	М	20	6	DHF II	primary infection
22	N35	F	25	5	DHF II	secondary infection
23	N40	F	28	4	DHF II	secondary infection
			·	Non-D	ENV-infected patients	
24	N16	М	35	7	Graves' disease	ND
25	N27	F	65	6	Unspecified viral infection	ND
26	N37	F	33	5	Influenza virus infection	ND
27	N39	М	23	9	Unspecified viral infection	ND
28	N43	F	41	15	Influenza virus infection	ND
DOE			M =1	Γ - 6	vale ND = not determined	

DOF = duration of fever. M = male, F = female. ND = not determined.

Clinical diagnosis is based on WHO criteria 2009.

Unspecified viral infection refers to the patients coming with suspected viral infection but clinical and laboratory diagnoses were not DENV infection.

The ratio of IgM: IgG is used to distinguished either primary or secondary infection. IgM: $IgG \ge 1.8$ is interpreted as primary DENV infection whereas the value < 1.8 is interpreted as secondary infection.

Plasma, PBMCs, saliva and urine were taken from both groups of patients during acute, early convalescent and late convalescent periods. Some patients lacked all or partial febrile specimen collections. Therefore, the first specimen collections

were in early convalescent period. In addition, some patients had more than one specimens collected during early or late convalescent period. However, the duration time between dates of these specimens was at least 7 days, enough to be included in this project. The total number of each specimen type in each time point (except for negative controls) is presented in Figure 12 and Appendix B.

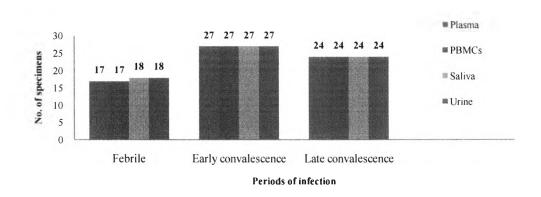


Figure 12: A number of specimens collected from 23 dengue-infected patients divided by each specimen type and period of infection (the result including double specimen collections).

Nested RT-PCR (E gene) result for DENV detection

Nested RT-PCR for E gene (partial sequence, 434 bp) was done in all specimens of 23 DENV infected patients to explore the longest day of DENV detection and generate the sequences for serotype and other molecular characterizations. Primers were taken from published article by Yenchitsomanus *et al.* and the PCR product spanned more than 95% of domain III of E gene reported as an important region for DENV pathogenesis [25, 56, 143].

All 23 DENV-infected patients were positive for nested RT-PCR (E gene primers). Dengue genome was presented in various specimen types and time points (Table 21, 22 and Figure 13). During the late convalescent period, only PBMCs and urine were positive by this method. The latest day of detectability was day 7 for plasma, day 8 for saliva, day 27 for PBMCs and day 46 for urine, respectively. Specimens from non-dengue-infected patients were all negative by nested RT-PCR using the E gene primers.

Table 21: Nested RT-PCR results of dengue and non-dengue infected patients in plasma, PBMCs, saliva and urine in different periods of infection (using E gene primers)

Code	Sex	DOF	Clinical	Days of	Plasma	PBMCs	Saliva	Urine
			Diagnosis	collection				
N2	F	4	DHF II	3	+	+	+	+
				21	-	+	-	-
				90	-	-	-	-
N3	М	7	DHF II	7	+	+	-	-
				22	-	-	-	-
				75	-	-	-	-
N4	F	5	DHF II	5	-	+	-	-
				21	-	-	-	_
N5	М	7	DHF II	6	+	+	-	-
				23	-	-	_	+
N6	F	6	DHF I	4	-	+	-	-
				23	-	-	_	_
				80	-	-	-	-
N8	М	5	DHF I	7	+	+	+	+
				25	-	-	_	-
N9	F	9	DHF II	9	_	+	-	-
				15	_	-	_	-
				30	_	-	_	_
				90	_	-	_	-
N10	M	5	DHF II	4	+	+	_	+
				27	_	+	_	_
				90	_	_	_	_
N12	F	6	DHF II	4	+	+	_	
				12	_	- -	_	+
				26	_	-	_	+
				75	_	-	_	_
N13	F		DHF I	8	_	+	_	+
		='	•	15	_	-		+
				29	_	-		_
				64	_	_		_
N17	F	7	DHF I	7	+	- +	+	+
	•	,	DIN 1	13		-		
				33		-	_	+
					•	<u>-</u>	-	

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N20	М	8	DHF I	6	ND	ND	-	+
				30	-	-	-	+
N21	М	7	DF	7	-	+	-	-
				14	-	-	-	+
				24	-	-	-	-
N22	М	7	DHF II	4	-	+	-	-
				24	-	-	- 1	-
N23	М	8	DHF I	7	+	-	+	+
				17	-	-	-	-
				45	-	-	-	-
N24	F	6	DSS	3	+	+	+	+
				13	-	-	-	-
				32	-	-	-	-
				49	-	-	-	-
N28	М	8	DHF II	6	+	+	-	-
				14	-	-	-	+
				46	-	-	-	+
				90	-	-	-	-
N29	F	8	DHF II	5	+	+	-	+
				19	-	-	-	+
				33	-	-	-	-
N30	M	9	DHF III	7	+	+	-	+
				20	-	-	-	-
				38	-	-	-	-
N33	М	6	DHF III	7	+	+	+	+
				18	-	-	-	+
				31	-	-	-	-
N34	М	6	DHF II	8	-	+	+	+
				14	-	-	-	+
				28	-	-	-	-
N35	F	5	DHF II	7	-	-	-	-
				13	-	-	-	+
				27	-	-	-	-
N40	F	4	DHF II	4	+	+	-	+
				21	-	+	-	-

Code	Sex	DOF	Clinical	Days of	Plasma	PBMCs	Saliva	Urine
Code	Sex	DOF	Diagnosis	collection	Piasma	PDIVICS	Saliva	Offile
N40	F	4	DHF II	71	-	-	-	-
N16	М	7	Graves'	6	-	-	-	-
			disease	13	-	-	-	-
				34	-	-	-	-
				64	-	-	-	-
N27	F	6	Unspecified	5	-	-	-	-
			viral	14	-	-	-	-
		I	infection	54	-	-	-	-
N37	F	5	Influenza	4	-	-	-	-
			virus	28	-	-	-	-
			infection					
N39	М	9	Unspecified	7	-	-	-	-
			viral	15	-	-	-	-
			infection	33	-	-	-	-
N43	F	15	Influenza	4	-	-	-	-
1			virus	23	-	-	-	-
			infection	33	-	-	-	-

^{+ =} positive, - = negative and ND = not determined. M = male and F= female.

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

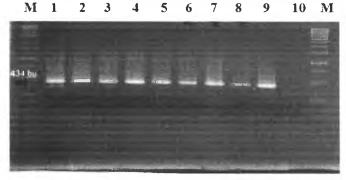


Figure 13: 1.5% agarose gel electrophoresis of nested RT-PCR product using E gene primers in specimens collected from dengue-infected patients in different time points. The expected product is 434 bp (shown in red arrow). M= 1 kb DNA marker, 1 = N5 PBMCs, 2 = N5/2 urine, 3 = N13 PBMCs, 4 = N13 urine, 5 = N13/2 urine, 6 = N12 PBMCs, 7 = N12/2 urine, 8 = N12/3 urine, 9 = positive control DENV4 and 10 = negative control (no template added).

Table 22: The summary of nested RT-PCR results (E gene primers) of plasma, PBMCs, saliva and urine collected from each patient at different time points. The results were divided by each specimen and time point.

	Plasma	PBMCs	Saliva	Urine
Febrile period	12/17	16/17	4/18	9/18
	(70.59%)	(94.12)	(22.22%)	(50.00%)
Early	2/27	5/27	3/27	15/27
convalescence	(7.41%)	(18.52%)	(11.11%)	(55.55%)
Late	0/24	1/24	0/24	3/24
convalescence	(0.00%)	(4.16%)	(0.00%)	(12.50%)

Dengue serotypes, genotypes and strains were analyzed in all 23 patients but only 13 patients (13/23, 56.52%) with positive nested RT-PCR (in any specimen type) for at least 2 time points were continuously studied for sequential genetic variations.

Serotype classification in DENV-infected patients

To classify the serotype of DENV in all dengue-infected patients, semi-nested or nested RT-PCR using the primers of Lanciotti *el al.* and Yenchitsomanus *et al.* was used [56, 58]. Moreover, direct sequencing of partial E gene was also done to classify serotype of DENV in each patient by blasting with GenBank.

The result showed that 20 patients (20/23, 86.96%) were infected with single DENV serotype infections and 3 patients were infected with multi or mixed DENV serotypes. For single serotypic infection, more patients were infected with DENV2 (11/20, 55.00%) than with DENV1 (6/20, 30.00%) or DENV3 (3/20, 15.00%). No patient was infected with DENV4. Concurrent multi-serotype infections were found in 3 patients - N33, N34 and N40 (3/23, 13.04%) with DENV2+DENV4, DENV1+DENV3 and DENV1+DENV2, respectively (Figure 14 and Table 23). The mixed infections in 3 patients were found in different specimens and time points. In N33 patient, 1st early convalescent plasma, PBMCs, saliva and 2nd convalescent urine were DENV4 while 1st convalescent urine was DENV2 + DENV4. In N34 patient, 1st early convalescent PBMCs, saliva and urine of N40 patient urine was DENV1 + DENV3. The febrile plasma, PBMCs and urine of N40 patient were DENV1 whereas early convalescent PBMCs was DENV1+DENV2 (Figure 15).

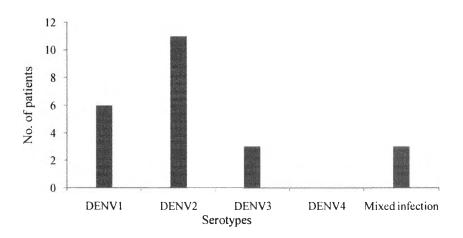


Figure 14: Serotype classification of 23 DENV-infected patients.

Table 23: DENV serotype results of 23 DENV-infected patients

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N2	F	4	DHF II	3	D2	D2	D2	D2
				21	-	D2	-	-
				90	-	-	-	-
N3	М	7	DHF II	7	D2	D2	D2	-
				22	-	-	-	-
				75	-	-	-	-
N4	F	5	DHF II	5	-	D2	-	-
				21	-	-	-	-
N5	М	7	DHF II	6	D2	D2	-	-
				23	-	-	-	D2
N6	F	6	DHF I	4	-	D2	-	-
				23	-	-	-	-
				80	-	-	-	-
N8	M	5	DHF I	7	D2	D2	D2	D2
				25	-	-	- 1	-
N9	F	9	DHF II	9	-	Di	-	-
				15	-	DI	-	-
				30	-	-	-	-
				90	-	-	-	-

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N10	M	5	DHF II	4	D2	D2	D2	D2
				27	_	D2	_	-
				90	_	-	-	-
N12	F	6	DHF II	4	DI	DI	-	-
				12	- i	-	-	DI
				26	-	-	-	DI
				75	-	-	-	-
NI3	F	5	DHF I	8	-	D2	-	D2
				15	-	-	-	D2
				29	-	-	-	-
				64	-	-	-	-
NI7	F	7	DHF I	7	D2	D2	D2	D2
				13	-	-	-	D2
				33	-	-	-	-
N20	М	8	DHF I	6	ND	ND	-	DI
				30	-	-	-	DI
N21	M	7	DF	7	-	D2	-	-
				14	-	-	-	D2
				24	-	-	-	-
N22	M	7	DHF II	4	-	D2	-	-
				24	-	-	-	-
N23	M	8	DHF I	7	D3	-	D3	D3
				17	-	-	-	-
				45	-	-	-	-
N24	F	6	DSS	3	D3	D3	D3	D3
				13	-	-	-	-
				32	-	-	-	-
				49	-		-	-
N28	M	8	DHF II	6	D3	D3	-	-
				14	-	-	-	D3
				46	-	-	-	D3
2100				90	-	-	-	-
N29	F	8	DHF II	5	DI	DI	-	DI
				19	-	-	-	DI
					-	<u>-</u>	-	

Code	Sex	DOF	Clinical	Days of	Plasma	PBMCs	Saliva	Urine
Couc	Sex	DOI	Diagnosis	collection	Tiasilia	1 DIVICS	Saliva	Office
N30	M	9	DHF III	7	DI	DI	-	DI
				20	-	-	-	-
				38	-	-	-	-
N33	M	6	DHF III	7	D4	D4	D4	D4+D2
				18	-	-	-	D4
			:	31	-	-	-	-
N34	M	6	DHF II	8	-	DI	DI	DI
				14	-	-	-	D1+D3
				28	-	-	-	-
N35	F	5	DHF II	7	-	-	-	-
				13	-	-	-	DI
}				27	-	-	-	-
N40	F	4	DHF II	4	DI	DI	-	D1
				21	-	D2+D1	-	-
				71	-	-	-	-

D1= DENV1; D2= DENV2; D3= DENV3; D4= DENV4. DOF= duration of fever.

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

All non-DENV infected specimens were negative for all RT-PCR for serotype classification (data not shown).

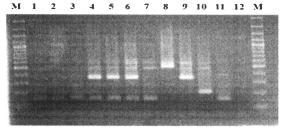


Figure 15: Serotype nested RT-PCR results using Yenchitsomanus protocol in N33 specimens. Lane 1 is N33 plasma. Lane 2 is N33 PBMCs, Lane 3 is N33 saliva. Lanes 4 & 5 are N33 urine. Lane 6 is new extraction of N33 urine. Lane 7 is N33/2 (day18 of illness) urine. Positive controls (DENV1-DENV4) are presented in lane 8-11, respectively. Lane 12 is negative control (DW). M = 100 bp (plus) DNA marker.

To find out which serotype represented major clones in each multi-serotype or mixed-serotype specimen, PCR products (E gene) from those specimens in 3 mixed

[&]quot;-"= negative and ND = not determined. M = male and F= female.

serotype infected patients were taken to do cloning and sequencing techniques. Ten to fifteen colonies from each experiment were picked for sequencing. Clones representing each serotype in each specimen were counted. The results revealed that all single-serotype-infected specimens in these 3 patients showed uniform and identical serotype in all relevant clones. Three mixed serotype infected specimens (1st early convalescent urine of N34 and early convalescent PBMCs of N40) presented the heterogeneous clones containing 2 serotypes. The major serotype in 1st early convalescent urine (N33) was DENV4. In 2nd convalescent urine of N34, the major serotype was DENV3 and the major serotype of early convalescent PBMCs of N40 was DENV2 (Table 24).

Table 24: The major and minor serotypes of DENV in 3 mixed-serotype-infected patients

	_		,	,		
Code	DOF	Specimens	No. of clones	Serotypes	No. of major clones (serotype)	No. of minor clones (serotype)
N33	6	plasma (7)	14	4	14 (DENV4)	0
		PBMCs (7)	11	4	11(DENV4)	0
		saliva (7)	12	4	12 (DENV4)	0
		urine (7)	15	4+2	11 (DENV4)	4 (DENV2)
		urine (18)	13	4	13 (DENV4)	0
N34	6	PBMCs (8)	14	1	14(DENVI)	0
		saliva (8)	13	1	13 (DENVI)	0
		urine (8)	13	1	13 (DENV1)	0
		urine (14)	16	1+3	14 (DENV3)	2 (DENV1)
N40	4	plasma (4)	12	1	12 (DENV1)	0
		PBMCs (4)	15	1	15 (DENV1)	0
		urine (4)	12	1	12 (DENV1)	0
		PBMCs (21)	13	1+2	12 (DENV2)	I (DENVI)
	1		1	I		I

DOF = duration of fever. The number in "()" represents the day of specimen collections.

Genotype and strain classifications of DENV in DENV-infected patients

E gene sequences (388 bp except primers) from positive specimens of 23 patients were taken to explore genotypes and strains by blasting each sequence with the Dengue database in the Viral Bioinformatics Resource Center (VBRC) and GenBank, respectively. In mixed-serotype-infected specimens, both major and minor serotypes were also investigated.

The genotype of all DENV1-infected patients were classified in genotype I. DENV2-infected patients were clustered into 2 different genotypes, Asian I (all patients, excluding N13) and cosmopolitan (N13 patient) whereas all DENV3-infected patients were classified in genotype II. DENV4 in mixed-serotype-infected patient was classified in genotype I (Table 25 and Figure 16). No mixed genotype infection was found in all single-serotype-infected patients.

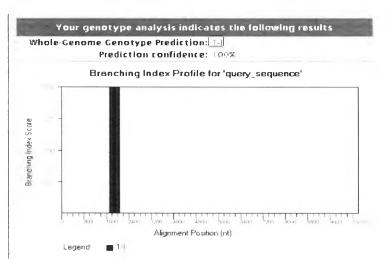


Figure 16: The example of genotype blast result of N12 specimens during febrile (N12 plasma and PBMCs), early convalescent (N12/2 urine) and late convalescent (N12/2 urine) periods. All specimens gave the same results of genotype I as marked in the red box. The result "1-I" means "serotype I genotype I".

In this study, there were 6 strains of DENV1 (ThD1_K0107_98, 00132/09, D1.Myanmar.059/01, 02128/07, 16007 and ThD1_0049_01), 6 strains of DENV2 (D2/Thailand/0606aTw, 16681, ThD2_0981_00, D2/Cambodia/0708aTw, D2/Singapore/0806aTw and D2/Vietnam/0804bTw), 3 strains of DENV3

(D3/Myanmar/0810aTw, Thai0308a/Tw and D3/Myanmar/0707aTw) and 1 strain of DENV4 (TN2511) circulating in our patients. The single strain infection was found in all patients with single serotype infection, excluding 2 patients (N2 and N10) presenting mixed strains of DENV2 infection. Moreover, multi strain infections of DENV1 were found in mixed-serotype-infected specimen of N34 and N40. There were strains ThD1_0049_01 and 16007 in N34 patient while strains 02128/07 and ThD1_K0107_98 were found in N40 patient (Table 25 and Figure 17).

Accession	Distribution	Man score	Total score	COVERNO	- value	<u>Han</u>	Links
c046415.1	Dengue virus 2 strain D2/Cambodia/0708aTw envelope protein TEI ge	-118	796	100%	0.0	39%	Pillia Lauren-Laur III
48837981	Dengue virus 2 envigerie for envelope protein i partial cds, isolate: RE	1(1	701	100%	9.0	35%	
ABT07980.1	Dengue virus 2 envigene for envelope protein, partial cds, isolate: RC	<u>701</u>	701	100%	0.0	99%	
#STAKE	: Dengua virus 2 eriv gene for envelope protein, partial cds, isolate: RC	<u>701</u>	701	400%	0.0	99%	
48533476.1	Dengue virus 2 envigene for envelope protein, partial cds, isolate: RC	701	701	100%	9.0	39%	
AR93/971.1	Dengue virus 2 envigene for envelope protein, partial ods, isplatet RL	<u>"01</u>	7(1	100%	(:.0	13%	
ABT37966.1	:Dengue virus 2 envigene for envelope protein, partial cds, isclate: RC	701	701	100%	0.0	99%	
ABE87965/1	Dengue virus 2 envigene for envelope protein, partial cds. isolate: RC	<u>:(·1</u>	701	100%	0.0	33%	
AEE97964 1	Dengue virus 2 envigene for envelope protein, partial cds, isolate: RC	701	701	100%	9.0	33%	
A85409h01	Dengua virus 2 envigene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	39%	
ARES0945.1	Dengue virus 2 envigene for envelope protein, partial cds. isolate: RC	201	791	100%	0.0	29%	
489800411	Dangue virus 2 envigene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
182971	Dengue virus 2 envigene for envelope protein, partial cds, isciate: RC	201	701	100%	3.0	39%	
465314421	Dengue virus 2 envigene for envelope protein, partial ods, isciare: PC	761	791	100%	9.0	33%	
4899774111	Dengue virus 2 envigene for envelope protein, partial cds, isolate: RC	701	701	100%	0,0	99%	
ABT07340.1	-Derique virus 2 envigene for envelope protein, partial cds, isplate: RC	701	701	100%	9.0	99%	

Figure 17: The example of DENV strain analysis of N17 specimens during febrile (N17 plasma, PBMCs, saliva and urine) and early convalescent (N17/2 urine) periods. All specimens presented the same DENV strains. The blast result is noted in the red box.

Table 25: Summary results of serotypes, genotypes and strains of DENV in 23 DENV-infected patients

Code	Sex	Clinical diagnosis	Serotypes	Genotypes	Similar strains
N2	F	DHF II	2	Asian I	ThD2_0981_00
		!			D2/Vietnam/0804bTw *
N3	М	DHF II	2	Asian I	ThD2_0981_00
N4	F	DHF II	2	Asian I	ThD2_0981_00
N5	M	DHF II	2	Asian I	ThD2_0981_00
N6	F	DHF I	2	Asian I	ThD2_0981_00
N8	М	DHF I	2	Asian I	ThD2_0981_00
N9	F	DHF II	l	I	ThD1_K0107_98

Code	Sex	Clinical diagnosis	Serotypes	Genotypes	Similar strains
NI0	M	DHF II	2	Asian I	ThD2_0981_00
					D2/Thailand/0606aTw*
NI2	F	DHF II	1	I	ThD1_K0107_98
N13	F	DHF I	2	Cosmopolitan	D2/Singapore/0806aTw
N17	F	DHF I	2	Asian I	D2/Cambodia/0708aTw
N20	М	DHF I	ı	1	00132/09
N21	М	DF	2	Asian I	ThD2_0981_00
N22	М	DHF II	ı	I	D1.Myanmar.059/01
N23	М	DHF I	3	II	D3/Myanmar/0810aTw
N24	F	DSS	3	П	Thai0308a/Tw
N28	М	DHF II	3	II	D3/Myanmar/0707aTw
N29	F	DHF II	ı	I	02128/07
N30	М	DHF III	1	I	02128/07
N33	М	DHF III	4 (major)	I	TN2511
			2 (minor)	Asian I	16681
N34	М	DHF II	1 (major)	I	ThD1_0049_01
			3 (major) ^s	II	ThD3_0140_84
			l (minor) ^{\$}	I	16007
N35	F	DHF II	1	I	02128/07
N40	F	DHF II	l (major)	I	02128/07
			2 (major)#	Asian I	D2/Thailand/0606aTw
	15		l (major)#	I	ThD1_K0107_98

M = male and F = female. * = different strains during convalescent period.

Major = major serotype in each specimen and the sequence was retrieved from direct sequencing. Minor = minor serotype in each specimen and the sequence was retrieved after doing cloning and sequencing techniques.

The consensus sequence of minor serotype is generated from overall sequences of selected colonies on the plate of mixed-serotype-contained specimen.

To explore serotypes, genotypes and strains of DENV in different time points of each patient, only 13 DENV-infected patients positive for nested RT-PCR (E gene) at least 2 time points were investigated. The results presented that serotypes, genotypes and strains of single-serotype-infected patients in different specimens and

⁵N34 urine (14) shows DENV3 as a major serotype and DENV1 as a minor serotype.

[&]quot;N40 PBMCs (21) shows DENV2 as a major serotype and DENV1 as a minor serotype.

time points were the same except 2 patients (N2 and N10). In N2 and N10 patients, serotypes and genotypes in different specimens and time points were identical but the difference of DENV strains were found during early and convalescent PBMCs in both 2 patients (Table 26). In 3 mixed-serotype-infected patients, different serotypes, genotypes and strains were definitely found. Moreover, mixed strains of DENV1 were found in N34 and N40 patients although the genotype of 2 patients was the identical.

Table 26: Serotypes, genotypes and strains of DENV in 13 prolonged dengue-infected patients

Code	Specimens	Serotype	genotype	Similar strain
N2	plasma (3)	2	Asian I	ThD2_0981_00
DOF=4	PBMCs (3)	2	Asian I	ThD2_0981_00
	saliva (3)	2	Asian I	ThD2_0981_00
	urine (3)	2	Asian I	ThD2_0981_00
	PBMCs (21)	2	Asian I	D2/Vietnam/0804bTw
N5	plasma (6)	2	Asian I	ThD2_0981_00
DOF=7	PBMCs (6)	2	Asian I	ThD2_0981_00
	urine (23)	2	Asian I	ThD2_0981_00
N10	plasma (4)	2	Asian I	ThD2_0981_00
DOF=5	PBMCs (4)	2	Asian I	ThD2_0981_00
	urine (4)	2	Asian I	ThD2_0981_00
	PBMCs (27)	2	Asian I	D2/Thailand/0606aTw
N12	plasma (4)	1	I	ThD1_K0107_98
DOF=6	PBMCs (4)	1	I	ThD1_K0107_98
	urine (12)	ı	I	ThD1_K0107_98
	urine (26)	1	I	ThD1_K0107_98
N13	PBMCs (8)	2	Cosmopolitan	D2/Singapore/0806aTw
DOF=5	urine (8)	2	Cosmopolitan	D2/Singapore/0806aTw
	urine (15)	2	Cosmopolitan	D2/Singapore/0806aTw
NI7	plasma (7)	2	Asian I	D2/Cambodia/0708aTw
DOF=7	PBMCs (7)	2	Asian I	D2/Cambodia/0708aTw
	saliva (7)	2	Asian I	D2/Cambodia/0708aTw
	urine (7)	2	Asian I	D2/Cambodia/0708aTw
	urine (13)	2	Asian I	D2/Cambodia/0708aTw
N20	urine (6)	1	I	00132/09
DOF=8	urine (30)	1	I	00132/09

Code	Specimens	Serotype	genotype	Similar strain
N21	PBMCs (7)	2	Asian I	ThD2_0981_00
DOF=7	urine (14)	2	Asian I	ThD2_0981_00
N28	plasma (6)	3	11	D3/Myanmar/0707aTw
DOF=8	PBMCs (6)	3	11	D3/Myanmar/0707aTw
	urine (14)	3	П	D3/Myanmar/0707aTw
	urine (46)	3	II	D3/Myanmar/0707aTw
N29	plasma (5)	i	I	02128/07
DOF=8	PBMCs (5)	1	I	02128/07
	urine (5)	1	I	02128/07
	urine (19)	1	I	02128/07
N33	plasma (7)	4	1	TN2511
DOF=6	PBMCs (7)	4	I	TN2511
	saliva (7)	4	1	TN2511
	urine (7)	4	Ī	TN2511
	urine (18)	4 (major)	Ī	TN2511
		2 (minor)	Asian I	16681
N34	PBMCs (8)	1	Ī	ThD1_0049_01
DOF=6	saliva (8)	1	Ī	ThD1_0049_01
	urine (8)	1	Ī	ThD1_0049_01
	urine (14)	3 (major)	II	ThD3_0140_84
		l (minor)	Ī	16007
N40	plasma (4)	1	I	02128/07
DOF=4	PBMCs (4)	1	Ī	02128/07
	urine (4)	1	I	02128/07
	PBMCs (21)	2 (major)	Asian I	D2/Thailand/0606aTw
		l (minor)	ī	ThD1_K0107_98
Definition		l (minor)	1	

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

DOF = duration of fever. The number in "()" indicates the day of specimen collections.

Major = a major serotype in each specimen and the sequence was retrieved from direct sequencing.

Minor = a minor serotype in each specimen and the sequence was retrieved after doing cloning and sequencing techniques.

DENV detection and viral load in different time points of infection

Real time SYBR Green I RT-PCR (qRT-PCR) was used to detect, monitor and explore the viral load of DENV in plasma, PBMCs, saliva and urine in different time points of 23 patients. The protocol and primers were adapted from the previous study of dos Santos *et al.* [61]. Primers were designed to detect all 4 serotypes of DENV at 5' UTR region. Stock DENV of 4 serotypes were firstly used as positive controls and for construction the standard curve for viral quantification. All positive controls were firstly done to validate the limit of detection and melting temperature (Tm) for the expected PCR product. The results showed that melting temperature of expected PCR products of 4 serotypes was in the range of 80.70°C - 81.86 °C (Figure 18). The limits of detection in this study were 5 x 10⁻³ PFU/ml (DENV1), 4.75 x 10⁻² PFU/ml (DENV2), 2.75 x 10⁻³ PFU/ml (DENV3) and 2.5 x 10⁻³ PFU/ml (DENV4), respectively (data not shown).

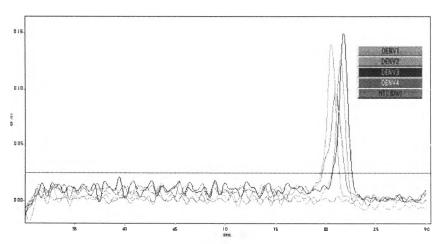
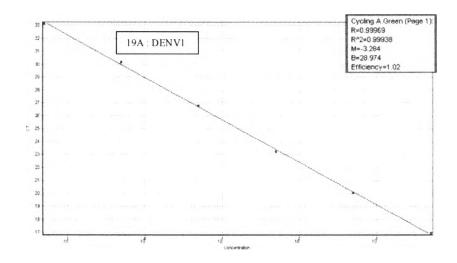
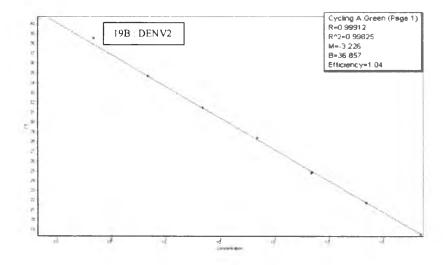
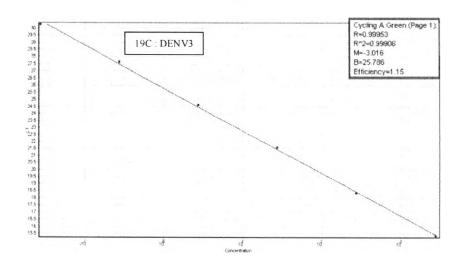


Figure 18: The melting curve analysis of positive controls (DENV1-DENV4).

To construct the standard curve for determining dengue viral load in clinical specimens, stock DENV1 was diluted from 5 x 10^3 PFU/ml to 5 x 10^{-2} PFU/ml, DENV2 from 4.75 x 10^5 PFU/ml to 4.75 x 10^{-2} PFU/ml, DENV3 from 2.75 x 10^3 PFU/ml to 2.75 x 10^{-2} PFU/ml and DENV4 from 2.5 x 10^4 PFU/ml to 2.5 x 10^{-3} PFU/ml. The standard curve of all 4 serotypes is presented in Figure 19.







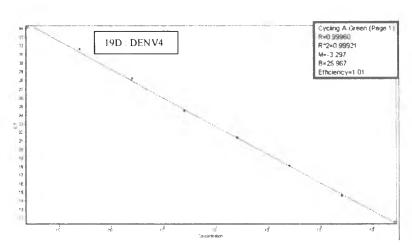


Figure 19: The standard curve of all 4 serotypes using DENV stocks (Figure 19A-19D).

The qRT-PCR was continuously used (after validation of the assay) to detect and quantification of DENV in plasma, PBMCs, saliva and urine of all patients during febrile, early convalescent and late convalescent periods. Starting templates for standard viral concentration and specimens were equal as 4 µl per reaction (25 µl). Results were presented in a cycle threshold (Ct), a melting curve analysis (Tm) and a viral load (PFU/ml). If the Ct of some specimens were out of the length of standard curve, viral RNA was either diluted and repeated or reported as "less than the last point of standard concentration" (PFU/ml). In mixed serotype infections, standard curve was based on the major serotype of infected specimens.

DENV genome could be detected in all DENV-infected patients. The positive results varied in each specimen and time point similar to nested RT-PCR results (Table 27. Figure 20 and 21). Plasma, PBMCs, saliva or urine was positive during febrile and early convalescent periods. However, the positive results were only found in PBMCs and urine during late convalescent period (Table 28). All non-dengue-infected patients (negative control group) were completely negative for qRT-PCR.

After determining the longest time of DENV detection by qRT-PCR, the result showed that 17 of 23 (73.92%) DENV-infected patients were positive for DENV detection at least 2 times points of specimen collections. DENV genome could be

detected as late as day 8 of illness in saliva, day 21 of illness in PBMCs, day 22 of illness in plasma and day 46 of illness in urine, respectively.

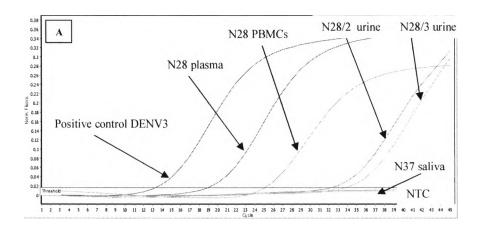
Table 27: qRT-PCR results of 23 DENV-infected patients

Code	Sex	DOF	Clinical	Day of	Plasma	PBMCs	Saliva	Urine
			Diagnosis	collection	17437714	l Birtes	Surrva	
N2	F	4	DHF II	3	+	+	+	+
				21	-	+	-	-
				90	-	-	-	-
N3	М	7	DHF II	7	+	+	+	+
				22	+	-	-	+
				75	-	-	-	-
N4	F	5	DHF II	5	+	+	+	+
				21	-	-	-	-
N5	М	7	DHF II	6	+	+	+	+
				23	-	-	-	+
N6	F	6	DHF I	4	+	+	-	-
				23	-	-	-	-
				80	-	-	_	-
N8	М	5	DHF I	7	+	+	+	+
				25	-	-	-	+
N9	F	9	DHF II	9	-	+	-	-
				15	-	+	-	-
				30	-	-	-	-
				90	-	-	-	-
N10	М	5	DHF II	4	+	+	+	+
				27	-	-	-	-
				90	-	-	-	-
N12	F	6	DHF II	4	+	+	-	+
				12	-	-	-	+
		ļ		26	-	-	-	+
				75	-	-	-	-
NI3	F	5	DHF I	8	-	+	-	+
				15	-	-	-	+
				29	-	-	-	-
				64	-	-	-	-
N17	F	7	DHF I	7	+	+	+	+

Code	Sex	DOF	Clinical Diagnosis	Day of collection	Plasma	PBMCs	Saliva	Urine
N17	F	7	DHF I	13	-	-	-	+
	,			33	-	-	_	-
N20	M	8	DHF I	6	ND	ND	+	+
				30	-	-	-	+
N21	М	7	DF	7	-	+	-	-
	}			14	-	-	-	+
				24	-	-	-	-
N22	M	7	DHF II	4	+	+	+	+
				24	-	-	-	+
N23	М	8	DHF I	7	+	-	-	+
				17	-	-	_	-
				45	-	-	-	-
N24	F	6	DSS	3	+	+	+	+
				13	-	-	-	-
				32	-	-	-	-
				49	-	-	-	-
N28	М	8	DHF II	6	+	+	-	-
				14	-	-	-	+
				46	-	-	-	+
				90	-	-	-	-
N29	F	8	DHF II	5	+	+	-	+
				19	-	-	-	+
				33	-	-	-	+
N30	M	9	DHF III	7	+	+	-	+
				20	-	-	-	-
				38	-		-	-
N33	M	6	DHF III	7	+	+	+	+
				18	+	-	-	+
				31	-		-	-
N34	M	6	DHF II	8	+	+	+	+
				14	-	-	-	+
				28	-	-		-
N35	F	5	DHF II	7	+	+	+	+
				13	-	-	-	-
				27	-		-	-

C- 1-	C	DOF	Clinical	Day of	Diamon	DD14C	Calian	Urine
Code	Sex	DOF	Diagnosis	collection	Plasma	PBMCs	Saliva	Urine
N40	F	4	DHF II	4	+	+	-	+
				21	-	+	-	-
				71	-	-	-	-
N16	М	7	Graves'	6	-	-	-	-
			disease	13	-	-	-	-
				34	-	-	-	-
				64	-	-	-	-
N27	F	6	Unspecified	5	-	-	-	-
			viral	14	-	-		-
			infection	54	-	-	-	-
N37	F	5	Influenza	4	-	-	-	-
			virus	28	-	-	-	-
			infection					
N39	М	9	Unspecified	7	-	-	-	-
			viral	15	-	-	-	-
			infection	33	-	-	-	-
N43	F	15	Influenza	4	-	-	-	-
			virus	23	-	-	-	-
			infection	33	-	-	-	-

M = male and F= female. += positive, -= negative and ND = not determined. DOF = duration of fever Definitions: febrile period (duration of fever), early convalescence (first day of fever recovery until day 25 of illness) and late convalescence (day 26 - day 90 of illness).



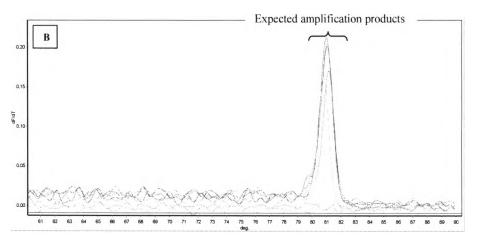


Figure 20: The amplification plot (A) and the melting curve analysis (B) of each qRT-PCR result derived from positive results of N28 specimens (N28 plasma, N28 PBMCs, N28/2 urine and N28/3 urine). The results were compared with positive control (DENV3) and negative control (N37 saliva: non dengue-infected patient). NTC= no template control.

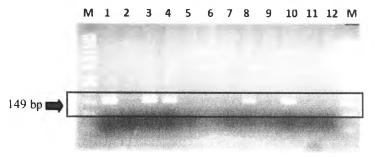


Figure 21: 2% gel electrophoresis of qRT-PCR products of N28 specimens. The expected band is noted as the red box. M= 50 bp DNA marker, I= positive control DENV3 (2.75 x 10⁴ PFU/ml), 2= positive control DENV3 (2.75 x 10⁻³ PFU/ml), 3= N28 plasma, 4= N28 PBMCs, 5= N28 urine, 6= N28 saliva, 7= N28/2 PBMCs, 8= N28/2 urine, 9= N28/3 plasma, 10= N28/3 urine, 11= NTC (no template control) and 12 = N37 saliva (negative control, non dengue-infected patient).

PBMCs Plasma Saliva Urine 15/17 Febrile period 9/18 16/17 14/18 (50.00%) (88.23%)(94.12%)(77.78%)7/27 8/27 4/27 19/27 Early (14.81%)convalescence (25.92%)(29.63%)(70.37%)0/24 0/24 0/24 4/24 Late convalescence (0.00%)(0.00%)(0.00%)(16.67%)

Table 28: qRT-PCR results of DENV-infected patients (The results are presented in each specimen and time point)

The viral load of each patient varied in different specimens and time points (Table 29 and Figure 22). In some specimens, the viral load was reported "less than the last standard point of standard concentration" such as < 0.05 PFU/ml in febrile PBMCs of N12 (DENV1) because the Ct was out of the last point of DENV1 standard curve (0.05 PFU/ml). During febrile period, dengue viral load in plasma or PBMCs was mostly higher than in saliva and urine, except 2 patients showing the high dengue viral load in urine (N4, N17). The high viral load was found in urine instead of plasma and PBMCs in early convalescent urine of some patient (N12, N13, and N35). In late convalescent period, DENV was detected in urine only and the viral load was lower than all specimens during febrile and early convalescent periods. In two patients (N9 and N23), the different viral load in all specimens could not be investigated because the Ct values were out of the range of standard curve. Therefore, the comparison of viral load among different time points was not explored.

The viral load in different specimens and time points of each patient depended on the day of specimen collections (Table 29). If the first specimens were collected during febrile period, viral load was higher in plasma or PBMCs than in saliva and urine as well as than in all convalescent specimens such as in N2 patient (Figure 22A). In addition, when all convalescent specimens were compared such as in N13 and N35, the high viral load was mostly found in the first urine during that period. Moreover, although the high viral load was found in plasma or PBMCs during febrile period, the viral load in convalescent specimen of some patients was higher than all febrile specimens such as in N12 patient. The high viral load in convalescent specimen was stable in a short period of time, then the viral load decreased until

undetectable when monitoring for a long period (Figure 22B). In other cases, if the first specimens were collected at the lasted day of fever or early convalescent period, the viral load was higher in urine than in all specimens during the first collection. Subsequently, the viral load in secondly collected specimens during convalescent period became decreased and lower than first specimen collections such as in N17 patient (Figure 22C). The comparison of viral load in N9 patient between 2 time points could not be investigated because the values were out of the range of standard curve. Nevertheless, if the results were monitored by the Ct values, the viral load early convalescent PBMCs was higher than in febrile PBMCs.

Table 29: The demonstration of dengue viral load in different specimens and time points (All specimens were started at the same volume of 4 μ l RNA)

Codo	DOF	Positive specimen	C	Tm	Ch	Viral load
Code	DOF	(day of collection)	Serotype	(°C)	Ct	(PFU/ml)
N2	4	plasma (3)	2	79.90	21.99	46,200
		PBMCs (3)	2	79.90	24.53	7,310
		saliva (3)	2	80.10	31.50	45.70
		urine (3)	2	80.06	36.82	0.93
		plasma (21)	ND	80.10	38.65	0.24
		PBMCs (21)	2	80.30	40.52	0.06
		urine (21)	ND	80.70	38.80	0.21
N3	7	plasma (7)	2	79.94	34.64	1.07
		PBMCs (7)	2	79.94	37.06	0.216
		saliva (7)	2	80.00	36.52	0.311
		urine (7)	ND	79.86	37.41	0.17
		plasma (22)	ND	79.90	39.08	0.0555
		urine (22)	ND	79.86	36.20	0.386
N4	5	plasma (5)	ND	79.84	37.07	0.215
		PBMCs (5)	2	79.86	36.20	1.91
		saliva (5)	ND	80.14	36.94	0.235
		urine (5)	ND	79.96	36.63	0.288
N5	7	plasma (6)	2	79.90	21.99	2.64
		PBMCs (6)	2	79.92	29.37	22.60

Code	DOF	Positive specimen	Saratuma	Tm	Ct	Viral load
Code	DOF	(day of collection)	Serotype	(°C)	Ct	(PFU/ml)
N5	7	saliva (6)	ND	80.37	35.00	0.62
		urine (6)	ND	80.30	37.13	0.16
		urine (23)	2	80.62	26.85	113.50
N6	6	plasma (4)	2	80.20	35.83	0.496
		PBMCs (4)	2	80.34	34.27	1.41
N8	5	plasma (7)	ND	80.04	28.25	1.80
		PBMCs (7)	2	79.90	28.34	1.69
		saliva (7)	ND	79.80	28.69	1.30
		urine (7)	ND	80.00	22.47	122
		urine (25)	ND	80.20	32.53	0.0795
N9	9	PBMCs (9)	1	80.40	35.58	< 0.05
		PBMCs (15)	1	80.20	32.53	< 0.05
N10	5	plasma (4)	2	80.00	8.10	2,130,000
		PBMCs (4)	2	79.98	16.04	34,900
		saliva (4)	ND	80.47	30.15	23.45
		urine (4)	2	80.25	28.30	61.05
N12	6	plasma (4)	1	80.26	29.37	0.052
		PBMCs (4)	1	80.20	31.15	< 0.05
		urine (4)	ND	80.10	34.19	< 0.05
		urine (12)	1	80.30	22.16	7.11
		urine (26)	1	80.56	27.80	0.152
N13	5	PBMCs (8)	2	78.88	34.82	1.71
		urine (8)	2	78.88	28.37	95.85
		urine (15)	2	79.55	35.90	0.86
N17	7	plasma (7)	2	80.25	40.79	15.50
					(diluted 1:10)	
		PBMCs (7)	2	79.92	34.77	38.75
		saliva (7)	2	80.02	37.81	7.64
		urine (7)	2	80.02	28.37	1,160
		urine (13)	2	80.47	30.15	449
N20	8	saliva (6)	ND	81.14	29.35	<0.05
		urine (6)	1	80.30	26.35	0.301
		urine (30)	1	80.44	29.97	< 0.05
N21	7	PBMCs (7)	2	80.54	32.13	0.342
		urine (14)	2	80.84	25.05	42.80

	DOE	Positive specimen	G .	Tm	Ci	Viral load
Code	DOF	(day of collection)	Serotype	(°C)	Ct	(PFU/ml)
N22	7	plasma (4)	ND	80.84	30.65	0.313
		PBMCs (4)	1	80.86	28.37	1.65
		saliva (4)	ND	80.60	27.71	2.68
		urine (4)	ND	80.94	31.62	0.155
		urine (24)	ND	80.64	30.29	0.405
N23	8	plasma (7)	3	81.00	39.53	< 0.0275
		urine (7)	3	80.80	39.16	< 0.0275
N24	6	plasma (3)	3	80.76	22.24	786
					(diluted 1:2)	
		PBMCs (3)	3	80.66	22.74	134
					(diluted 1:2)	
ĺ		saliva (3)	3	80.94	36.51	< 0.0275
		urine (3)	3	80.46	43.67	< 0.0275
N28	8	plasma (6)	3	81.00	18.97	267
		PBMCs (6)	3	81.04	25.10	2.41
		urine (14)	3	81.16	32.28	< 0.0275
		urine (46)	3	80.80	33.78	< 0.0275
N29	8	plasma (5)	1	80.70	18.18	3,580
					(diluted 1:2)	
		PBMCs (5)	1	80.80	21.44	197
		urine (5)	1	80.80	31.30	0.248
		urine (19)	1	81.16	30.39	0.461
		urine (33)	ND	81.24	38.07	< 0.05
N30	9	plasma (7)	1	80.56	21.17	61.4
		PBMCs (7)	1	80.60	24.79	4.92
}		urine (7)	1	80.60	37.67	< 0.05
N33	6	plasma (7)	4	80.54	30.17	0.0533
		PBMCs (7)	4	80.54	31.42	0.0222
		saliva (7)	4	80.90	33.79	0.00425
		urine (7)	4 +2	80.54	29.64	0.077
		plasma (18)	ND	80.60	33.77	0.00429
		urine (18)	4	80.44	28.25	0.203
N34	6	plasma (8)	ND	80.50	35.62	< 0.05
		PBMCs (8)	1	80.10	36.05	< 0.05
		saliva (8)	1	80.66	32.79	0.0908
		urine (8)	I	80.46	30.16	0.537

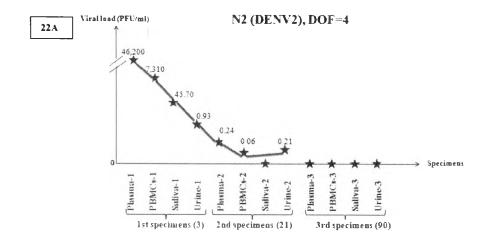
Code	DOF	Positive specimen (day of collection)	Serotype	Tm (°C)	Ct	Viral load (PFU/ml)
N34	6	urine (14)	1+3	81.06	31.10	< 0.0275
N35	5	plasma (7)	ND	80.90	33.17	< 0.05
		PBMCs (7)	ND	80.90	32.92	< 0.05
		saliva (7)	ND	80.70	34.40	< 0.05
		urine (7)	ND	80.80	32.99	< 0.05
		urine (13)	l	80.87	25.99	2.19
N40	4	plasma (4)	I	80.86	22.07	15.08
					(diluted 1:2)	
		PBMCs (4)	1	81.06	22.90	4.30
		urine (4)	1	80.94	28.79	0.0775
		PBMCs (21)	1+2	79.76	29.92	< 0.0475

DOF = duration of fever. Tm = Melting temperature (°C). Ct = cycle threshold.

The bold characters represent major serotype in mixed infected specimens.

The number in "()" represents the day of specimen collections. This table presents only positive results by illustrating the melting curve analysis (Tm) and cycle threshold of amplification curve (Ct). The expected melting curve analysis of PCR product is approximately 79.90-81.40 °C for all 4 serotypes (the data from ten-fold dilution of each stock DENV). Melting curve data out of this range was acceptable after confirming by 2% agarose gel electrophoresis and comparing with positive control.

The last standard viral concentrations of DENV1 to DENV4 were 5×10^{-2} PFU/ml (DENV1), 4.75 $\times 10^{-2}$ PFU/ml (DENV2), 2.75 $\times 10^{-2}$ PFU/ml (DENV3) and 2.5 $\times 10^{-3}$ FPU/ml (DENV4).



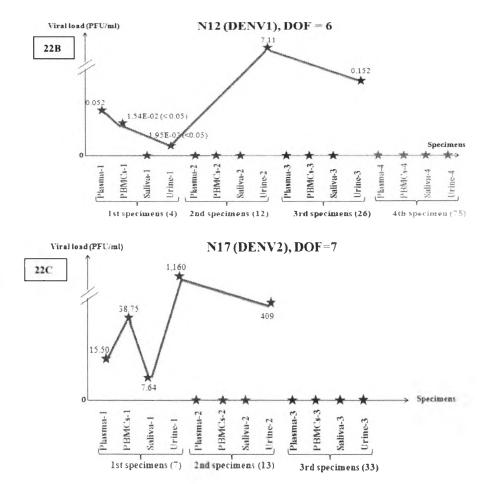


Figure 22: The viral load of DEN- infected patients (N10, 12 and N17). N10 (figure 22A) presented the viral loads in plasma, PBMCs, saliva and urine collected during day 4, 27 and 90 of illness. N12 (figure 22B) presented the viral loads in plasma, PBMCs, saliva and urine collected during day 4, 12, 26 and 75 of illness. N17 (figure 22C) presented the viral loads in plasma, PBMCs, saliva and urine collected during day 7, 13 and 33 of illness. The viral load scales are separately in each figure that can not be used to compare the finding among each patient.

When comparing the results between qRT-PCR and nested RT-PCR by using different primer sets (5'UTR and E gene, respectively) in 23 patients, the tendency of positive results after using qRT-PCR was higher than using nested RT-PCR. Additionally, the number of patients positive for DENV at least 2 time points were also higher when the results of qRT-PCR were analyzed (See Appendix C). The qRT-PCR results were mostly correlated with the results of nested RT-PCR while some specimens were positive only in either qRT-PCR or nested RT-PCR. The longest time of DENV detection in each specimen was varied in each RT-PCR

technique. After examining the results of both protocols, the longest times of DENV detection in each specimen were day 7 of illness in saliva, day 22 of illness in plasma, day 27 of illness in PBMCs, and day 46 of illness in urine (Table 30).

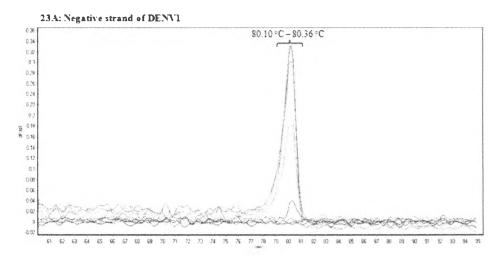
Table 30: The longest time of positive DENV detection in each specimen by comparing two methods of RT-PCR (The bold type presents the longest time of DENV detection in each specimen)

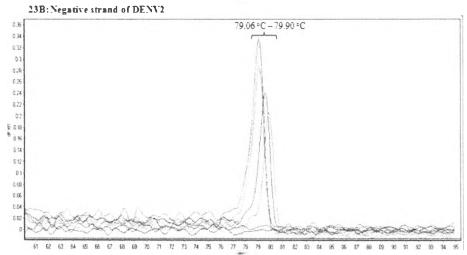
	Plasma	PBMCs	Saliva	Urine
qRT-PCR	day 22	day 21	day 7 of	day 46
	of illness	ofillness	illness	of illness
Nested RT-PCR	day 7	day 27	day 8 of	day 46 of
	of illness	of illness	illness	illness

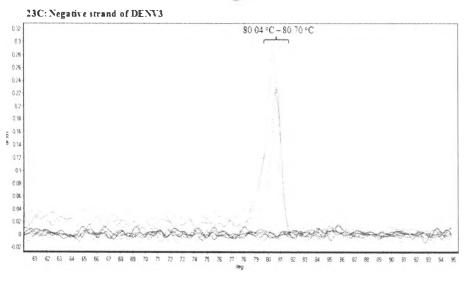
Negative strand detection of DENV in different specimens and time points using tagged real time RT-PCR (tagged qRT-PCR)

Tagged real time RT-PCR (tagged qRT-PCR) was used to detect the negative strand of DENV in all positive qRT-PCR specimens during febrile, early convalescent and late convalescent periods. The results were reported as "detected" or "not detected" after confirming by melting temperature (Tm) and gel electrophoresis (when the Tm results were inconclusive).

This assay was firstly validated to determine the limit of detection using recombinant plasmids containing negative strand PCR product of positive control DENV1-DENV4. The limits of detection were 2.39 copies/µl (DENV1), 5.60 copies/µl (DENV2), 3.37 copies/µl (DENV3) and 4.29 copies/µl (DENV4) (data not shown). The melting curve analysis of negative strand DENV using diluted positive controls from 1:2 to 1:512 (cDNA of each stock DENV1-DENV4) was in the range of 79.06°C – 80.70 °C (Figure 23).







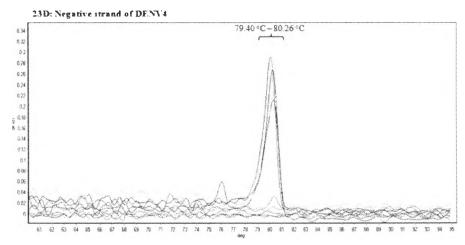


Figure 23: The melting curve analysis of negative strand DENV1-DENV4 (23A - 23D). The cDNA of each stock DENV was diluted from 1:2 to 1:512. All 4 serotypes gave the same length of PCR products. The melting temperatures of each DENV ranged from $79.06^{\circ}C - 80.70^{\circ}C$ ($80.10^{\circ}C - 80.36^{\circ}C$ (DENV1), $79.06^{\circ}C - 79.90^{\circ}C$ (DENV2), $80.04^{\circ}C - 80.70^{\circ}C$ (DENV3) and $79.40^{\circ}C - 80.26^{\circ}C$ (DENV4)).

Of 96 positive qRT-PCR specimens in 23 patients in different time points, there were 16 specimens in 9 patients positive for negative strand detection (16.67%) suggesting the evidence of viral replication (Table 31, Figure 24 and 25). During febrile period, the negative strand of DENV was found in plasma (6/15), PBMCs (5/16) and urine (1/14). In early and late convalescent periods, the negative strand of DENV was detected in urine (3/19 and 1/4, respectively) (Figure 26). No negative strand detection of DENV was found in saliva. The longest times of negative strand detection were day 7 of illness in plasma and PBMCs and day 26 of illness in urine.

Table 31: Tagged qRT-PCR results in positive q RT-PCR specimens

Code	DOF	Positive specimen	Serotype	Negative strand detection result	Tm (°C)
N2	4	plasma (3)	2	detected	79.84
		PBMCs (3)	2	detected	79.84
		saliva (3)	2	not detected	ND
		urine (3)	2	not detected	ND
		plasma (21)	ND	not detected	ND
		PBMCs (21)	2	not detected	ND

		Positive		Negative strand detection	Т.
Code	DOF	specimen	Serotype	result	Tm
					(°C)
N2	4	urine (21)	ND	not detected	ND
N3	7	plasma (7)	2	not detected	ND
		PBMCs (7)	2	not detected	ND
		saliva (7)	2	not detected	ND
		urine (7)	ND	not detected	ND
		plasma (22)	ND	not detected	ND
		urine (22)	ND	not detected	ND
N4	5	plasma (5)	ND	not detected	ND
		PBMCs (5)	2	not detected	ND
		saliva (5)	ND	not detected	ND
		urine (5)	ND	not detected	ND
N5	7	plasma (6)	2	not detected	ND
		PBMCs (6)	2	not detected	ND
		saliva (6)	ND	not detected	ND
		urine (6)	ND	not detected	ND
		urine (23)	2	not detected	ND
N6	6	plasma (4)	ND	not detected	ND
		PBMCs (4)	2	not detected	ND
N8	5	plasma (7)	2	not detected	ND
		PBMCs (7)	2	not detected	ND
		saliva (7)	2	not detected	ND
		urine (7)	2	not detected	ND
		urine (25)	ND	not detected	ND
N9	9	PBMCs (9)	l	not detected	ND
		PBMCs (15)	1	not detected	ND
N10	5	plasma (4)	2	detected	79.40
		PBMCs (4)	2	detected	79.20
		saliva (4)	2	not detected	ND
		urine (4)	2	not detected	ND
N12	6	plasma (4)	1	not detected	ND
		PBMCs (4)	1	not detected	ND
		urine (4)	ND	not detected	ND
		urine (12)	1	detected	80.30
		urine (26)	1	detected	80.20
N13	5	PBMCs (8)	2	not detected	ND

		Positive		Negative strand detection	T
Code	DOF	specimen	Serotype	result	Tm
		-			(°C)
N13	5	urine (8)	2	not detected	ND
		urine (15)	2	not detected	ND
N17	7	plasma (7)	2	not detected	ND
		PBMCs (7)	2	not detected	ND
		saliva (7)	2	not detected	ND
		urine (7)	2	not detected	ND
		urine (13)	2	not detected	ND
N20	8	saliva (6)	ND	not detected	ND
		urine (6)	1	not detected	ND
		urine (30)	1	not detected	ND
N21	7	PBMCs (7)	2	not detected	ND
		urine (14)	2	not detected	ND
N22	7	plasma (4)	ND	not detected	ND
		PBMCs (4)	1	not detected	ND
		saliva (4)	ND	not detected	ND
		urine (4)	ND	not detected	ND
		urine (24)	ND	not detected	ND
N23	8	plasma (7)	3	not detected	ND
		urine (7)	3	not detected	ND
N24	6	plasma (3)	3	detected	80.14
		PBMCs (3)	3	detected	80.50
		saliva (3)	3	not detected	ND
		urine (3)	3	detected	80.54
N28	8	plasma (6)	3	detected	80.54
		PBMCs (6)	3	not detected	ND
		urine (14)	3	not detected	ND
		urine (46)	3	not detected	ND
N29	8	plasma (5)	1	detected	79.80
		PBMCs (5)	1	not detected	ND
		urine (5)	i	not detected	ND
		urine (19)	1	detected	80.10
		urine (33)	ND	not detected	ND
N30	9	plasma (7)	1	detected	80.16
		PBMCs (7)	1	detected	80.34
		urine (7)	1	not detected	ND

		Positive		Negative strand detection	Tm		
Code	DOF	specimen	Serotype	result			
					(°C)		
N33	6	plasma (7)	4	not detected	ND		
		PBMCs (7)	4	not detected	ND		
		saliva (7)	4	not detected	ND		
		urine (7)	4+2	not detected	ND		
		plasma (18)	ND	not detected	ND		
		urine (18)	4	not detected	ND		
N34	6	plasma (8)	ND	not detected	ND		
		PBMCs (8)	1	not detected	ND		
		saliva (8)	ı	not detected	ND		
		urine (8)	1	not detected	ND		
		urine (14)	1+3	not detected	ND		
N35	5	plasma (7)	ı	not detected	ND		
		PBMCs (7)	1	not detected	ND		
	1	saliva (7)	1	not detected	ND		
		urine (7)	1	not detected	ND		
		urine (13)	1	detected	80.00		
N40	4	plasma (4)	1	not detected	ND		
		PBMCs (4)	1	detected	79.80		
		urine (4)	1	not detected	ND		
		PBMCs (21)	1+2	not detected	ND		
		Stock DENVI	I	detected	80.10-80.36*		
		stock DENV2	2	detected	79.06-79.90*		
		stock DENV3	3	detected	80.04-80.70*		
		stock DENV4	4	detected	79.40-80.26*		
ND = not determined DOF = duration of forces							

ND = not determined. DOF = duration of fever.

The number in "()" represents the day of specimen collections.

The interpretation of positive result is based on the melting curve analysis and gel electrophoresis.

^{*}Tm results of stock DENV1-DENV4 were derived from the diluted 1:2 until 1:512 of cDNA in each serotype.

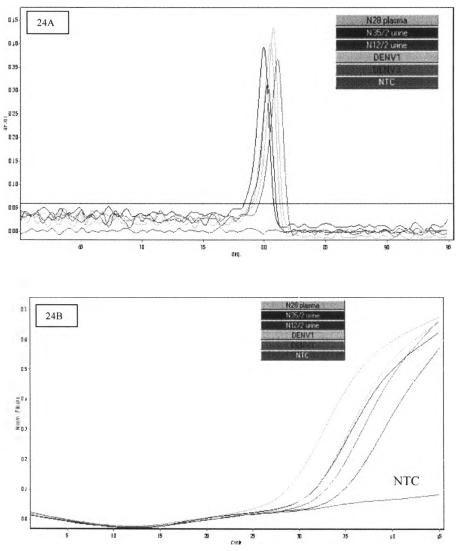


Figure 24: The melting curve analysis (24A) and the amplification plot (24B) of negative strand detection results in positive qRT-PCR specimens (N35/2 urine, 2^{nd} convalescent urine, N28 plasma, febrile plasma and N12/2 urine, early convalescent urine) compared with positive control of DENV1 and DENV3negative strands.

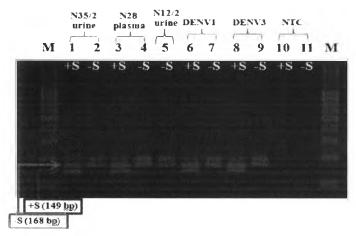
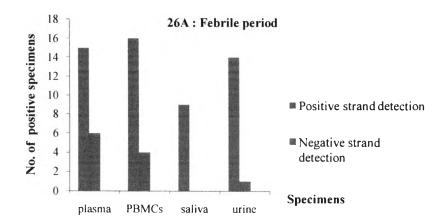
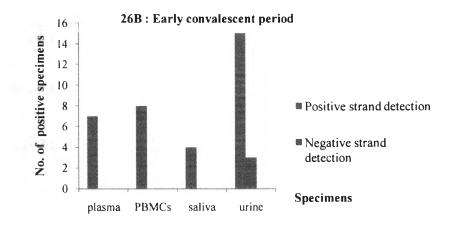


Figure 25: The 2% agarose gel electrophoresis of negative strand (168 bp) detections by tagged qRT-PCR of N35/2 urine (2nd convalescent urine), N28 plasma (febrile plasma), N12/2 urine (early convalescent urine), positive control DENV1 and positive control DENV3. The results were compared with qRT-PCR results (positive strand detection, 149 bp) except N35/2 urine. "+S" refers positive strand detection whereas "-S" refers negative strand detection. M = 100 bp DNA marker. NTC = no template control.





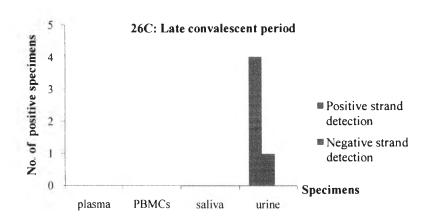
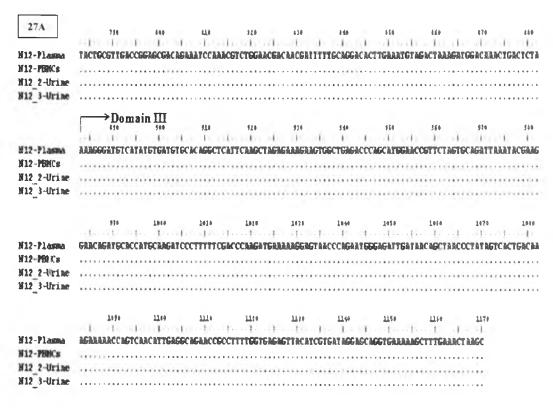


Figure 26: The comparison of positive and negative strand detection results of DENV-infected patients by qRT-PCR and tagged qRT-PCR during febrile period (26A), early convalescent period (26B) and late convalescent period (26C).

Genetic variation of DENV in different periods of infection

To determine whether genetic variations of DENV in different specimens and time points were identical, direct sequencing of partial E gene sequences (> 95% of nucleotide sequences locating at domain III) of 13 patients, except primers (388 bp) were compared. This sequence length was selected because the domain III region has been reported that it correlates with viral pathogenesis, evolution and adaptation [25]. Nucleotide and deduced amino sequence (using translation tool in BioEdit program) alignments were done using ClustalW algorithm in BioEdit program (version 7.0).

In DENV1-infected patients (N12, N20 and N29), nucleotide and amino acid sequence alignments from direct sequencing among specimens in different time points of each patient were identical, except N20 patient. In N20 patient, the result showed that the nucleotide variation between febrile and late convalescent urine was found at position 1073 (T→C) accounting for 0.25%. This variation resulted in the amino acid change at position 358 (V358A) locating on the domain III of E gene (Figure 27 and Table 32).



N12 plasma and N12 PBMCs (februle), N12 2 urine (early convalescence) and N12 3 urine (late convalescence)

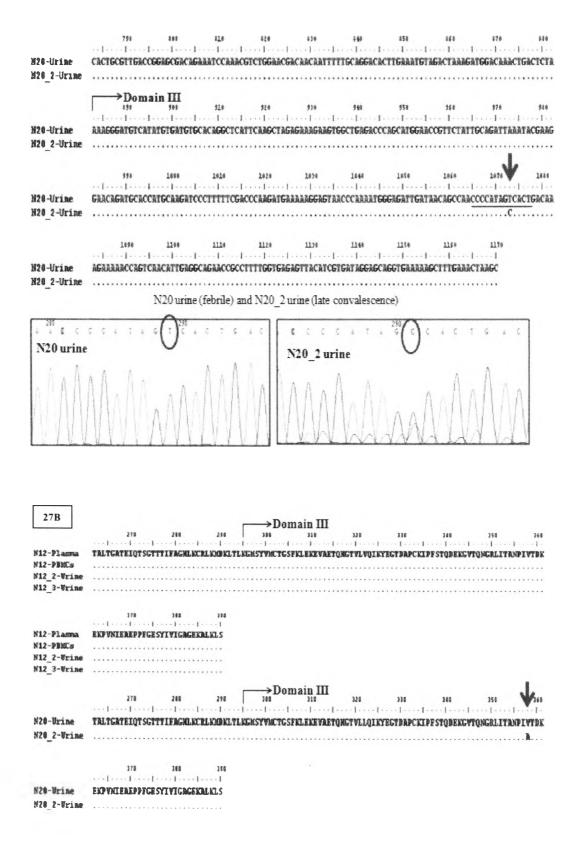
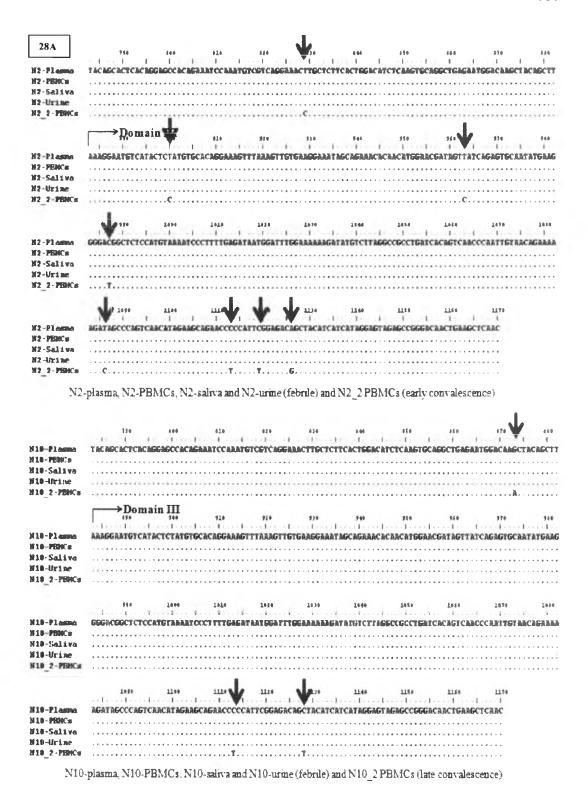


Figure 27: Nucleotide (27A) and amino acid (27B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa, respectively) derived from positive specimens of DENV1-infected patients (N12 and N20) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). Nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,485 bp and 495 aa) in GenBank accession no. AY732472.1 starting from the position 783 to 1170 (nucleotides) and accession no. ABB70708.1 starting from the position 262 to 390 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 883 to 1180 and the amino acid position 295 to 395, respectively [143, 144]. N20 specimens showed the one position of nucleotide variation at position 1073 (T→C) (marked as the red arrow at figure 25A). This variation resulted in V358A (marked as the red arrow at figure 27B) on the domain III of E gene.

In DENV2-infected patients (N2, N5 N10, N13, N17 and N21), nucleotide and amino acid sequence alignments among specimens in different time points of each patient were the same if early or late convalescent specimen was urine. Interestingly, nucleotide variations between febrile and convalescent PBMCs were found in 2 patients (N2 and N10). The variations were 2.06% in N2 and 0.77% in N10 compared with all similar sequences in each patient, respectively. In N2 patients, the variant positions of N2 2-PBMCs (early convalescence) were found at positions 829 ($T \rightarrow C$), 900 (T \rightarrow C), 963 (T \rightarrow C), 987 (C \rightarrow T), 1086 (T \rightarrow C), 1113 (C \rightarrow T), 1119 (C \rightarrow T) and 1126 (A→G). All variations resulted in silent mutations, excluding the position 1126 $(A \rightarrow G)$ causing the amino acid change at position 376 (S376G). In N10 patient, variant positions of N10 2-PBMCs (late convalescence) were found at positions 873 $(G \rightarrow A)$, 1113 $(C \rightarrow T)$ and 1128 $(C \rightarrow T)$. However, nucleotide changes in N10 2-PBMCs did not cause amino acid change (silent mutation) (Figure 28 and Table 32). Variations of both patients mostly occurred in domain III of E gene similar to DENV1-infected patients. Additionally, these results presented 2 convalescent PBMCs sharing the same position of nucleotide variation at 1113 (C→T) but this position did not cause amino acid change.



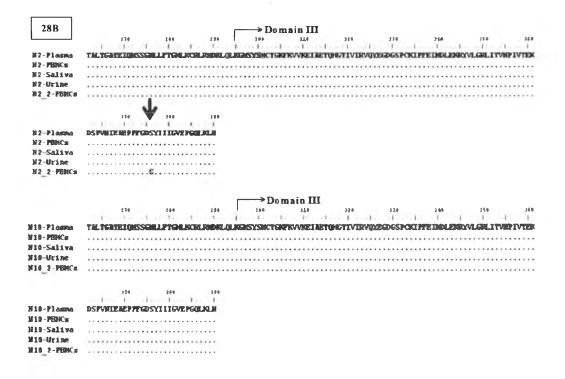


Figure 28: Nucleotide (28A) and amino acid (28B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa, respectively) derived from positive specimens of DENV2-infected patients (N2, and N10) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). The nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,485 bp and 495 aa) in GenBank accession no. DQ181872.1 starting from the position 783 to1170 (nucleotides) and accession no. AFN87732.1 starting from the position 262-390 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 883 to 1180 and amino acid position 295 to 395, respectively [143, 144]. There were 6 positions of N2_2-PBMCs difference from all specimens during febrile period. The variation were at positions 829 (T \rightarrow C), 900 (T \rightarrow C), 963 (T \rightarrow C), 987 (C \rightarrow T), 1086 (T \rightarrow C), 1113 (C \rightarrow T), 1119 (C \rightarrow T) and 1126 (A \rightarrow G) marked as the red arrows. All nucleotide variations did not affect the amino acid change (silent mutation) except the mutation at position 1126 (A \rightarrow G) caused S376G in N2_2-PBMCs (marked as the red arrow). Additionally, there were 3 positions of N10_2-PBMCs difference from all specimens during febrile period. The variations marked as the red arrows are positions 873 (G \rightarrow A), 1113 (C \rightarrow T) and 1128 (C \rightarrow T) that did not affect the amino acid change (silent mutation).

The nucleotide and amino acid sequence variations in different specimens and time points of DENV3-infected patient (N28) were identical (Figure 29).

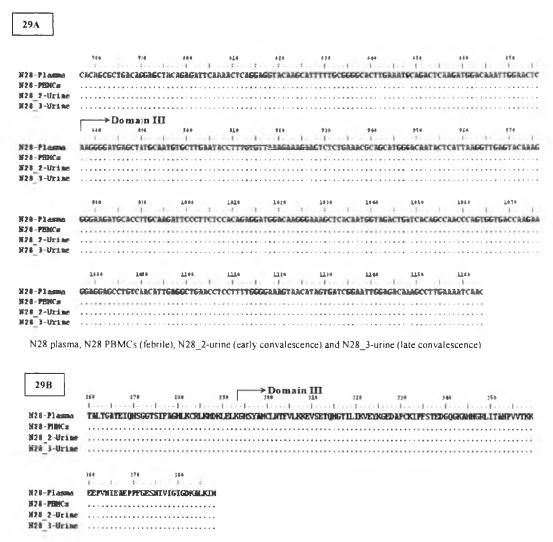
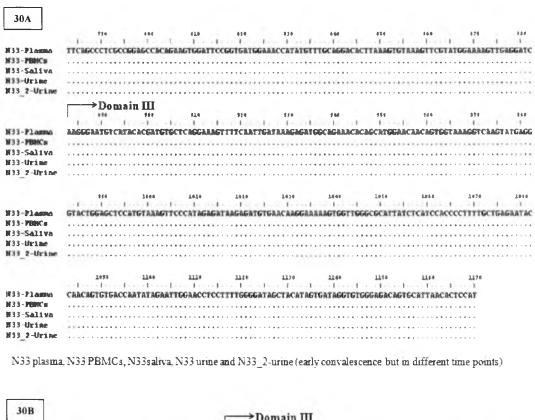


Figure 29: Nucleotide (29A) and amino acid (29B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa) derived from positive specimens of DENV3-infected patient (N28) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). The nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,479 bp and 493 amino acids) in GenBank accession no. JF968066.1 starting from position 777 to 1164 (nucleotides) and accession no. AF171764.1 starting from position 260 to 388 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 877 to 1171 and amino acid position 293 to 393, respectively [25, 143]. The nucleotide and amino acid sequences among specimens collected during febrile, early convalescent and late convalescent periods were the same.

In 3 mixed-serotype-infected patients, the nucleotide and amino acid sequence alignments of N33 specimens were not changed. Although 1st convalescent urine of N33 presented mixed serotype infections (DENV2 + DENV4) by serotype RT-PCR, the direct sequencing result presented the clear chromatogram of single serotype (DENV4) to compare with other N33 specimens (DENV4) (Figure 30).



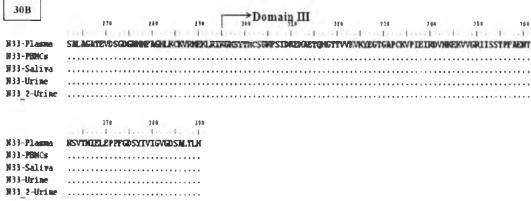


Figure 30: Nucleotide (30A) and amino acid (30B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa, respectively) derived from positive specimens of DENV4-infected patient (N33) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). The nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,485 bp and 495 amino acids) in GenBank

accession no. AY786197.1 starting from position 783 to 1170 (nucleotides) and accession no. AAU89351.1 starting from position 262 to 390 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 883 to 1180 and amino acid position 295 to 395, respectively [25, 143]. The nucleotide and amino acid sequences among specimens collected during different time points of early convalescent periods were the same.

The nucleotide and amino acid sequences alignments in different specimens and time points were not done in both N34 and N40 patients due to clearly different serotypes of E gene sequences resulting from the mixed-serotype infection in different specimens and time points.

Table 32: Summary results of nucleotide and amino acid variations of dengue-infected patients

			Nucleotide	Amino acid
Patients	Specimen	Time of collection	changes	changes
			(positions)*	(positions)*
N2	PBMCs	early convalescence (21)	829 (T→C)	-
(DENV2)			900 (T → C)	-
			963 (T→C)	-
			987 (C→T)	-
			1086 (T→C)	-
			1113 (C→T)	-
			1119 (C→T)	-
			1126 (A→G)	\$376G
N10	PBMCs	late convalescence (27)	873 (G→A)	•
(DENV2)			1113 (C→T)	-
			1128 (C→T)	-
N20 (DENV1)	urine	late convalescence (30)	1073 (T→C)	V358A

^{*} The nucleotide and amino acid positions are based on the E gene sequence of each serotype after blasting with GenBank. The nucleotide and amino acid changes were compared with febrile specimens. The number in "()" represents the day of specimen collection.

Genetic diversity of DENV in each specimen and time points in DENV-infected patients

The genetic diversity of DENV in different time points were explored by sequencing 10-15 colonies derived from each specimen of individual patient. Nucleotide and amino acid sequence variations of all clones in each specimen were analyzed by aligning with direct sequencing result using BioEdit program (version 7.0).

Nucleotide sequence alignments showed that all specimens of each patient contained both major sequences (similar to direct sequencing result) and minor sequences or variants (Figure 31A and 32A) suggesting the occurrence of heterogeneous DENV population or quasispecies. The different of nucleotide variations in each clone comparing with consensus sequence (direct sequencing) varied from one to twenty-eight positions composing of single nucleotide mutation and deletion. Moreover, these mutations were frequently found at domain III of E gene.

Amino acid sequence variations were found among clones in each specimen of individual patient in the presence of both major and minor (variant) sequences confirming the presence of heterogeneous population or quasispecies similar to nucleotide alignment results (Figure 31B and 32B). The frequency of different amino acid mutation types varied in each specimen composing of missense, nonsense, silent to frame-shift mutation (resulting from one single nucleotide mutation), which depended on each type of nucleotide variations. Most amino acid sequence variations of each clone in the same specimen were found at the domain III of E gene suggesting the relationship between amino acid variations and host selection pressure.

31A	
1 3174	740 866 616 828 820 840 350 668
N12-Plasma direct	TACTGCGTTGRCCGGRGCGGCRGARATCCANACGTCTGGRRCGRCACGATTTTTGCAGGACACTTGRRATGTRGACTAN
N12-Plasma 1	c
N12-Plasma 2	
N12-Plasma_3	AA
N12-Plasma 4	
W12-Plasma 3	
N12-Plasma_6	
N12-Plasma_7	
N12-Plasma_8	***************************************
N12-Plassna_9	••••••••••••••••••••••••
N12-Plasma_10	
N12-Plasma 11	
W12-Plasma_12	Domain III
	\$70 480 890 900 910 920 930 940
N12-Plasma direct	AGATGGACRANCTGACTCTRANAGGGATGTCHTATGTGTTGTGCACAGGCTCATTCRAGCTAGAGARAGGAGTGGCTGAG
N12-Plasma 1	AURITORCARRETORCICIARAROURISICATATOTORIOTORCAROCTERIACAROCTAROROGRAPIO
N12-Plasma 2	
N12-Plasma 3	
N12-Plasma 4	
N12-Plasma 5	
N12-Plasona 6	
N12-Plasma 7	c.
N12-Plasma 8	
M12-Plasma 9	
N12-Plasma 10	
N12-Plasma 11	
N12-Plasma 12	***************************************
	950 960 910 930 990 1000 1016 1020
	『マ・・・『・・・・』 「・・・『・・・・『・・・・『・・・・『・・・・『・・・・』 「・・・・『・・・・』 「・・・・『・・・・』・・・・『・・・・』 「・・・・』 「・・・・』 「・・・・』 「・・・・』 「・・・・・・・・
N12-Plasma_direct	ACCCRECATGGANCCETTCTAGTGCNGATTNANYACGANGGANCAGATGCNCCATGCNAGATCCCTTTTTCGRCCCCNAGN
N12-Plasma_1	
N12-Plasma_2	
N12-Plasma_3	6
N12-Plasma_4 N12-Plasma_5	
N12-Plasma 6	***************************************
N12-Plasma 7	***************************************
N12-Plasma 8	***************************************
N12-Plasma 9	***************************************
N12-Plasma 10	*****************************
	- A
N12-Plasma 11	
_	A.
N12-Plasma_11	A.
N12-Plasma_11	1690 1846 1050 1866 1870 1869 1046 1100
N12-Plasma_11 N12-Plasma_12	and the office of the office for a filter of the office of the filter of the filter of the filter of the filter
N12-Plasma_11 N12-Plasma_12 N12-Plasma_direct	TGRARANGGAGTARCCCAGRATGGGAGTTGRTARCAGCTRACCCTRYAGTCRCTGRCAAAGRARARCCAGTCRACATTG
N12-Plasma_11 N12-Plasma_12 N12-Plasma_direct N12-Plasma_1	and the office of the office for a filter of the office of the filter of the filter of the filter of the filter
N12-Plasma_11 N12-Plasma_12 N12-Plasma_direct N12-Plasma_1 N12-Plasma_2	TGRARANGGAGTARCCCAGRATGGGAGTTGRTARCAGCTRACCCTRYAGTCRCTGRCAAAGRARARCCAGTCRACATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3	TGRARANGGAGTARCCCAGRATGGGAGTTGRTARCAGCTRACCCTRYAGTCRCTGRCAAAGRARARCCAGTCRACATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 4	TGRARANGGAGTARCCCAGRATGGGAGTTGRTARCAGCTRACCCTRYAGTCRCTGRCAAAGRARARCCAGTCRACATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 4 N12-Plasma 5	TGARRANGGAGTARCCCAGRATGGGAGATTGATARCAGCTARCCCTATAGTCACTGACARAGRARANCCAGTCARCATTG
N12-Plasma 12 N12-Plasma direct N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 5 N12-Plasma 6	TGRARANGGAGTARCCCAGRATGGGAGTTGRTARCAGCTRACCCTRYAGTCRCTGRCAAAGRARARCCAGTCRACATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 2 N12-Plasma 3 N12-Plasma 4 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7	TGRARANGERSTANCCCAGRATGGGAGATTGRTARCAGCTRACCCTATAGTCACTGRCAAAGRARANGCCAGTCARCATTG
N12-Plasma 12 N12-Plasma direct N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 5 N12-Plasma 6	TGRARAMGEMETANCCCAGRATGGGAGATTGNT RACAGCTANCCCTATAGTCACTGACAAAGRARAMCCAGTCARCATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 5 N12-Plasma 5 N12-Plasma 7 N12-Plasma 7 N12-Plasma 7	TGRARANGERSTANCCCAGRATGGGAGATTGRTARCAGCTRACCCTATAGTCACTGRCAAAGRARANGCCAGTCARCATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 4 N12-Plasma 6 N12-Plasma 6 N12-Plasma 6 N12-Plasma 8 N12-Plasma 8 N12-Plasma 9	TGRARANGGAGTANCCCAGRATGGGAGATTGAT RACAGCTANCCCTATAGTCACTGACARAGRARANCCAGTCARCATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 8 N12-Plasma 8 N12-Plasma 9 N12-Plasma 9 N12-Plasma 10	TGRARANGGRETARCCCAGRATGGGAGATTGRTARCAGCTRACCCTATAGTCRCTGRCAAAGRARARCCAGTCAACATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 2 N12-Plasma 3 N12-Plasma 4 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 8 N12-Plasma 8 N12-Plasma 8 N12-Plasma 10 N12-Plasma 10 N12-Plasma 11	TGRARANGGRETARCCCAGRATGGGAGATTGRTARCAGCTRACCCTATAGTCRCTAGAGRARARCCAGTCARCATTG .G.
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 2 N12-Plasma 3 N12-Plasma 4 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 8 N12-Plasma 8 N12-Plasma 8 N12-Plasma 10 N12-Plasma 10 N12-Plasma 11	TGRARANGGRETARCCCAGRATGGERGATTGRTARCMCTRACCCTATAGTCRCTAGRARANACCAGTCRACATTG .G
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 2 N12-Plasma 3 N12-Plasma 4 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 8 N12-Plasma 8 N12-Plasma 10 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11	TGRARANGENGTANCCCAGANTGGGAGATTGNTANCAGCTANCCCTATAGTCACTCACAAAGAAAAACCAGTCANCATTG .G
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 3 N12-Plasma 3 N12-Plasma 5 N12-Plasma 6 N12-Plasma 7 N12-Plasma 8 N12-Plasma 9 N12-Plasma 10 N12-Plasma 11 N12-Plasma 12	TGRANAMESMETANCCCAGANTGGGAGATTGNT ANCIGCT NACCCTATAGTC ACTCACAAAGAAAAACCAGTCAACATTG . G G G G G LII.0 LII.0 LII.0 LI 10 LI
N12-Plasma 11 N12-Plasma direct N12-Plasma direct N12-Plasma 1 N12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 8 N12-Plasma 9 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12	TGRARAMEGRETARCCCAGRATGEGREGATTERTARCMCTRACCCTRTAGTCRCRAAGRARAMCCAGTCRACRTTG .GGGGGGGGGGGGGGGGGGGGGGGGGG.
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 H12-Plasma 3 N12-Plasma 4 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 8 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12	TGRARANGGRETANCCCAGRATGGEAGATTGRTARCAGCTRACCCTATAGTCRCTAGAGRARANCCAGTCARCATTG .G
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 H12-Plasma 3 N12-Plasma 4 N12-Plasma 6 N12-Plasma 7 N12-Plasma 8 N12-Plasma 8 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 12	TGRARANGERSTANCCCAGANTGGGAGATTGNTANCAGCTRACCCTATAGTCRCCAAAGRARANGCCAGTCANCATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma 1 N12-Plasma 1 N12-Plasma 3 N12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 1 N12-Plasma 1 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 12 N12-Plasma 1 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3	TGRANAMESMETANCCCAGANTGGGAGATTGNT ANCINCCTARACCCATAGTCACCTCACAAAGAAAAACCCAGTCAACCATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 9 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 2 N12-Plasma 3 N12-Plasma 2 N12-Plasma 3	TGRARAMEGRETARCCCAGRATGEGRATTGRTARCMCTRACCCTATAGTCRCTAGRARAMCCAGTCRACATTG .G
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 H12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 8 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 12 N12-Plasma 3 N12-Plasma 3 N12-Plasma 5 N12-Plasma 6 N12-Plasma 6 N12-Plasma 12	TGRANANGGRETANCCCAGANTGGEAGATTGRTANCAGCTRACCCTATAGTCRCTAGAGRANANCCAGTCARCATTG .G
N12-Plasma 11 N12-Plasma 12 N12-Plasma 12 N12-Plasma direct N12-Plasma 2 H12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 13 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7	TGRARANGERSTANCCCAGRATGGGAGATTGRTARCAGCTRACCCTATAGTCRCCAAAGRARARCCAGTCARCATTG
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 H12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 8 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 12 N12-Plasma 3 N12-Plasma 3 N12-Plasma 5 N12-Plasma 6 N12-Plasma 6 N12-Plasma 12	TGRARAMEGRETARCCCAGRATGEGREGATTGRTARCMCTRACCCTATAGTCRCTAGRARAMACCAGTCRACCATTG .G
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 N12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 9 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 3 N12-Plasma 3 N12-Plasma 4 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 3 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 7 N12-Plasma 7	TGRARAMEGRETARCCCAGRATGEGRATTGRTARCMCTRACCCTATAGTCRCTGRCAAAGRARAMCCAGTCARCATTG .G
N12-Plasma 11 N12-Plasma 12 N12-Plasma direct N12-Plasma 1 N12-Plasma 2 H12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 8 N12-Plasma 10 N12-Plasma 11 N12-Plasma 11 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 12 N12-Plasma 3 N12-Plasma 3 N12-Plasma 6 N12-Plasma 6 N12-Plasma 6 N12-Plasma 7 N12-Plasma 7 N12-Plasma 7 N12-Plasma 8 N12-Plasma 8	TGRARAMEGRETARCCCAGRATGEGREGATTGRTARCMCTRACCCTATAGTCRCTAGRARAMACCAGTCRACCATTG .G

	790 848 510 520 520 546 550 560
N12-PBMCs direct	TACTGCCTTGBCCGGACAGABBTCCBBACGTCTGGBACGACAGCGTTTTTTGCGGGACACTTGBBATGTAGACTAB
	THUTGEOFT GRECOGRADE CHERRATE CARACTET GRANCE CARCEATTITT GEALD ACTION AND THE AC
N12-PBHCs_2	
N12-PBMCs_7	
N12-PBMCs A	
N12-PBMCs B	
N12-PBMCs_D	
W12-PBMCs E	
N12-PBMCs F	.6.,,,,,
N12-PBMCs_H	
W12-PBMCs_J	***************************************
N12-PBMCs_13	
N12-PBMCs 14	
	Domain III
	876 884 898 988 918 928 928 948
N12-PBMCs_direct	Agat ggreraletgretet krangggatgtertatgtgatgtgenerggetertertyerigetrangetragagnargargtggetgrg
N12-PBMCs_2	
N12-PBMCs_?	***************************************
N12-PBMCs_A	
N12-PBMCs B	
N12-PBMCs_D	
N12-PBMCs_E	
N12-PBMCs_F	
N12-PBHCs_H	
N12-PHMCs_J	·
N12-PBMCs_13	
W12-PBMCs 14	
	950 950 970 950 990 1000 1010 1010
	and the state of the first of t
N12-PBMCs_direct	ACCCAGCATGGABCCGTTCTAGTGCAGATTAANTACGAAGGAACAGATGCACCATGCAAGATCCCTTTTTCGACCCAAGA
N12-PBMCs 2	
N12-PBNCs_7	
N12-PBMCs A	
N12-PBMCs B	
W12-PBMCs D	
N12-PBMCs E	·····T
N12-PBMCs F	***************************************
N12-PBMCs_H	***************************************
N12-PBMCs_J	• • • • • • • • • • • • • • • • • • • •
N12-PBMCs_13	T
N12-PBMCs 14	G
	1030 1040 1050 1050 1070 1050 1098 1100
	The Double Territorial Control of the Property
N12-PBMCs_direct	TGANANGGAGTANCCCAGAATGGGAGATTGATAACAGCTAACCCTATAGTCACTGACAAAGAAAAACCAGTCAACATTG
N12-PBMCs 2	······C
W12-PBMCs_7	
N12-PEMCs_A N12-PEMCs_B	
76/7	
N12-PBMCs D N12-PBMCs E	T
N12-PENCE E	
N12-PRMCs H	······································
_	
N12-PBMCs J	
N12-PBMCs_13	•••••••••••••••••••••••••••••••••••••••
W12-PBMCs 14	GG.
	1110 1120 1130 1140 1150 1150 1170
N12-PBMCs direct	ACCURATE CONTROLLER CO
N12-PBMCs 2	AGGCAGARCCGCCTTTTGGTGAGAGTTACATCGTGATAGGAGCAGGTGARARAGCTTTGARACTARGC
N12-PBMCs 7	
N12-PBMCs A	
N12-PBMCs B	G. ,
N12-PBMCs D	
N12-PB9Cs E	
N12-PBNCs F	***************************************
N12-PRMCs H	***************************************
N12-PBMCs J	***************************************
N12-PBHCs 13	***************************************
N12-PBMCs 14	······································

	790 800 810 820 830 840 450 860
7710 0 15-day - 14	TACTGCGTTGACCGGGGCGACAGAAATCCAARCGTCTGGGRCGACAACGATTTTTGCAGGACACTTGAAATCTAGACTAA
N12_2-Urine_direct N12_2-Urine_1	TRUTGCGTTGRUUGGRUCGGRUUGGRUUGGGRUUGGTTTTTTGURGGRURUTTGARATGTALA
N12_2-Urine_1 N12_2-Urine_2	***************************************
W12 2-Urine 3	
N12 2-Urine 4	
M12 2-Urine 5	
N12 2-Urine 6	J
N12 2 Urine 7	
N12 2-Urime 8	
N12 2-Urine 9	
N12 2-Urine 10	
W12 2-Urine 11	
	Domain_III
	676 486 450 500 510 520 530 540
N12_2-Urime_direct	AGATGGRCRANCTGRCTCTARANGGGRTGTCATATGTGATGTGCRCRGGCTCRTTCRAGCTRGRGRAAGRRGTGGCTGRG
N12_2-Urine_1	
N12_2-Urime_2	
N12_2-Urine_3	
N12 2-Urine 4	
N12 2-Urine 5	
N12_2-Urine_5	
N12_2-Urine 7	
W12 2-Urine B	
W12 2-Urine 9	
N12 2-Urine 10	
W12_2-Urine_11	
	850 960 970 9s0 990 <u>1010 1010</u>
N12 2-Urine direct	ACCCAGCATGGAACCGTTCTAGTGCAGATTABATACGAAGGAACCAGATGCACGATGCAAGATCCCTTTTTCGACCCAAGA
W12 2-Urine 1	MCCADCATOGAACCOTTCTREYTOCROSETARAN RCGRANGORACENTATOCACCATOCARNATCCCTTTTTCORCCCARDA
N12 2-Urine 2	
N12 2 Urine 3	
W12 2-Urine 4	
N12 2-Urine 5	
M12 2-Urime 6	TG
N12 2-Urine 7	
N12 2-Urine 8	
N12 2-Uxine 9	
N12 2-Uriae 10	T
N12_2-Urime_11	
	1030 1040 1050 1980 1076 1080 1098 1100
N12_2-Urime_direct	TGAAAAAGGAGTAACCCAGAATGGGAGATTGATAACAGCTAACCCTATAGTCACTGACAAGAAAAACCAGTCAACATTG
N12_2-Urine_1 N12_2-Urine_2	
N12_2-Urine_2 N12_2-Urine_3	***************************************
N12 2-Urine 4	
N12 2-Urine 5	
N12 2-Urine 6	
N12 2-Urise 7	***************************************
N12 2 Urine 8	***************************************
N12 2-Urine 9	
W12 2-Urine 10	
N12 2-Urine 11	
-	
	1110 1120 1140 1150 1160 1170
	- le la lacela descha d
N12_2-Urime_direct	aggcagaaccgccttttggtgagagttacatcgtgataggagcaggtgaaaaaagctttgaaactaagc
N12 2-Urine 1	
N12 2-Urime 2	
N12 2-Urine 3	
N12 2-Urine 4	***************************************
N12 2 Urine 5	······································
N12_2-Urime_6 N12_2-Urime_7	***************************************
N12 2-Urine 8	***************************************
N12 2-Urine 9	
N12 2-Urine 10	······································
N12 2-Urine 11	······································

	530 960 970 950 950 940 920 990
N12 3-Urine direct	TACTECCTTGACCGGAGCGACAGAAATCCAAACGTCTGGAACGACAACGATTTTTGCAGGACACTTGAAATGTAGACTAA
M12 3-Urine 1	
H12 3-Urine 2	
Time and	
H12_3-Urine_5	
N12 3-Urine 6	
R12 3-Urine 8	
N12 3-Urine 9	
N12 3-Urine 13	c
N12 3-Urine 14	
W12 3-Urine 15	
W12 3-Urine 20	
W12 3-Urine 21	
Ten.	
W12_3-Urine_22	Domain III
	Domain III
	270 820 850 500 910 520 530 540
	- A franchischer Levis Providence possible education of particular advantage and a
N12 3-Urine direct	AGATGGRCARACTGACTCTARAAGGGATGTCATATGTGATGTG
N12 3-Urine 1	
R12 3-Urine 2	
N12 3-Urine 5	***************************************
M12 3-Urine 6	
H12_3-Urine #	c.,
W12_3-Urine_9	
W12_3-Urine_13	
N12_3-Urime 14	
N12 3-Urine 15	
N12 3-Urine 20	
N12 3-Urine 21	
W12 3-Urine 22	A
MIZ 3 OLINE ZZ	
	350 380 375 340 399 1000 1019 1020
	-1 -1 -1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
N12_3-Urine_direct	ACCCAGCATGGAACCGTTCTAGTGCAGATTAAATACGAAGGAACAGATGCACCATGCAAGATCCCTTTTTCGACCCAAGA
N12 3-Urine 1	***************************************
N12 3-Urine 2	T
N12 3-Urine 5	
N12 3-Urine 6	
N12 3-Urine 8	***************************************
N12 3-Urine 9	
W12_3-Urine_13	
N12_3-Urine_14	TT
W12 3-Urine 15	
N12 3-Urine 20	
W12 3-Urine 21	7
W12 3-Urine 22	
	1020 1040 1050 1050 1070 1080 1000 1180
H12 3-Urine direct	TGARARAGGRGTRACCCRGARTGGGRGATTGATRACAGCTRACCCTATAGTCACTGRCARAGRARACCAGTCRACATTG
W12 3-Wrine 1	
	c
N12 3-Wrine 2	***************************************
W12_3-Wrine_5	
N12_3-Wrine_6	
N12 3-Urine 8	***************************************
Table 1997	
W12 3-Wrine 9	***************************************
Table 1997	
W12 3-Wrine 9 W12 3-Wrine 13	c.
N12 3-Wrine 9 N12 3-Wrine 13 N12 3-Wrine 14	c.
N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15	c.
N12 3-Wrine 9 N12 3-Wrine 13 N12 3-Wrine 14 N12 3-Wrine 15 N12 3-Wrine 20	c
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_14 M12_3-Wrine_15 R12_3-Wrine_20 M12_3-Wrine_21	c.
N12 3-Wrine 9 N12 3-Wrine 13 N12 3-Wrine 14 N12 3-Wrine 15 N12 3-Wrine 20	c
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_14 M12_3-Wrine_15 R12_3-Wrine_20 M12_3-Wrine_21	c.
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_14 M12_3-Wrine_15 R12_3-Wrine_20 M12_3-Wrine_21	C
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_14 M12_3-Wrine_15 R12_3-Wrine_20 M12_3-Wrine_21	C
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_14 M12_3-Wrine_15 R12_3-Wrine_20 M12_3-Wrine_21	C
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_14 M12_3-Wrine_15 N12_3-Wrine_20 N12_3-Wrine_21 N12_3-Wrine_22	C LLIO LLIO LLIO LLO LLO LLO LLO LLO LLO L
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_14 M12_3-Wrine_15 R12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 W12_3-Wrine_22	C. LILO 11:6 11:20 11:40 11:50 11:60 11:79 AGGCAGRACCGCCTTTTGGTGAGGAGTTACATCGTGATAGGGAGCAGGTGARATAGCTTTGATACTTAGGC
N12_3-Wrine_9 N12_3-Wrine_13 N12_3-Wrine_14 N12_3-Wrine_15 N12_3-Wrine_20 N12_3-Wrine_21 N12_3-Wrine_22 N12_3-Wrine_1 N12_3-Wrine_1 N12_3-Wrine_1 N12_3-Wrine_1	C. LL10 LL14 LL10 LL40 LL50 LL60 LL79 AGGCAGARCCGCCTTTTGGTGAGGGTTACATCGTGATAGGAGCAGGTGAARAGCTTTGARACTAAGC
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_15 M12_3-Wrine_15 M12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 M12_3-Wrine_15 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_2 M12_3-Wrine_5	C LILO 11:4 11:50 11:40 11:50 11:60 11:70 AGGCAGRACCGCCTTTTGGTGAGGTTACATCGTGATAGGAGCAGGTGARARAGCTTTGRARCTAGGC
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_15 M12_3-Wrine_15 M12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 M12_3-Wrine_22 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_5 M12_3-Wrine_5 M12_3-Wrine_6	C LILO LISO LISO LISO LISO LISO LISO LISO
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_15 M12_3-Wrine_15 M12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_5 M12_3-Wrine_6 M12_3-Wrine_6 M12_3-Wrine_8	C. C. LILO 11:6 11:20 11:40 11:50 11:60 11:79 AGGCAGRACCGCCTTTTGGTGAGGAGTTACATCGTGATAGGGAGCCAGGTGARARAGCTTTGARACTAGGC G.
N12_3-Wrine_9 N12_3-Wrine_13 N12_3-Wrine_14 N12_3-Wrine_15 N12_3-Wrine_20 N12_3-Wrine_21 N12_3-Wrine_22 N12_3-Wrine_1 N12_3-Wrine_1 N12_3-Wrine_1 N12_3-Wrine_5 N12_3-Wrine_5 N12_3-Wrine_6 N12_3-Wrine_6 N12_3-Urine_8 N12_3-Urine_9	C. LILO 114 1150 1140 1150 1150 1179 AGGCAGARCCGCCTTTTGGTGAGGTTACATCGTGATAGGAGCTGGAARAAGCTTTGAARCTAAGC
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_15 N12_3-Wrine_15 N12_3-Wrine_20 N12_3-Wrine_21 N12_3-Wrine_22 W12_3-Wrine_12 W12_3-Wrine_1 W12_3-Wrine_2 W12_3-Wrine_5 W12_3-Wrine_6 W12_3-Wrine_8 W12_3-Wrine_9 W12_3-Wrine_9 W12_3-Wrine_13	C. C. LL10 LL16 LL50 LL60 LL50 LL60 LL79 AGGCAGGRCCGCCTTTTGGTGAGGTTACATCGTGATAGGAGCTGAAARAGCTTTGARACTAAGC G. G. C.
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_15 M12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 M12_3-Wrine_22 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_2 M12_3-Wrine_6 M12_3-Wrine_6 M12_3-Urine_8 M12_3-Urine_9 M12_3-Wrine_13 M12_3-Wrine_14	C LILO LISO LISO LISO LISO LISO LISO LISO
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_15 M12_3-Wrine_15 M12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_5 M12_3-Wrine_6 M12_3-Wrine_8 M12_3-Wrine_9 M12_3-Wrine_13 M12_3-Wrine_13 M12_3-Wrine_13 M12_3-Wrine_14 M12_3-Wrine_15	C. C. LILO LIS LISO LISO LISO LISO LISO LITE AGGCAGARACCGCCTTTTGGTGAGGTTACATCGTGATAGGAGCAGGTGARARAGCTTTGARACTAAGC
M12_3-Wrine_9 M12_3-Wrine_13 M12_3-Wrine_15 M12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 M12_3-Wrine_22 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_5 M12_3-Wrine_6 M12_3-Wrine_8 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_15 M12_3-Wrine_15 M12_3-Wrine_20	C. LILO 114 1130 1140 1150 1160 1179 AGGCAGRACCGCCTTTTGGTGAGGTTACATCGTGATAGGAGCTGGARARAGCTTTGAARCTAAGC
M12_3-Wrine_9 M12_3-Wrine_13 R12_3-Wrine_15 M12_3-Wrine_15 M12_3-Wrine_20 M12_3-Wrine_21 M12_3-Wrine_22 M12_3-Wrine_1 M12_3-Wrine_1 M12_3-Wrine_5 M12_3-Wrine_6 M12_3-Wrine_8 M12_3-Wrine_9 M12_3-Wrine_13 M12_3-Wrine_13 M12_3-Wrine_13 M12_3-Wrine_14 M12_3-Wrine_15	C. LL10 LL10 LL10 LL20 LL40 LL50 LL60 LL70 AGGCAGRACCGCCTTTTGGTGAGGAGTTACATCGTGATAGGGAGCAGGTGARAAAGCTTTGAARCTAAGC GGG.

31R Domain III 200 290 210 320 N12-Plasma direct TALTGATELOTSGTTT IF AGHLKCRLKADKLTLKGASSYNCTGSFKLEIGSVÆTUNGTVLVOLKVEGTDAPCKI PFSTÓD N12-Plasma 1 N12-Plasma 2 W12-Plasma 3 M12-Plasma 4 W12-Plasma 5 N12-Plasma 6 N12-Plasma 7 N12-Plasma 8 N12-Plasma 9 N12-Plasma 10 W12-Plasma 11 N12-Plassma 12 370 280 266 EKGVTQNGRLITANPIVTDKEKPVNIEAEPPFGESYIVIGAGEKALKLS--N12-Plasma direct N12-Plasma 1R.,--N12-Plasma 2 N12-Plasma 3 N12-Plasma 4 N12-Plasma 5 K. EXPRM. DXXQLTLXSLT. KNQSTLRQMRLLVR---, TSXX. QVK..XXX N12-Plasma 6 N12-Plasma 7 K.EXPRM.DXXQLTLXSLT.KNQSTLRQHRLLVR---.TSXX.QVK.PXXX N12-Plasma 8 N12-Plasma 9 N12-Plasma 10 K. EXPRIM. DXXQLTLXSLT. KHQSTLRQHRLLVR---. TSXX. QVK. PXXX N12-Plasma 11 N12-Plasma 12v...... Domain III 100 210 700 210 720 330 240 N12-PERCs direct Taltgateigtsgtttifmghlrcrlkodkltikgrsyvkctgspklerevaetgrgtvlvglkyegtdapckippstgd N12-PBMCs 2 W12-PBMCs_7 W12-PBMCs A N12-PBMCs B W12-PBMCs D N12-PBMCs E N12-PBMCs F N12-PBMCs H N12-PBHCs_J M12-PBMCs_13 N12-PBMCs 14 250 360 270 240 N12-PENCs direct EXCEVTQUEGRELITAREPIVITOREXEVITIE REPPFGESYTVI GAGERGLEGES --N12-PBMCs 2 K.EXPRH.DXXXXLTLXSLT.KNQSTLRQHRLLVR---.TSXX.QVK..XXC 🛊 M12-PBMCs 7 N12-PBMCs A ************************************* N12-PEMCs B N12-PBMCs D N12-PBMCs E M12-PBMCs F K.EXPRH.DXXQLTLXSLT.KNQSTLRQNRLLVR---.TSXX.QVK..XXC N12-PRMCs H N12-PBMCs_J K.EXPRN.DXXQLTLXSLT.104QSTLRQMRLLVR---.TSXX.QVK..XXC * N12-PBMCs_13 N12-PBMCs 14

	Domain_III
	278 288 299 219 229 229 240
N12 2-Urine direct	TALTGATEIQTSGTTTIPAGALKCRLIOOKLTIKGASYVACTGSFRLEREVAETQAGTVI.VQIKGEGTDAPCKIPFSTQD
W12 2-Urine 1	
W12 2-Urine 2	E.IV
N12 2-Urine 3	
W12 2-Urine 4	
N12 2-Urine 5	
W12 2-Urine 6	
M12 2-Urime 7	
W12_2-Urime_8	X
W12_2-Urime_9	
W12 2-Uriae 10	
N12 2-Urine_11	
	750 760 376 350 290
N12 2-Urime direct	EKGVT (BIGRL I T NIP I VTD KEKPVITE NE P PEGESYI VI G AGEKOLIKLS
N12 2-Urime 1	
N12 2-Urine 2	
N12 2-Urime 3	
N12 2-Urine 4	K.EXPRM.DXXQLTLXSLTRIXIQSTLRQBTRLLVR, TSXX.QVK., XRX *
N12 2-Urine 5	
N12 2-Urine 6	1,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,
N12 2-Urine 7	***************************************
N12 2-Urine 8	K. EXPRH. DXXQLTLXSLT. KNQSTLRQNRLLVR TSXX. QVK., XXX
N12 2-Urine 9	
N12 2-Urine 10	V
W12 2-Urine 11	
	Domain III
	270 240 150 777 226 226 220 340
N12 3-Urine direct	TALTGATE LOTSGTTT IF AGALKORLIGIOKLTLKGRSYVMCTGSFKLEKEVAETGHGTVLVO IKYEGTDAPCKI PFSTOD
1125 0 01210 121000	
M12 3-Mrine 1	INDIANISM AND
N12 3-Urine 1 N12 3-Urine 2	
N12 3-Urine 2	
N12 3-Urime 2 N12 3-Urime 5	
N12 3-Urine 2	
N12 3-Urime 2 N12 3-Urime 5 N12 3-Urime 6	
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8	
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9	
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13	
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14	
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15	
M12 3-Urime 2 M12 3-Urime 5 M12 3-Urime 6 M12 3-Urime 8 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15 M12 3-Urime 20	RWTNOLX. CHRXCAQAHSSXR.KNE.RPSNEPFXCRE.NTKEQBBHARSL.RPKM
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 20	RWTNOLX. CHOXCAQAHSSOR. KNE.RPSHEPFXCRE.NTKEQBHHARSL. RPKM
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 20	RWTROLX CHROCAGANSSOR KNERPSNEPFXCRENTKEGRHHARSE RPKM CHROCAGANSSOR KNERPSNEPFXCRENTKEGRHHARSE RPKM X K I G RPKM
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 20	RYTTOLLX . CHOXCAQAHSSOR . KHE.RPSHEPFXCRE.NTKEQBHHARSL . RPKM . CHOXCAQAHSSOR . KHE.RPSHEPFXCRE.NTKEQBHHARSL . RPKM . X . K . I G . RPKM
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 21 N12 3-Urine 22	RWTNCLX. CHRXCAQAHSSXR.KWLRPSNEPFXCRLNTKEQHHUARSL.RPKM
M12 3-Urime 2 M12 3-Urime 5 M12 3-Urime 6 M12 3-Urime 8 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15 M12 3-Urime 20 M12 3-Urime 21 M12 3-Urime 22 M12 3-Urime 22 M12 3-Urime 22	RWTNCLX. CHRXCAQAHSSXR.KNE.RPSNEPFXCRE.NTKEQBBHARSL.RPKM
M12 3-Urime 2 M12 3-Urime 5 M12 3-Urime 6 M12 3-Urime 8 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15 M12 3-Urime 20 M12 3-Urime 21 M12 3-Urime 22	RWTNOLX. CHROCCAQAHSSOR. KNERPSHEPFXCRENTKEQBBHARSL. RPKM CHROCCAQAHSSOR. KNERPSHEPFXCRENTKEQBBHARSL. RPKM X K I. G. RPKM 250 240 170 240 790 EXGVTQRCRLITAMPIVTDREKPVHIBAEPPFGESYIVIGAGEKALRUS T
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 21 N12 3-Urine 21 N12 3-Urine 22 N12 3-Urine 22 N12 3-Urine 3	RYTINGLX. CHROCCAGARISSOR. KHERPSHEPFXCRENTIKEGRHHARSL. RPKM CHROCCAGARISSOR. KHERPSHEPFXCRENTIKEGRHHARSL. RPKM CHROCCAGARISSOR. KHERPSHEPFXCRENTIKEGRHHARSL. RPKM X K I. G. RPKM 759 260 170 260 790 EKGVTGRGRLITAMPIVTDREKPVNIEAEPPFGESYIVIGAGEKALKI.S T. K.EXPRN. DXQGLTLXSLT. KNQSTLRQDRLLVR, TSXX. QVK. XRX
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 21 N12 3-Urine 21 N12 3-Urine 22 N12 3-Urine 22 N12 3-Urine 22 N12 3-Urine 6	RYTINGLX. CHROCCAQAHSSOR. KHERPSHEPFXCRENTKEQBHHARSL. RPKM CHROCCAQAHSSOR. KHERPSHEPFXCRENTKEQBHHARSL. RPKM X. K. I
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 21 N12 3-Urine 21 N12 3-Urine 22 N12 3-Urine 22 N12 3-Urine 2 N12 3-Urine 6 N12 3-Urine 5 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 6	RYTICLX . CHEXCAGANSSXR . KHERPSHEPFXCRENTKEQBHHHARSL . RPKM . CHEXCAGANSSXR . KHERPSHEPFXCRENTKEQBHHHARSL . RPKM X K. I
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 13 N12 3-Urine 13 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 21 N12 3-Urine 21 N12 3-Urine 22 N12 3-Urine 22 N12 3-Urine 6 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 6 N12 3-Urine 6 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9	RWTNCLX. CHRXCAQAHSSXR.KNERPSHEPFXCRENTKEQHHARSL.RPKM
M12 3-Urime 2 M12 3-Urime 5 M12 3-Urime 6 M12 3-Urime 8 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15 M12 3-Urime 20 M12 3-Urime 21 M12 3-Urime 22 M12 3-Urime 22 M12 3-Urime 22 M12 3-Urime 6 M12 3-Urime 6 M12 3-Urime 6 M12 3-Urime 8 M12 3-Urime 9 M12 3-Urime 9 M12 3-Urime 13	RWTNCLX. CHRXCAQAHSSXR.KNERPSNEPFXCRENTKEQBHHARSL.RPKM
M12 3-Urime 2 H12 3-Urime 5 H12 3-Urime 6 H12 3-Urime 6 H12 3-Urime 9 H12 3-Urime 13 H12 3-Urime 15 H12 3-Urime 15 H12 3-Urime 20 H12 3-Urime 21 H12 3-Urime 22 H12 3-Urime 22 H12 3-Urime 2 H12 3-Urime 6 H12 3-Urime 5 H12 3-Urime 6 H12 3-Urime 6 H12 3-Urime 6 H12 3-Urime 7 H12 3-Urime 9 H12 3-Urime 9 H12 3-Urime 13 H12 3-Urime 13 H12 3-Urime 13	RWTNOLX. CHRXCAQAHSSXR. KNE.RPSNEPFXCRE.NTKEQBBHARSL. RPKM
M12 3-Urime 2 M12 3-Urime 5 M12 3-Urime 6 M12 3-Urime 6 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15 M12 3-Urime 20 M12 3-Urime 21 M12 3-Urime 21 M12 3-Urime 22 M12 3-Urime 2 M12 3-Urime 6 M12 3-Urime 9 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15	RWTNOLX. CHROCCAGAHSSOR. KHERPSHEPFXCRENTKEGRRHARSE. RPKM CHROCCAGAHSSOR. KHERPSHEPFXCRENTKEGRRHARSE. RPKM X K. I. G. RPKM 250 260 170 260 790 EKGVTGRGRLITAMPIVTDREKPVHIEBEPPFGESYIVIGBGEKM.RLS- T. K.EXPRM. DXQGLTLXSLT. KNGSTLRGDRLLVR TSXX. GVK. XRX K. EXPRM. DXQGLTLXSLT. KNGSTLRGDRLLVR TSXX. GVK. XRX K. EXPRM. DXQGLTLXSLT. KNGSTLRGDRLLVR TSXX. GVK. XRX
N12 3-Urine 2 N12 3-Urine 5 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 20 N12 3-Urine 21 N12 3-Urine 21 N12 3-Urine 22 N12 3-Urine 2 N12 3-Urine 6 N12 3-Urine 6 N12 3-Urine 6 N12 3-Urine 8 N12 3-Urine 8 N12 3-Urine 9 N12 3-Urine 13 N12 3-Urine 13 N12 3-Urine 14 N12 3-Urine 15 N12 3-Urine 15 N12 3-Urine 15 N12 3-Urine 15 N12 3-Urine 20	RWTNOLX. CHROCCAQAHSSOR. KHERPSHEPFXCRENTKEQBHHARSE. RPKM CHROCCAQAHSSOR. KHERPSHEPFXCRENTKEQBHHARSE. RPKM X. K. I. G. RPKM 250 240 170 240 790 EKGVTQHRRLITAMPIVTDREKPVNIEAEPPFGESYIVIGAGEKALKES- T. K. EXFRM. DXQQETLXSLT. KNQSTLRQDRLLVR TSXX. QVK. XRX K. EXFRM. DXQQETLXSLT. KNQSTLRQDRLLVR TSXX. QVK. XRX K. EXFRM. DXQQETLXSLT. KNQSTLRQDRLLVR TSXX. QVK. XRX
M12 3-Urime 2 M12 3-Urime 5 M12 3-Urime 6 M12 3-Urime 6 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15 M12 3-Urime 20 M12 3-Urime 21 M12 3-Urime 21 M12 3-Urime 22 M12 3-Urime 22 M12 3-Urime 6 M12 3-Urime 9 M12 3-Urime 9 M12 3-Urime 13 M12 3-Urime 13 M12 3-Urime 14 M12 3-Urime 15	RWTNOLX. CHROCCAQAHSSOR. KNE.RPSNEPFXCRE.NTKEQBBHARSL. RPKM CHROCCAQAHSSOR. KNE.RPSNEPFXCRE.NTKEQBBHARSL. RPKM X K. I. G. RPKM 250 340 170 240 790 EKGVTQBGRLITAMPIVTDREKPVHIEBEPPFGESYIVIGAGEKM.RLS T. K.EXPRM. DXQQLTLXSLT. KNQSTLRQDRLLVR TSXX. QVK. XRX K. EXPRM. DXQQLTLXSLT. KNQSTLRQDRLLVR TSXX. QVK. XRX K. EXPRM. DXQQLTLXSLT. KNQSTLRQDRLLVR TSXX. QVK. XRX

Figure 31: Nucleotide (31A) and amino (31B) sequence alignments of all clones derived from each N12 specimens, N12 plasma (4), N12 PBMCs (4), N12 urine (12) and N12 urine (26), (DENVI infected patients) present heterogeneous population or quasispecies in different time points. Direct sequencing of each specimen is used as a reference sequence (major population) to compare with all variant sequences or minor populations (10-15 sequences per specimen). The positions of nucleotide and amino acid are based on direct sequencing alignments in figure 27A and 27B. Domain III of E gene is starting from position 883 to 1,180 of nucleotide and position 295-395 of amino acid sequences (complete E gene). Deletion position is presented as "-". In-frame stop codon is presented as " *". The presence of frame-shift mutations is marked as "*". The number in "()" represents the day of specimen collection.

32A	798 4	14	83.0	420	470	3 60	850	860
M2-Plasma_direct	TREAGCACTERCAGERGE	CACABAA	RICCALATET	CLTCMGLARA	CITICITE	ACTEGACATO	LC WW. LPC WP	GCTGA
M2-Plasma_5								
N2-Plasma_8								
N2-Plasma M								
N2-Plasma N								
M2-Plasma O								
M2-Plasma Q								
					• • • • • • • • • •			
M2-Plasma_S				• • • • • • • • •	• • • • • • • • • •	• • • • • • • • •		
N2-Plasma_S1				• • • • • • • • •				
N2-Plasma U								
M2-Plasma V					<i></i> .			
W2-Plasma W								
		_						
		Doina						
		I	₹,,,	500	910	110	970	144
							(4	
N2-Plasma direct	GARTGERCARGCTACAGO	TTALLGG	BATGTCATAC	TCTATGTGCA	CAGGALAGTT	TRANSTTETS	argerating	CAGAA
N2-Plasma 5								
N2-Plasma 8						C		
N2-Plasma M								
N2-Plasma N								
N2-Plasma 0								
M2-Plasma_0								
M2-Plasma_S								
M2-Plasma S1								
M2-Plasma U							6	
M2-Plasma V								
M2-Plasma W								
M2-Planna W			• • • • • • • • • •					
	950 9	60	970	540	110	1000	1010	1020
	f ()	11	- tt-	- 1 1		11	1 I	150
N2-Plasma direct	ACACAACATGGAACGAT	MITTATCA	GAGTGCAATA	TGARGGGRC	GECTCTCCAT	STARRATCCC	TTTTGAGATI	LATEGA
M2-Plasma 5								
N2-Plasma 8								
N2-Plasma N								
_								
N2-Plasma N			• • • • • • • • •	• • • • • • • • • •	• • • • • • • • • •	• • • • • • • • •		
N2-Plasma_0			• • • • • • • • • •					
N2-Plasma ()								
N2-Plasma S								
N2-Plasma S1								
N2-Plasma U								
N2 -Plasma V								
_			• • • • • • • • • •					
N2 Plasma W		• • • • • • • •	• • • • • • • • •	• • • • • • • • • •	• • • • • • • • •	• • • • • • • • • •		
	1020 14	40	1050	1060	1670	1080	1840	1100
	the said and the said							
N2-Plasma direct	TTTGGAAAAAAGATATG	PCTTAGGC	CECCTEATCE	CHGTCARCCC	ANTIGTANCE	GALARIGATA	SCCCASTCA	CRIRG
N2-Plasma 5								
N2-Plasma 8								
N2-Plasma K								
_	• • • • • • • • • • • • • • • • • • • •							
N2-Plasma_N								
N2-Plasma_0								
N2-Plasma (<i></i>	1	1				
M2-Plasma S		<i></i> .						
N2-Plasma S1								
N2-Plasma U								
N2-Plasma V								
_	• • • • • • • • • • • • • • • • • • • •							
N2-Plasma W	• • • • • • • • • • • • • • • • • • • •		• • • • • • • • • •			• • • • • • • • •		
	1114 11	120	1170	1140	1150	1160	1170	
	Tread on the						1	
N2-Plasma direct	AMECHEANCCCCCATTC							
N2-Plasma 5								
_								
N2-Plasma 8								
N2-Plasma_M	• • • • • • • • • • • • • • • • • • • •							
N2-Plasma_N								
N2-Plasma_0				G				
M2-Plasma Q								
N2-Plasma S								
N2-Plasma S1								
N2-Plasma U								
	• • • • • • • • • • • • • • • • • • • •							
N2-Plasma V		• • • • • • • •	• • • • • • • • •					
M2-Plasma W								

	750 000 438 020 430 646 650 460
N2-PBMCs direct	THERECHETCHEGGREGETHERETECHERETECTERGETHERETECTECTECTECTECTECTECTECTECTECTECTECTEC
M2-PENCS 1	THE INCIDENCE HER BEAUTICE AND THE TENEFORM TO THE TENEFORM THE TENEFORM TO THE THEORY OF THE TENEFORM TO THE THEORY OF THE TENEFORM TO THE TENEFORM TO THE THEORY OF THE TENEFORM TO THE TENE
N2-PBMCs 4	
M2 - PBMCs 5	
M2-PBMCs A	
M2-PBMCs C	
M2-PBMCs D	
M2-PERCs E M2-PERCs F	
M2-PBMCs G	
N2 PBMCs 22	
N2-PBMC= 23	
M2 - PERCs 24	
M2-PEMCs 25	
	Domain III
	\$70 48 \$900 \$1A \$20 \$70 \$40
M2-PBMCs direct	GRATGGACRAGCTACAGCTTARAGGARTGTCATACTCTATGTGCACAGGARAGTTTARAGTTGTGARGGARATAGCAGAR
N2 PBMCs 1	
H2 PBMCs 4	
M2-PBRCs_5	
M2-PERCS A	
N2-PBMC= C N2-PBMC= D	
M2-PENCE E	
M2 PEMCs P	
M2-PHMCs G	
M2 PENCs 22	
M2 FENCE 23	
M2-PENCs 24	6
N2-PBMCs_25	
	984 980 970 900 990 1000 1010 1020
M2-PHMCs direct	ACRCAM ATGGRACGAT AGT TATCAGAGTGCAAT ATGRAGGGGACGGCTCTCCATGTAARATCCCTTTTGAGATAATGGA
M2 - PEMCs 1	
N2-PBNCs 4	
M2-PBMCs_5	••••••
M2-PENCs A M2-PBNCs C	•••••••••••••••••••••••••••••••••••••••
M2-PBMCs D	6
N2-PENCS E	
M2 - PRINCE F	
M2-PBMCs_G	
M2-PEMCs 22	
M2-PBMCs_23	
N2-PBMCs 24 N2-PBMCs 25	
MS-MONES 23	
	1030 1040 1050 1050 1070 1070 1070
M2 PBMCs direct	TTTGGRARAMGETATGTCTTAGGCCGCCTGATCACAGTCARCCCARTTGTAMCAGARARGATAGCCCAGTCANCATAG
N2-PBMCs_1 N2-PBMCs_4	
M2-PRINCE 5	······································
N2-PBHCs A	
M2-PBMCs C	***************************************
M2 - PENCs_D	а
N2-PBMCs E N2-PBMCs F	
M2-PBMCs G	
M2 PBMCs 22	
M2-PEMCs 23	
N2 PRINCE 24	
M2 PBMCs 25	
	1110 1120 1120 1140 1150 1160 1176
M2-PBMCs direct	ARGCAGARCCCCCATTCGGAGACAGCTACATCATCATCATCATGAGAGCCGGGACAACTGAAGCTCAAC
M2-PEDICE 1	
M2-PBMCs 4	***************************************
M2-PEMCs_5	c.
M2 - PEDICE A	***************************************
M2-PENCs C M2-PENCs D	***************************************
M2-PERCE E	TT
M2-PERCS P	
N2-PEDOCE G	***************************************
M2-PBMCs_22	
M2-PEDICs 23	······································
M2-PB96cm 24	***************************************

	796	800	810	010	976	840	450	850
N2-Saliwa direct	TACAGCACTCACAGG				ADACTTCCTCT			
M2-Saliva 1	I MC MOC MC I C MC MOO!	OCC MC NOWS	IMI CCHMMI	O'I CO'I CHOO	MARKETI GC I CI	Carcatoraca	ICI CAMOTOCA	DUCTOR
N2-Saliva !								
N2-Saliva 3								
W2-Saliwa 4								
N2-Saliva 9								
M2-Saliva 6								
W2-Saliva 7								
N2-Saliva 8						T .		
M2-Saliwa 9								
W2-Saliva 10								
N2-Saliva 11								
M2-Saliva 12								
		Doma	in III					
	676	884	Fugu	940	910	920	539	944
		de la lata		1				10
N2-Saliva direct	GARTGGACAAGCTAC	AGCTTARAGG	GAATGTCAT	ACTCTATGT	GCACAGGAAAG	TTAAAGTTG	TGAAGGAAATA	GCAGAA
N2-Saliva_i								
M2 Saliwa :								
N2-Saliva 3								
N2-Saliva 5								
N2-Saliva 6								
M2-Saliva 7								
M2-Saliwa II							E	
N2-Saliwa 9								
M2-Saliwa 10					T			
W2-Saliwa 11								
N2-Saliwa 12								
	954	968	970	111	210	1000	1010	1010
	- I F I	1 1	1 1	1	1 1 1	- 1 - 3		1
M2-Saliva_direct	ACRCARCATGGARCG	ATAGTTATCI	AGAGTECAA	Tatgaaggg	GACGGCTCTCC	atgtraratc	CCTTTTGAGAT	AATGGA
H2-Saliva_1								
M2-Saliva_2								
M2-Saliva 3	************							
N2-Saliva 4	************						• • • • • • • • • • •	
N2-Saliva 5			******					
H2-Saliva_6 H2-Saliva 7								
M2-Saliva 8				* * * * * * * * *			*******	
N2-Saliva 9								
N2-Saliva 10								
N2-Saliva 11	************						* * * * * * * * * * * *	
M2-Saliva 12								
	1030	1840	1050	1060	1678	1080	1090	1100
				1				1204
N2-Saliva direct	TTTGGAAAAAAGATA	GTCTTAGG	CCGCCTGAT	CACAGTCAA	CCCAATTGTAA	CAGAAAAAGA	TAGCCCAGTCA	ACATAG
N2-Saliva_1								
H2-Saliva 2	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · ·						
N2-Saliva 3	* * * * * * * * * * * * * * * * * * * *					*******	* * * * * * * * * * *	
N2-Saliva_4 N2-Saliva_5	*************		• • • • • • • •					
N2-Saliva 6	***********	*******				*******	* * * * * * * * * * *	
N2-Saliva 7	*************					* * * * * * * * * *	* * * * * * * * * * *	
N2-Saliva 8	*************							
N2-Saliva 9								
N2-Saliva 10	***************							
N2-Saliva 11	************							
N2-Saliva 12	* * * * * * * * * * * * * * * * * * * *							
The state of the s								
	1119	1110	1130	1140	1156	1168	1176	
Control of the control		.1.,	1	1	1 1 1		1	
N2-Saliva_direct	AAGCAGAACCCCCAT!	CGGAGACA	CTACATCA	TCATAGGAG	TAGAGCCGGGA	CARCTGARGC	TCAAC	
N2-Saliva 1	***********							
N2-Saliva_2	***********							
N2-Saliva 3								
N2-Saliva 4	***********							
N2-Saliva 5	************							
N2-Saliva 6 N2-Saliva 7	*************							
N2-Saliva 8	*************							
N2-Saliva 9								
N2-Saliva 10	**************							
N2-Saliva 11	***********							
W2-Saliva 12	*************							

	750 868 826 426 420 440 850 860
N2-Urime direct	THE RECLETION OF RECOVERED ATTECHNET CONTROL OF THE STATE
N2-Urine 1	
N2-Urine 2	
M2-Urime_3	
N2-Urime_4	
M2-Urime_6	
N2-Urine_?	
N2-Urime_8	
N2-Urine_9	
N2-Urine 18	•••••
W2-Urine 11 W2-Urine 12	
N2-Urine L	
N2-Urime 22	
M2-UIIME 22	
	Domain III
	170 100 100 100 100 100 100 100 100 100
N2-Urime direct	CHICCH CARCIACIACIACIACICATA ANG ANTO CATACICA ANG TO CARCALACTUTA ANG TIGHT CHICAGA ANT ACCIONA
N2-Urime 1	
N2-Urine 2	
M2-Urime 3	c
N2-Urime_4	
N2-Urime_6	
N2-Urime_?	
M2-Urime_8	
N2-Urime_9	
N2-Urine_10	
M2-Urime_11	
M2-Urime 12 M2-Urime L	
N2-Urine 22	
M2-011BE 22	
	150 160 170 100 110 1000 1010 1020
M2-Urime direct	MORNING MESTAGEN ASTATIONS ASSESSED AND MESTAGE ASSESSED
M2-Urime 1	
M2-Urine 2	
M2-Urine 3	
M2-Urine_4	
M2-Urime_6	
N2-Urime_7	
M2-Urine_6	C
N2-Urime_9	•••••••••••••••••••••••••••••••••••••••
M2-Urime_10	
N2-Urine 11	•••••••••••••••••••••••••••••••••••••••
N2-Urime 12 N2-Urime L	***************************************
M2-Urine 22	***************************************
MY-OLIME 22	***************************************
	1020 1040 1050 1050 1070 1030 1050 1100
N2-Urine direct	TTTGGAAAAAAAGATATGTCTTAGGCCGCCTGATCACAGTCARCCCARTTGTAACAGAAAAAGATAGCCCAGTCARCATAG
H2-Urine_1	T
N2-Urime_2	***************************************
M2-Urime_3	
M2-Urine_4	
N2-Urime_6	***************************************
N2-Urine_7	***************************************
N2-Urime_8 N2-Urime_9	***************************************
M2-Urine 10	•••••••••••••••••••••••••••••••••••••••
N2-Urime 11	***************************************
N2-Urine 12	***************************************
N2-Urine L	***************************************
M2-Urine 22	
-	
	1118 1120 1130 1140 1150 1150 1170
	teles of sevel seeds of ever for extension of the extension for extensio
M2-Urime_direct	ARGCAGRACCCCCRTTCGGAGACAGCTACATCATCATAGGAGTAGAGCCGGGACAACTGAAGCTCAAC
M2-Urime_1	······································
N2-Urime_2	
M2-Urine_3	
N2-Urime_4 N2-Urime 6	
N2-Urine 7	•••••••••••••••••••••••••••••••••••••••
M2-Urine 8	••••••••••
N2-Urine 9	
N2-Urine 10	
N2-Urine 11	***************************************
N2-Urine 12	***************************************
M2-Urine L	***************************************
M2-Urine 22	*******************

	730 800 810 820 820 840 450 860
N2 2-PBMCs direct	TACAGCACTCACAGGAGCCACAGAAATCCAAATGTCGTCAGGAAACCTGCTCTTCACTGGACATCTCAAGTGCAGGCTG
N2 2-PBMCs 1	1
N2 2-PENCs 2	
M2 2-PBMCs 3	
N2 2-PBMCs 4	
W2 2-PBNCs 5	
H2 2-PBMCs 6	
N2 2-PEMCs 7	G
N2 2-PERICS F	,0
N2 2-PBMCs 10	
N2_2-PEMCs_11	
M2_2-PBMCs_12	
N2 2-PBNCs 21	••••••••••••••••••••••••••••••
N2 2-PBNCs 22	CGG
	Domain III
	974 48 900 918 924 518 540
N2 2-PBHCs direct	GRRTGGRCARGCTACHGCTTARAGGRATGTCATACTCCRTGTGCRCAGGRARGTTTARAGTTGTGRAGGRARTAGCRGAI
N2 2-PBMCs 1	T
N2 2 - PBMCs 2	
N2 2-PBMCs 3	
N2 2-PBMCs 4	- G
N2 2-PBMCs 5	
N2 2-PHMCs 6	
162 2 - PRMCs 7	
N2 2-PEMCs 8	***************************************
N2 2-PBNCs 10	
N2 2-PBMCs 11	
N2 2-PBMCs 11	
	•••••
N2 2-PBHCs 21	
N2 2-PERICS 22	
	958 960 970 960 990 1000 1010 1050
	death of the facility of the facility of
N2_2-PBMCs_darect	MCACANCATGGANCGATAGTCATCAGAGTGCAATATGAAGGGGATGGCTCTCCATGTAAAATCCCTTTTGAGATAATGG
N2_2-PENCs_1	
N2 2-PBMCs 2	
M2 2-PEMCs 3	
N2 2-PBMCs 4	
N2 2-PBMCs 5	
N2 2-PBMCs 6	
M2 2-PRMCe 2	
N2 2-PBMCs 7	***************************************
M2 2-DERMOR B	***************************************
N2 2-PBMCs 10	
H2 2-PBMCs 0 H2 2-PBMCs 10 H2 2-PBMCs 11	
N2 2-PEMCs 10 N2 2-PEMCs 11 N2 2-PEMCs 11 N2 2-PEMCs 12	
N2 2-PENCs 10 N2 2-PENCs 11 N2 2-PENCs 11 N2 2-PENCs 12 N2 2-PENCS 21	
N2 2-PEMCs 10 N2 2-PEMCs 11 N2 2-PEMCs 11 N2 2-PEMCs 12	
N2 2-PENCs 10 N2 2-PENCs 11 N2 2-PENCs 11 N2 2-PENCs 12 N2 2-PENCS 21	
N2 2-PENCs 10 N2 2-PENCs 11 N2 2-PENCs 11 N2 2-PENCs 12 N2 2-PENCS 21	
N2 2-PENCs 2 N2 2-PENCs 10 N2 2-PENCs 11 N2 2-PENCs 12 N2 2-PENCs 21 N2 2-PENCs 21 N2 2-PENCs 22	1070 1040 1050 1060 1070 1000 1090 1100 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
N2 2-PENCs 0 N2 2-PENCs 10 N2 2-PENCs 11 N2-2-PENCs 12 N2 2-PENCs 21 N2 2-PENCs 22 N2 2-PENCs 22	1879 1849 1858 1860 1870 1888 1890 1100 TITGGRARARGATRICICITAGGCCGCCTGRICACAGTCARCCCRATTGTARCAGARARAGACAGCCCAGTCARCRIM
N2 2-PBMCs 0 N2 2-PBMCs 10 N2 2-PBMCs 11 N2-2-PBMCs 12 N2 2-PBMCs 21 N2 2-PBMCs 22 N2 2-PBMCs 22 N2 2-PBMCs 22	1870 1840 1859 1840 1870 1180 1180 1180 TTTGGAARARGATATGTCTTAGGCCGCCTGATCACAGTCAACCCCAATTGTAACAGARAAGGACAGCCCAGTCAACATAC
N2 2-PENCS 2 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 22	1826 1848 1858 1860 1870 1888 1890 1100 TTTGGRARAAGRIRAGCTTAGGCCGCTGRTCACAGTCARCCCARTTGTARCAGARAAGACAGCCCAGTCARCRIM T.
N2 2-PENCs 10 N2 2-PENCs 10 N2 2-PENCs 11 N2 2-PENCs 12 N2 2-PENCs 21 N2 2-PENCs 22 N2 2-PENCs 22 N2 2-PENCs 22 N2 2-PENCS 1 N2 2-PENCS 1 N2 2-PENCS 1 N2 2-PENCS 3	1870 1840 1859 1840 1870 1180 1180 1180 TTTGGAARARGATATGTCTTAGGCCGCCTGATCACAGTCAACCCCAATTGTAACAGARAAGGACAGCCCAGTCAACATAC
M2 2-PENCS 0 M2 2-PENCS 10 M2 2-PENCS 11 M2-2-PENCS 12 M2 2-PENCS 21 M2 2-PENCS 22 M2 2-PENCS 22 M2 2-PENCS 3	1870 1840 1858 1840 1870 1888 1890 1100 TTTGGRARARGRIRTGTCTTAGGCCGCCTGRTCACAGGTCARCCCARTTGTARCAGGARARAGACAGCCCAGTCARCATM T
M2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2-2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 M2 2-PENCS 22 M2 2-PENCS 2 N2 2-PENCS 3	1070 1040 1050 1040 1070 1000 1090 1100 THIGGRARARAGATRIGECTIAGGCCGCCTGRTCACCAGTCAACCCCAGTTGRACAGARARAGACAGCCCAGTCAACATAA T
N2 2-PENCS 2 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 2 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 5	1870 1840 1858 1840 1870 1888 1890 1100 TTTGGRARARGRIRTGTCTTAGGCCGCCTGRTCACAGGTCARCCCARTTGTARCAGGARARAGACAGCCCAGTCARCATM T
M2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2-2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 M2 2-PENCS 22 M2 2-PENCS 2 N2 2-PENCS 3	1870 1840 1858 1840 1870 1888 1890 1100 TTTGGRARARGRIRTGTCTTAGGCCGCCTGRTCACAGGTCARCCCARTTGTARCAGGARARAGACAGCCCAGTCARCATM T
N2 2-PENCS 2 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 2 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 5	1879 1849 1858 1860 1870 1888 1899 1100 TTTGGRARARGETRTGTCTTAGGCCGCCTGRTCACCGTCARCCCARTTGTARCAGARARAGACAGCCCAGTCARCATM T.
N2 2-PENCS 2 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 2 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 4 N2 2-PENCS 5 N2 2-PENCS 6 N2 2-PENCS 7	1070 1840 1859 1460 1670 1608 1690 1160 TTTGGGARARAGATATGTCTTAGGCCGCCTGATCACAGTCARCCCAATTGTARCAGARARAGACAGCCCAGTCARCATM T
N2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 6 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 7	1820 1840 1858 1868 1870 1888 1850 1180 TTTGGRARARGATATGTCTTAGGCCGCCTGATCACAGTCARCCCCAGTCARCATAGT T
N2 2-PENCS 2 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 2 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 6 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 8 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 8 N2 2-PENCS 10 N2 2-PENCS 10	1826 1848 1858 1866 1876 1888 1890 1100 TTTGGRARARAGATRTGTCTTAGGCCGCTGRTCACAGTCARCCCARTTGTARCAGARARAGACAGCCCAGTCARCATM T.
N2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 4 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 6 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 11	1676 1846 1858 1846 1876 1888 1890 1146 THTGGRARARAGATRTGTCTTAGGCCGCCTGRTCACCCCAGTCAACCCCAGTCACCCAGTCAACCAGARARAGACAGCCCAGTCAACATAA T
N2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 1	1870 1840 1858 1860 1870 1888 1890 1180 THTGGRARARAGATHTGTCTTAGGCCGCCTGHTCACAGTCARCCCAATTGTARCAGARARAGACAGCCCAGTCARCHTM T
N2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 4 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 6 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 11	1676 1846 1858 1846 1876 1888 1890 1146 THTGGRARARAGATRTGTCTTAGGCCGCCTGRTCACCCCAGTCAACCCCAGTCACCCAGTCAACCAGARARAGACAGCCCAGTCAACATAA T
N2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 1	1879 1848 1858 1868 1878 1888 1859 1100 TTTGGRARARGHTRTGTCTTRGGCCGCCTGRTCRCGGTCRCCCCRTTGTRACMGARARGMCRGCCCAGTCRACATM T.
N2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 1	1870 1840 1858 1860 1870 1888 1890 1140 THTGGRARARAGATATGTCTTAGGCCGCCTGATCACAGTCAACCCCAATTGTAACAGARAAAGACAGCCCAGTCAACATM T
N2 2-PENCS 0 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 1 N2 2-PENCS 1 N2 2-PENCS 1 N2 2-PENCS 2 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 1 N2 2-PENCS 1 N2 2-PENCS 1 N2 2-PENCS 1 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 21 N2 2-PENCS 22	1879 1848 1858 1868 1878 1898 1899 1100 TTTGGRARARGRITHTGTCTTAGGCCGCCTGHTCACAGTCARCCCARTTGTARCAGARARAGACAGCCCAGTCARCATM T.
N2 2-PENCS 2 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 6 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 11 N2 2-PENCS 22	1826 1848 1858 1868 1878 1888 1890 1100 TTTGGRARMAGRITATGTCTTMGGCCGCCTGATCMCAGTCAACCCCAATTGTAMCAGARARAGACMGCCCAATTCAMCATM T. 1110 1118 1120 1148 1158 1158 1158 1159 AMGCMGRARCCTCCATTTGGRGCGGGCTACCATCATCATCATGGGGGTTGAGCCCGGGRCAACTGRAGCTCAAC
N2 2-PENCS 2 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 2 N2 2-PENCS 2 N2 2-PENCS 3 N2 2-PENCS 3 N2 2-PENCS 5 N2 2-PENCS 5 N2 2-PENCS 7 N2 2-PENCS 7 N2 2-PENCS 8 N2 2-PENCS 10 N2 2-PENCS 10 N2 2-PENCS 11 N2 2-PENCS 12 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 21 N2 2-PENCS 22 N2 2-PENCS 22 N2 2-PENCS 22	1876 1848 1855 1846 1876 1888 1890 1140 TITGGRARARGATRIGICTINGGCCGCCTGGTCACCGCAGTCARCCCCAGTCARCATM T. 1110 1110 1110 1110 1110 1146 1150 1160 1170 ARGCIGRACCTCCATTIGGGCGCCTRCATCATCATGGGGTTGGGCCGGGCCAGCTCAGCCCCCCCC
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N2-Plasma_direct	TALTGATEIQHSSGNULFTGALKCRURDHLQLKGNSYSHCTGRPKVVREIAETQHGTIVIRVQYEGDGSPCKIFFEIDD
N2-Plasma_5	
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N2-Plasma H	***************************************
N2-Plasma N	
M2-Plasma O	
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W2-Plasma S	
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	Domain III
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N2-PBMCs 1 N2-PBMCs 4	TALTGATE LOGIS SGALLPT CALKCELBOOKLOLKGASYSHCTGARKAVARE LAET (HIGT LV LEVOLYZEGDGS PCKL PYE DO
N2-PBMCs 1 N2-PBMCs 4 N2-PBMCs 5	TALTGATE LORS SCHILLPT CALKCELENDRLOLKGINSYSHCTCREKVVIGE I RETOHGT LVIRVOYZEGDGS PCKI PPE IDD
N2-PBMCs 1 N2-PBMCs 4 N2-PBMCs 5 N2-PBMCs A	TALTGATE LORS SCHILLPT CALKCELENDRLOLKGINSYSHCTCREKVVIGE I RETOHGT LVIRVOYZEGDGS PCKI PPE IDD
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N2-PENCS 1 N2-PENCS 4 N2-PENCS 5 N2-PENCS A N2-PENCS C N2-PENCS D N2-PENCS E N2-PENCS E N2-PENCS G N2-PENCS G N2-PENCS 23 N2-PENCS 24 N2-PENCS 25 N2-PENCS 25 N2-PENCS 5 N2-PENCS 6	TALTGATE I ONS SON LEPT SMLKCELENDEL QUERONS Y SMCTCREEK VINE I NET (DAGT I VIRVO) Y 250 CKI PPE TOD P
N2-PENCS 1 N2-PENCS 4 N2-PENCS 5 N2-PENCS A N2-PENCS D N2-PENCS D N2-PENCS E N2-PENCS E N2-PENCS 22 N2-PENCS 23 N2-PENCS 24 N2-PENCS 25 N2-PENCS 25 N2-PENCS 5 N2-PENCS 6 N2-PENCS 7 N2-PENCS 6 N2-PENCS 7 N2-PENCS 7 N2-PENCS 8 N2-PENCS 8 N2-PENCS 8 N2-PENCS 8	TALTGATE I ONS SGRILLPT ONLIKCELENDEL QUERONS Y SHICT GREEK VIVE I NET ONG TI I VID VOY PEGGS P CKI PPET DO P
N2-PENCS 1 N2-PENCS 4 N2-PENCS 5 N2-PENCS A N2-PENCS D N2-PENCS E N2-PENCS E N2-PENCS G N2-PENCS 22 N2-PENCS 23 N2-PENCS 24 N2-PENCS 24 N2-PENCS 25 N2-PENCS 3 N2-PENCS 5 N2-PENCS 6 N2-PENCS 7 N2-PENCS 6 N2-PENCS 6 N2-PENCS 7 N2-PENCS 6 N2-PENCS 7	TALTGATE I DAS SGALLPT GALKCELRODEL QLERONS YSACTGRETKUVRE I NET (DAGT I VIEV (TYDEGOES P CKI PPET DO P S 150 150 170 180 180 190 180 190 180 180 18
N2-PENCS 1 N2-PENCS 4 N2-PENCS 5 N2-PENCS A N2-PENCS D N2-PENCS E N2-PENCS E N2-PENCS G N2-PENCS 22 N2-PENCS 23 N2-PENCS 24 N2-PENCS 25 N2-PENCS 25 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 6 N2-PENCS C	TALTGATE I ONS SGRILLPT ONLIKCELENDEL QUERONS Y SHICT GREEK VIVE I NET ONG TI I VID VOY PEGGS P CKI PPET DO P
N2-PENCS 1 N2-PENCS 4 N2-PENCS 5 N2-PENCS A N2-PENCS D N2-PENCS E N2-PENCS E N2-PENCS G N2-PENCS 22 N2-PENCS 23 N2-PENCS 24 N2-PENCS 24 N2-PENCS 25 N2-PENCS 3 N2-PENCS 5 N2-PENCS 6 N2-PENCS 7 N2-PENCS 6 N2-PENCS 6 N2-PENCS 7 N2-PENCS 6 N2-PENCS 7	TALTGATE I DAS SGALLPT GALKCELRODEL QLERONS YSACTGRETKUVRE I NET (DAGT I VIEV (TYDEGOES P CKI PPET DO P S 150 150 170 180 180 190 180 190 180 180 18
N2-PENCS 1 N2-PENCS 4 N2-PENCS 5 N2-PENCS A N2-PENCS D N2-PENCS E N2-PENCS E N2-PENCS G N2-PENCS 22 N2-PENCS 23 N2-PENCS 24 N2-PENCS 25 N2-PENCS 25 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 5 N2-PENCS 6 N2-PENCS C	TALTGATE LONG SCALL PT GALKCEL PROBLEL ROBES X SHCTCR PK VVRE LAET (HGT LV DRVITY DGDGS P CKI PFE DDD P S S LENGYVLGR. IT VNP LVTENOS PVNTE HE PPFGD SYT LIGVE PGQLKLM C C N A

	Domain III
	270 200 290 110 110 320 390 240
N2-Saliva_direct	TALTGATEIQHSSGHLLFTGHLKCRLINDKLQLKGHSYSMCTGRCKVVREIAETQHGFIVIRVQYEGDGSPCKIPFEIDD
W2-Saliva_1	
N2-Saliva 2	
N2-Saliva 3	
N2-Saliva 4	F
M2-Saliva 5	
W2-Saliva 6	
N2-Saliva 7	
- mark	
N2-Saliva_8	
N2-Saliva_9	
N2-Saliva 10	
N2 Saliva 11	
N2-Saliva 12	
-	
	250 250 270 280 290
	E
M2-Saliva_direct	LEIGRYVLGRE, I TVIIP IVTIEIGOS PVNTE REPPP GOSYT I I GVE PGULKEN
W2-Saliva_1	* SZONGRZYSTAT SZXEKSTATSZXEXSKÓRGSZ *
N2-Saliva 2	
W2-Saliva 3	
M2-Saliva 4	
N2-Saliva 5	
N2-Saliva 6	
N2-Saliva_7	• • • • • • • • • • • • • • • • • • • •
M2-Saliva_8	
N2-Saliva 9	
N2-Saliva 10	
W2-Saliva 11	
N2-Saliva 12	
MS-24TIA4 15	
	Domain III
	276 288 290 268 710 228 720 240
	614 746 734 746 770 774 776 786
310 10	adjusting all and as less to a facility of a standard and and
N2-Urine direct	TALTGATEIQNSSGNULFTGNUKCRLENDKLQLKGNSYSNCTGKFKVVKEIAETQHGTIVIRVQYEGDGSFCKIPFEIDD
M2-Urine direct	
N2-Urine_1	TALTGATE I QNSS GNELLFT GNELKCRUPNOKLQLKGNSYSMCTGKFKVVKEIAETQHGT I VIR VQYEGDGSPCKIPFEIDD
N2-Urine 1 N2-Urine 2	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSNCTGRFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V.
M2-Urine_1 M2-Urine_2 M2-Urine_3	TALTGATEIQNSSGNLLFTGNLKCRLENDKLQLKGNSYSNCTGRFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V
N2-Urine 1 N2-Urine 2	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSNCTGRFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V.
M2-Urine_1 M2-Urine_2 M2-Urine_3	TALTGATEIQNSSGNLLFTGNLKCRLENDKLQLKGNSYSNCTGRFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V. T.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 18	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V. T.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V. T.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12	TALTGATEIQNSSGNLLFTGALKCRLRADKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine L	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V. T.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine L	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V. T.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine L	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V. T.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine L	TALTGATEIQNSSGNLLFTGALKCRLRADKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T H.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine L	TALTGATEIQNSSGNLLFTGHLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V H 350 360 370 310 314 320
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2	TALTGATEIQNSSGNLLFTGNLKCRLENDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 11 N2-Urine 12 N2-Urine 22 N2-Urine 22	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 22 N2-Urine 22 N2-Urine 22	TALTGATEIQNSSGNLLFTGNLKCRLENDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 11 N2-Urine 12 N2-Urine 22 N2-Urine 22	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 22 N2-Urine 22 N2-Urine 22	TALTGATEIQNSSGNLLFTGNLKCRLRMDRLQLKGNSYSMCTGRFKVVREIAETQHGTIVIRVQYEGDGSPCKIPFEIND V T M LEIGYVLGRLITVIP IVTEXDSPVNIEAEPFF CDSYIIIGVEPGURUM V T.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 3 N2-Urine 3 N2-Urine 4	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V T M.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 3 N2-Urine 4 N2-Urine 6	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T M.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 3 N2-Urine 3 N2-Urine 4	TALTGATEIQNSSGNLLFTGNLKCRLRMDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIND V T M.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 3 N2-Urine 4 N2-Urine 6	TALTGATEIQNSSGNLLFTGNLKCRLENDKLQLKGNSYSMCTGKPKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIDD V T M
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 22 N2-Urine 2 N2-Urine 1 N2-Urine 3 N2-Urine 1 N2-Urine 1 N2-Urine 1 N2-Urine 6 N2-Urine 6 N2-Urine 6 N2-Urine 6 N2-Urine 6 N2-Urine 7 N2-Urine 7	TALTGATETQNSSGNLLFTGGLKCRLRADKLQLKGNSYSMCTGRPKVVKETAETQHGTIVTRVQYEGDGSPCKTPFETAD
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 8 N2-Urine 10 N2-Urine 11 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 3 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 6 N2-Urine 7 N2-Urine 0 N2-Urine 9	TALTGATEIQNSSGALLFTGALKCRLFADKLQLKGRSYSMCTGKFKVVREIAETQHGTIVIRVQYEGDGSPCCIPFEIMD V T. H.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 8 N2-Urine 10 N2-Urine 11 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 1 N2-Urine 1 N2-Urine 6 N2-Urine 6 N2-Urine 6 N2-Urine 7 N2-Urine 7 N2-Urine 9 N2-Urine 9 N2-Urine 9 N2-Urine 9	TALTGATEIQNSSGALLFTGALKCRLRADKLQLKGRSYSMCTGRFKVVREIAETQHGTIVIRVQYEGDGSPCCIPFEIND V
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 8 N2-Urine 10 N2-Urine 11 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 3 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 6 N2-Urine 7 N2-Urine 0 N2-Urine 9	TALTGATEIQNSSGALLFTGALKCRLFADKLQLKGRSYSMCTGKFKVVREIAETQHGTIVIRVQYEGDGSPCCIPFEIMD V T. H.
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 8 N2-Urine 10 N2-Urine 11 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 1 N2-Urine 1 N2-Urine 6 N2-Urine 6 N2-Urine 6 N2-Urine 7 N2-Urine 7 N2-Urine 9 N2-Urine 9 N2-Urine 9 N2-Urine 9	TRITGATEIQNSSGRILFTGHEKCRLRIDKLQLKGRSYSMCTGKFKVVKEIAETQHGTIVTRVQYEGDGSPCKIPFEIDD T ST H LENGYVLGRLITVIPIVTEXOSPVNIEMEPPPGDSYIIIGVEPGQLIGUN V T T
N2-Urine 1 N2-Urine 2 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine 12 N2-Urine 2 N2-Urine 2 N2-Urine 2 N2-Urine 3 N2-Urine 3 N2-Urine 4 N2-Urine 4 N2-Urine 6 N2-Urine 7 N2-Urine 7 N2-Urine 9 N2-Urine 9 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 11 N2-Urine 1	TRITGATEIQNSSGRILFTGHLKCRLRIDKLQLKGRSYSMCTGKFKVVKEIAETQHGTIVTRVQYEGDGSPCKIPFEIDD T T S50 360 270 214 129 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
N2-Urine 1 N2-Urine 2 N2-Urine 3 N2-Urine 4 N2-Urine 6 N2-Urine 8 N2-Urine 9 N2-Urine 10 N2-Urine 11 N2-Urine 12 N2-Urine L N2-Urine 2 N2-Urine 2 N2-Urine 1 N2-Urine 3 N2-Urine 3 N2-Urine 6 N2-Urine 3 N2-Urine 6 N2-Urine 6 N2-Urine 6 N2-Urine 7 N2-Urine 9 N2-Urine 9 N2-Urine 10 N2-Urine 10 N2-Urine 1	TRITGATEIQNSSGRILFTGHEKCRLRIDKLQLKGRSYSMCTGKFKVVKEIAETQHGTIVTRVQYEGDGSPCKIPFEIDD T ST H LENGYVLGRLITVIPIVTEXOSPVNIEMEPPPGDSYIIIGVEPGQLIGUN V T T

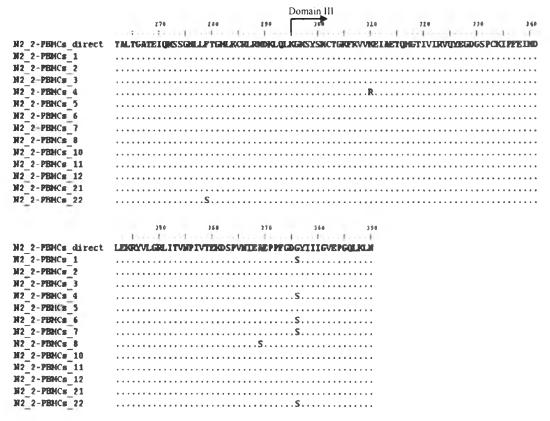


Figure 32: Nucleotide (32A) and amino (32B) sequence alignments of all clones derived from each N2 specimens, N2 plasma (3), N2 PBMCs (3), N2 saliva (3), N2 urine (3) and N2 PBMCs (21), (DENV2 infected patients) present heterogeneous population or quasispecies in different time points. Direct sequencing of each specimen is used as a reference sequence (major population) to compare with all variant sequences or minor populations (10-15 sequences per specimen). The positions of nucleotide and amino acid are based on direct sequencing alignments in figure 28A and 28B. Domain III of E gene is starting from position 883 to 1,180 of nucleotide and position 295-395 of amino acid sequences (complete E gene). Deletion position is presented as "*". In-frame stop codon is presented as "*". The presence of frame-shift mutations were marked as "*". The number in "()" represents the day of specimen collection.

Moreover, some clones contained deletion and in-frame stop codon suggesting the presence of defective viral genome as totally 9.54% of all clones analyzed (in all patients). In single-nucleotide-deleted sequences, frame-shift mutations were found such as in some clones of N12 specimens and febrile saliva of N2 (Figure 31B and 32B). A number of defective clones were different among specimens and time points. Defective clones were commonly found in plasma, PBMCs, saliva and urine of DENV-infected patients. Nevertheless, specimens in some patients such as N20 and

N33 did not contain defective viral genome (data not shown). During febrile period, defective viral genome was mostly found in plasma or PBMCs than in urine and saliva while it was found in PBMCs and urine during convalescent period. Moreover, the presence of defective viral genome randomly occurred and did not depend on serotypes, genotypes and strains of DENV.

Normalized Shannon entropy (S_n) was used as a tool for determining the diversity and complexity of DENV quasispecies in both nucleotide and amino acid levels. The reference range was 0 (no diversity) to 1 (maximum diversity). In mixedserotype-infected specimens, S_n value was calculated in both major serotypes and in minor serotype if there were sufficient clones to investigate. In this study, the S_n value ranged from 0.08 to 0.207 and 0.07 to 0.207 at nucleotide and amino acid levels, respectively in all patients (Table 33). The S_n value did not depend on each serotype. Moreover, the S_n value varied in specimens and time points suggesting the presence of different diversity (quasispecies) of DENV population. In DENVIinfected patients, the high S_n value of nucleotide level was found in febrile plasma and PBMCs indicating the occurrence of high quasispecies complexity during febrile period. Interestingly, the high S_n value was also found in early and late convalescent urine (N12 and N20). In DENV2-infected patients, the high S_n value was mostly found in febrile PBMCs or plasma. However, some patient such as N13 presented the high S_n value in 2nd convalescent urine. DENV3-infected patient (N28) presented the high S_n value in early and late convalescent urine. In mixed-serotype-infected patients (N33, N34 and N40), the high S_n value was found in multi-serotype-infected specimens (analyzed in the major serotype only).

The different S_n values at amino acid level in each specimen and time point confirmed the presence of heterogeneous population or quasispecies and indicated the diversity of E protein, especially on domain III of DENV which was the major antigenic site for host immune recognition. The S_n value at amino acid level was mostly consistence with the results of nucleotide level and varied in each specimen and time point (Table 33). In some specimens, the results were contradicted with the results of nucleotide level suggesting the presence of silently mutated clones resulting in the conservation of E protein among DENV populations such as in early convalescent urine of N12, febrile plasma of N29, 2^{nd} early convalescent urine of

N13, late convalescent urine of N28, 2nd early convalescent urine of N34 and early convalescent PBMCs of N40.

According to previous studies mentioning that the variations of E gene, particularly at domain III affect pathogenesis of DENV infection and are under selection pressure of host immune response, the estimation of dN/dS ratio was investigated using SLAC (single likelihood ancestor counting) method in the webbased Datamonkey (www.datamonkey.org) to explore whether amino acid variations, especially on the domain III of E gene were under the selection pressure [139, 140]. In mixed-serotype-infected specimens, only major serotype populations were examined as well. Moreover, some nucleotide clones containing deletion or in-frame stop codon and minor DENV serotype populations in mixed-serotype-infected specimens were omitted because of the limitation of program requirement.

The results demonstrated that amino acid variations at domain III in all specimens of each patient during febrile, early and late convalescent periods were under either positive (dN/dS > 1), purifying (dN/dS < 1) or neutral selection (dN/dS=1) suggesting that host immune pressure plays a role in genetic variations of DENV populations (Table 33). The range of dN/dS was from 0.20-2.73 in all specimens and time points. Most specimens in all time points were under purifying selection whereas some specimens were under either positive or neutral selection. The patterns of selection pressure in each specimen of individual patient were different depending on each specimen type and time point. For example, amino acid sequence variations of most specimens in N2 patient were under neutral selection whereas the amino acid variations of all specimens in N20 patient were under positive selection. Moreover, mutations of amino acid sequences in all specimens of some patients (N17 and N34) were under purifying selection. The positive selection affecting amino acid changes were mostly found in febrile plasma (N12, N29, N28 and N40) and PBMCs (N5, N10 and N28) as well as in febrile urine (N2, N20 and N40). During early and late convalescent periods, the positive selection was found in PBMCs (N13) and urine (N5, N13, N20, N28 and N33). This finding indicates that host immune response may drive amino acid variations and involve in persistent DENV population. Interestingly, this study demonstrated the purifying selection codon in febrile plasma of N17 at amino acid position 380. The dN/dS ratio could not be investigated in all minor serotype population of mixed-serotype-infected specimens because there were not enough unique sequences to calculate by this algorithm.

Table 33: Diversity (complexity) parameters of nucleotide and amino acid sequences of 13 DENV-infected patients in different specimens and time points

	DOF	Specimens	No. of Nucleotide level			An	nino acid	level	No of	dN/dS	
Code			clones	SS	DS	Sn	SS	DS	S_n	defective	an/as
					}		}			clones	
DENVI-infected patients											
N12	6	plasma (4)	12	3	9	0.184	3	9	0.184	3	1.60
		PBMCs (4)	11	2	9	0.207	2	9	0.207	4	0.53
		urine (12)	11	2	9	0.207	3	7	0179	2	0 90
	1	urine (26)	12	2	10	0 197	2	8	0.175	5	0.57
N20	8	urine (6)	12	7	5	0 112	7	5	0.112	0	1 19
		urine (30)	12	5	7	0 151	6	6	0.132	0	2 07
N29	8	plasma (5)	12	4	8	0 169	6	6	0.132	0	1.80
		PBMCs(5)	12	4	8	0.169	5	7	0.151	0	0.83
		urine (5)	12	5	7	0 151	5	7	0.151	l	0 84
		urine (19)	12	5	7	0.151	8	4	0.095	1	0.41
	DENV2-infected patients										
N2	4	plasma (3)	11	7	4	0.105	7	4	0.105	0	1.00
		PBMCs(3)	13	4	9	0 164	6	7	0.134	2	1.00
		saliva (3)	12	9	3	0 070	9	3	0.070	1	1 00
		urine (3)	13	9	4	0 080	10	3	0.061	0	1.81
		PBMCs (21)	13	7	6	0117	7	4	0.103	0	0.21
N5	7	plasma (6)	12	7	5	0.112	7	5	0.112	3	1.00
		PBMCs (6)	12	5	7	0151	6	6	0.132	1	2.73
		urine (23)	13	5	8	0.150	6	5	0.117	ı	1.40
N10	5	plasma (4)	13	6	7	0 134	8	5	0.099	4	0.20
		PBMCs (4)	12	10	2	0.047	10	2	0.047	0	1 01
		urine (4)	12	11	- I	0 024	11	ī	0.024	0	1.00
'		PBMCs (27)	13	8	5	0 099	10	3	0.061	0	0.56
N13	5	PBMCs (8)	15	3	12	0 166	4	11	0.161	3	1 44
		urine (8)	12	5	7	0 151	5	7	0.151	3	1 00
		urine (15)	12	2	10	0 197	6	6	0.132	3	0 56
N17	7	plasma (7)	12	5	7	0 151	6	6	0.132	2	0.47#
		PBMCs (7)	13	4	9	0.164	5	8	0.132	0	0.47
		saliva (7)	12	10	2	0.104	11	0	0.024	0	0.39
		urine (7)	12	7	5	0 112	7	5	0.024	1	0.60
		urine (13)	12	5	7	0 1 5 1	7	5	0.112	1	0.40
N21	7	PBMCs (7)	12	4	8	0 169	8	4	0.092	0	0.40
		urine (14)	13	9	3	0 072	9	3	0.092		
		21110 (17)			L		1	,	0.072	0	1.80
DENV3-infected patient N28 8 plasma (6) 13 3 10 0.178 6 7 0.134 0 1.85											
1420	0	PBMCs (6)	13	3	10	0 178	6	7	0 134	0	1 85
		FBMC \$ (0)	12	5	7	0 151	8	4	0.092	1	1.14

Code		Specimens	No of	Nucleotide level			Amino acid level			No. of	
	DOF		clones	SS	DS	S"	SS	DS	Sn	defective clones	dN/dS
N28	8	urine (14)	12	3	9	0.184	5	7	0.134	1	0.85
		urine (46)	12	3	9	0.184	6	6	0.132	0	1.17
All N33 specimens were DENV4 except N33 urine (7) was DENV4+DENV2 (major serotype was DENV4)											
N33	6	plasma (7)	14	12	2	0.036	12	2	0.036	0	0.20
		PBMCs (7)	11	9	2	0.036	10	1	0.028	0	1.00
		salıva (7)	12	6	6	0.132	7	5	0.112	0	0.80
		urine (7) [†]	11	5	6	0.151	6	5	0 112	0	1.76
İ		urine (7)	4	3	1	0.141	3	1	0.141	0	ND
		urine (18)	13	8	4	0.091	9	3	0.072	0	0.26
	All N34 specimens were DENV1 except N34 urine (14) was DENV3+DENV1 (major serotype was DENV3)										
N34	6	PBMCs(8)	14	3	11	0.172	5	9	0.147	4	0.46
		saliva (8)	13	4	9	0.164	8	5	0.099	0	0.65
		urine (8)	13	4	9	0.164	6	7	0 134	1	0.53
		urine (14) [†]	14	3	11	0.172	9	5	0.088	0	0.18
		urine (14)*	2	ND	ND	ND	ND	ND	ND	ND	ND
All N40 specimens were DENV1 except N40 PBMCs (21) was DENV1+DENV2 (major serotype was DENV2)											
N40	4	plasma (4)	12	7	5	0 112	7	5	0.112	3	1.80
		PBMCs(4)	15	3	12	0 166	5	10	0.145	3	0.31
		urine (4)	12	7	5	0 112	7	5	0.112	2	1.35
		PBMCs (21) [†]	12	2	10	0.197	5	6	0.142	2	0.11
		PBMCs (21)	1	ND	ND	ND	ND	ND	ND	ND	ND

DOF= duration of fever. ND = not determined.

The number in "()" presents the day of specimen collection.

 S_n = Normalized Shannon entropy.

SS = No. of similar sequences to direct sequencing.

DS= No. of different sequence patterns to direct sequencing.

Defective clone refers to the clone containing nucleotide deletion or in-frame stop codon.

dN/dS > 1 suggests under positive selection and dN/dS < 1 suggests under purifying selection.

[†]=Major population in mixed serotype infections. [•]=Minor population in mixed serotype infections.

[&]quot;Negative selection site was found in this specimen (p<0.1) at amino acid position 380 (codon 119 of 129 amino acid, Ile).

Phylogenetic tree analysis to demonstrate the association of DENV population in different specimens and time points

To characterize the association of each DENV population in different specimens and time points in each patient, phylogenetic tree of nucleotide sequences was done using MEGA program (version 5.0) based on neighbor-joining (NJ) method with Kimura 2-parameter model (1,000 replicates) [141, 142].

The phylogenetic trees of nucleotide sequences from all clones showed the intra- and inter- genetic variations among specimens in each patient (Figure 33-45). Sequences in all patients were similar but not absolutely identical confirming the presence of quasispecies or heterogeneous population, which supported nucleotide and amino acid sequence alignments in the previous experiment. The association among sequences randomly occurred in each patient and there was no specific pattern of this phenomenon. Nucleotide sequences of all clones in most patients were similarity without identical such as in N2, N10, N17, N20, N28, N29, and N40 patients. However, results of some patients showed the identical sequences in the same specimen such as in early convalescent urine of N5, N21 and N33. Moreover, nucleotide sequences of some patients composed of similar and identical sequences in different specimens at the same time point such as in N13 patient (1st early convalescent PBMCs and urine, marked as the red box in Figure 37) and in N34 patient (1st early convalescent PBMCs and saliva, marked as the red box in Figure 44). Nucleotide sequences of one patient (N12) illustrated similar and identical sequences in different specimens and time points (febrile PBMCs and early convalescent urine, marked as the red box in Figure 36) implying the occurrence of persistent DENV population in different time points. Interestingly, the unique DENV sequence was found in early convalescent PBMCs of N2 locating in different clusters of all sequences from N2 specimens (marked as the red box in Figure 33) and one sequence of early convalescent PBMCs was related to most febrile sequences (marked as the red arrow in Figure 33) indicating either coinfection or superinfection and persistent DENV population in PBMCs.

The nucleotide sequences of 3 mixed-serotype-infected patients clearly presented the distinguished serotype (major and minor serotypes) and each serotype

composed of both related and identical sequences indicating DENV quasispecies as well (Figure 43-45).

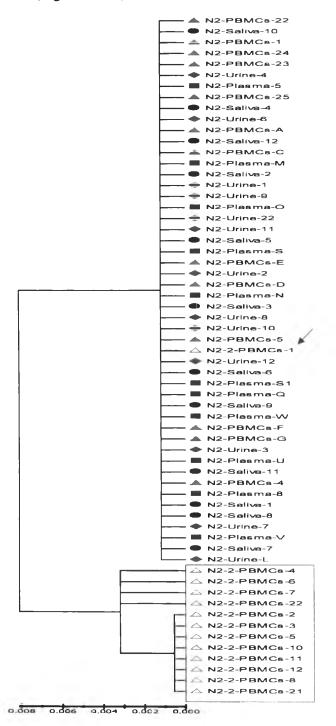


Figure 33: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N2 (DENV2-infected patient). There are febrile (N2 plasma, PBMCs, saliva and urine) and early convalescent (N2-2PBMCs) specimens. The unique DENV population in different time points is marked as the red box.

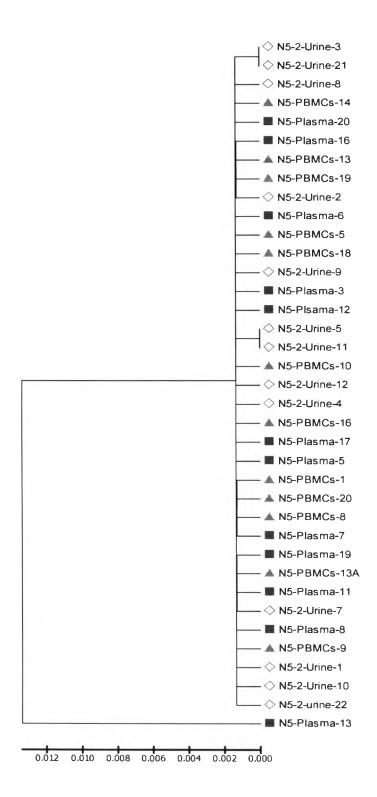


Figure 34: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N5 (DENV2-infected patient). There are febrile (N5 plasma and PBMCs) and early convalescent (N5-2 urine) specimens.

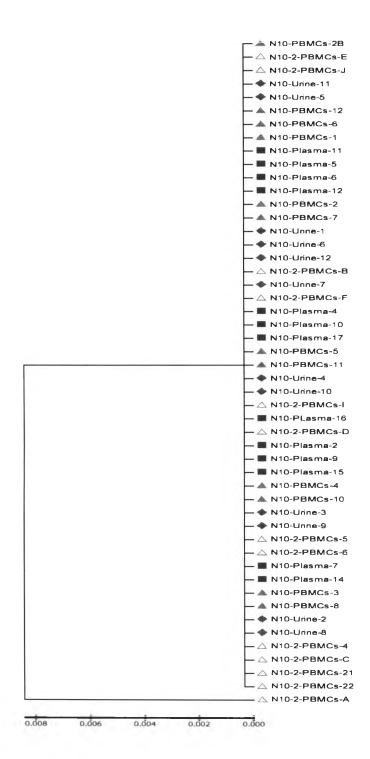


Figure 35: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N10 (DENV2-infected patient). There are febrile (N10 plasma, PBMCs and urine) and late convalescent (N10-2 PBMCs) specimens.

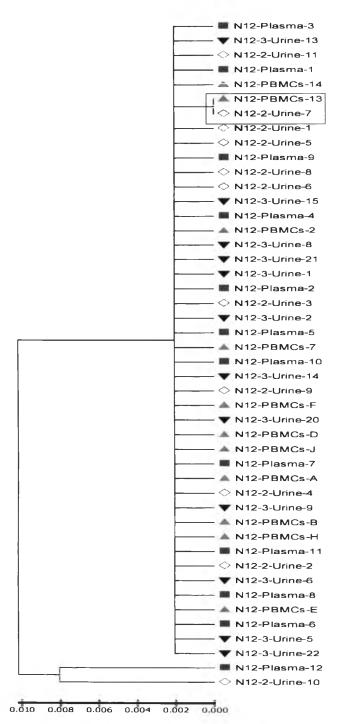


Figure 36: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N12 (DENV1-infected patient). There are febrile (N12 plasma, PBMCs), early convalescent (N12-2 urine) and late convalescent (N12-3 urine) specimens. The same DENV population in different specimens and time points is marked as the red box.

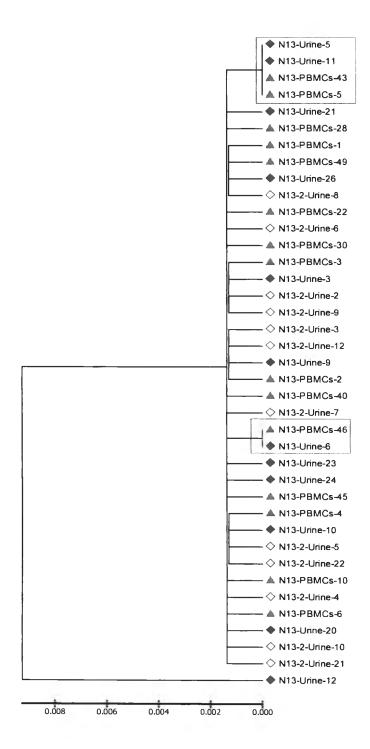


Figure 37: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N13 (DENV2-infected patient). There are 1st early convalescent (N13 PBMCs and urine) and 2nd early convalescent (N13-2 urine) specimens. The same DENV population in different specimens is marked as the red box.

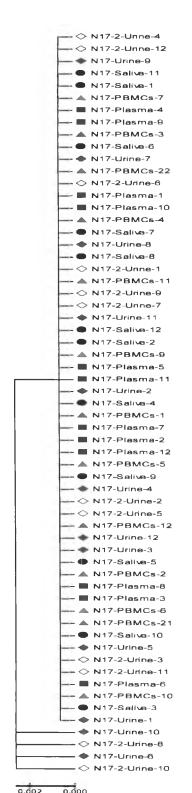


Figure 38: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N17 (DENV2-infected patient). There are febrile (N17 plasma. PBMCs, saliva and urine) and early convalescent (N17-2 urine) specimens.

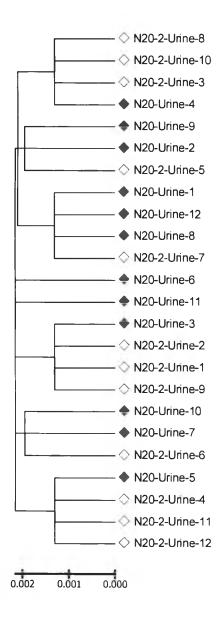


Figure 39: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N20 (DENV- infected patient). There are febrile (N20 urine) and late convalescent (N20-2 urine) specimens.

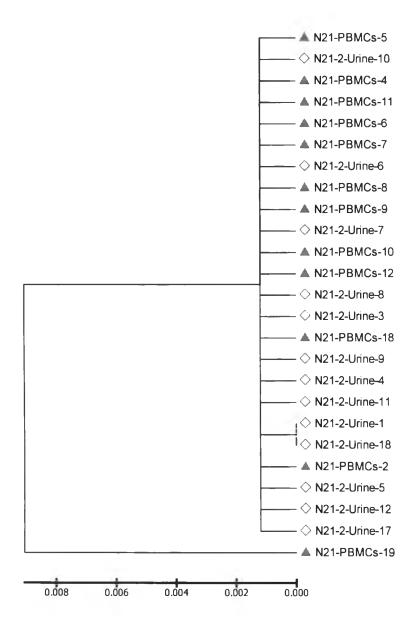


Figure 40: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N21 (DENV2-infected patient). There are febrile (N2 PBMCs) and early convalescent (N21-2 urine) specimens.

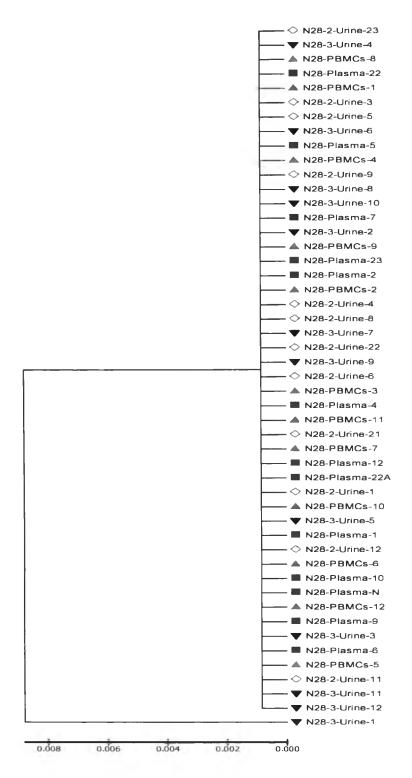


Figure 41: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N28 (DENV3-infected patient). There are febrile (N28 plasma and PBMCs), early convalescent (N28-2 urine) and late convalescent (N28-3 urine) specimens.

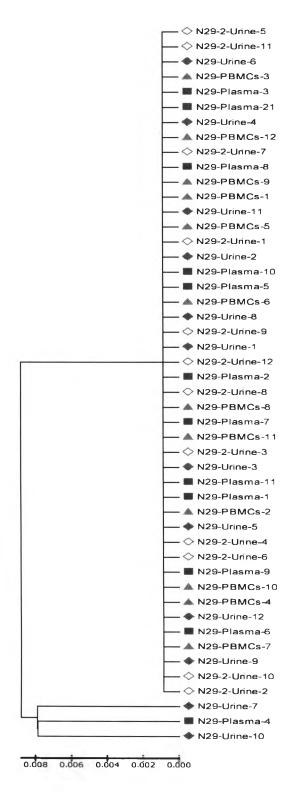


Figure 42: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N29 (DENV1-infected patient). There are febrile (N29 plasma, PBMCs and urine) and early convalescent (N29-2 urine) specimens.

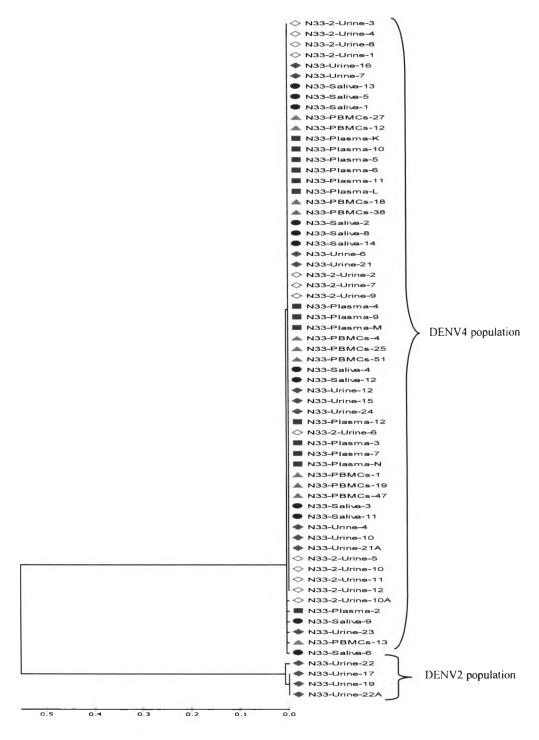


Figure 43: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N33 (DENV4+DENV2-infected patient). There are 1st early convalescent (N33 plasma, PBMCs and saliva: DENV4 and urine: DENV4+DENV2) and 2nd early convalescent (N33-2 urine: DENV4).

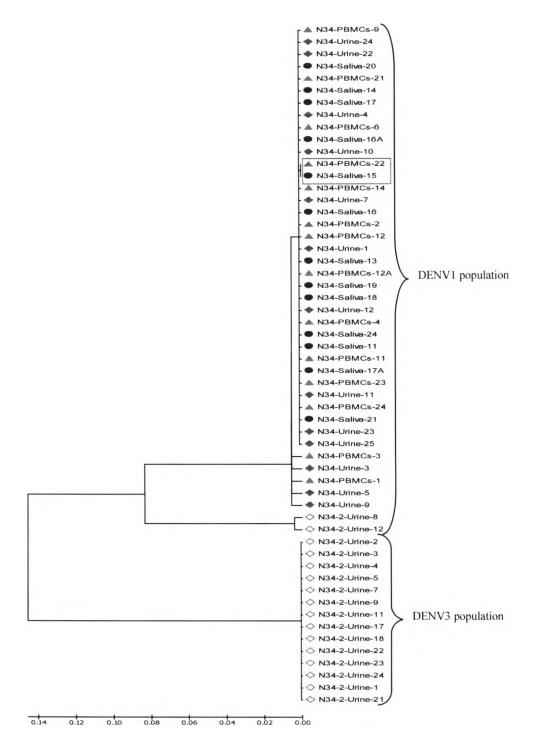


Figure 44: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N34 (DENV1+DENV3-infected patient). There are 1st early convalescent (N34 PBMCs, saliva and urine: DENV1) and 2nd early convalescent (N34-2 urine: DENV3+DENV1). The same DENV population in different specimens is marked as the red box.

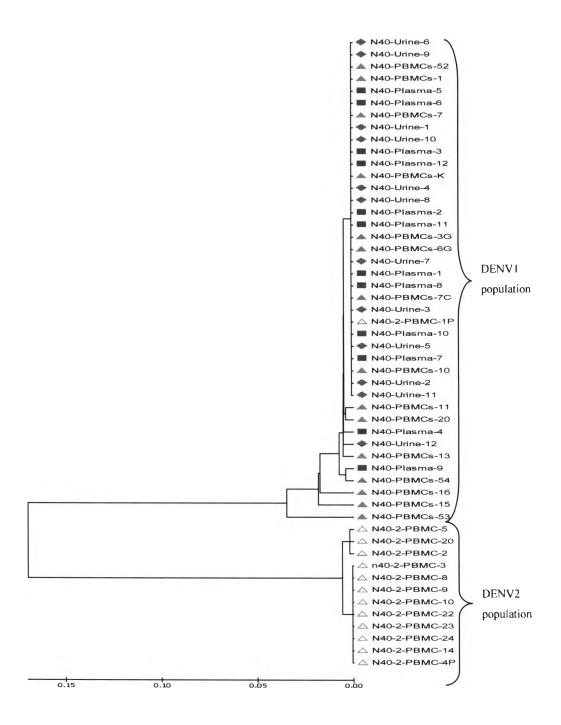


Figure 45: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N40 (DENV1+DENV2-infected patient). There are febrile (N40 plasma, PBMCs and urine: DENV1) and early convalescent (N40-2 PBMCs: DENV2+DENV1).