CHAPTER V

RESULT

1. Demographic data

1.1 HIV-1-seropositive Thais

We recruited 40 subjects into the study and confirmed their HIV status by serology. Since Mellors *et al.* showed that a single measurement of plasma HIV RNA level could predict the subsequent risk of progression to AIDS (95). We therefore grouped 855 HIV-infected donors from the Anonymous Clinic of Thai Red Cross AIDS Research Centre according to their viral loads into quartiles. The HIV-RNA of these 855 HIV-infected donors were ranged from 50 to > 500,000 copies/ml with median of 9,958. The $CD4^+$ T lymphocytes were ranged from 300 to 2,222 cells/mm^3 with median of 430. The HIV-RNA values from HIV-infected subjects with $CD4^+$ T cell counts more than 300 cells/mm^3. were analysed and then adopted the ranges of HIV-RNA to stratified our donors into 4 groups (10 donors per group) (less than 320, 320 to 3,351, 3,352 to 14,176, and more than 14,676 copies/ml) (Table 6 and 7). In the lowest viral load quartile, we could recruit only 5 subjects. The clinical characteristics were summarised in Table 8 and 9 and the averages were summarised in table 13. All 35 HIV-1-seropositive subjects were asymptomatic and antiretrovirals naïve. These subjects included 21 women and 14 men, with a mean age of 34.8 years old (ranging from 18 to 66 years old). Majority of subjects was heterosexuals (31/35 or 88.6 %), 2/35 (5.7%) subjects were homosexual, one (2.9%) subject was intravenous drug user and one (2.9%) subject was HIV infected blood recipient. At the time of enrollment, these subjects have been diagnosed HIV-positive for 1 to 11 year, with a mean of 4.8 years. There were no subjects whose HIV subtype was identified. The $CD4^+$ T lymphocytes were ranged from 306 to 1,318 cells/mm^3 with median of 502. Plasma viral load were ranged from < 50 to 453,456 copies/ml with median of 6,648. There were 18 subjects whose HLA types were identified (Table 9). A written informed consent was obtained from all subjects.

Table 6 Method of quartiles calculation.

Total number (n) = 855		
Minimum	Smallest observation = 1st observation	VL = 50
1st quartile	(n+1)/4 = 214th observation	VL = 319
2nd quartile	(n+1)/2 = 428th observation	VL = 3,351
3rd quartile	3(n+1)/4 = 642th observation	VL = 14,676
Maximum	Largest observation = 855 th observation	VL > 500,000

VL, viral load (copies/ml)

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Table 7 Range of viral load quartiles.

Q1 :	VL < 320 copies/ml. (<50 to 319 copies/ml.)
Q2 :	VL = 320 to 3,351 copies/ml.
Q3 :	VL = 3,352 to 14,676 copies/ml.
Q4 :	VL > 14,676 copies/ml. (14,677 to >500,000 copies/ml.)

Q, quartile; VL, viral load (copies/ml)

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Table 8 Clinical information of HIV-1-seropositive Thais.

Quartile group	Subject no.	Initial name (IN)	HIV status	Gender	Age (year)	Mode Tx	Known seroconversion	$CD4^+$ T cell (cells/mm^3)	Viral load (copies/ml)
Q1 VL<319 copies/ml	1	VMK	positive	F	27	Heterosexual	2003	1,318	< 50
	2	RSW	positive	F	66	Heterosexual	1994	369	< 50
	3	VPT	positive	F	28	Heterosexual	2004	621	159
	4	SMK	positive	M	35	IVDU	2000	792	204
	5	CHL	positive	F	60	Heterosexual	1996	920	260
Q2 VL=320-3,351 copies/ml	1	PMH	positive	F	18	Heterosexual	2002	431	477
	2	NKS	positive	M	30	Heterosexual	2001	624	1,609
	3	RML	positive	M	33	Heterosexual	2000	486	1,846
	4	RPK	positive	F	25	Heterosexual	1997	476	1,846
	5	TKK	positive	M	28	Heterosexual	2001	553	1,993
	6	NKP	positive	M	41	Heterosexual	2003	572	2,180
	7	VPL	positive	F	24	Heterosexual	2001	360	2,317
	8	JRS	positive	F	55	Heterosexual	2000	418	2,409
	9	SPT	positive	M	55	Heterosexual	2000	386	2,538
	10	ACS	positive	M	40	Blood infusion	2002	526	2,580
Q3 VL=3,352-14,676 copies/ml	1	OKC	positive	M	28	Homosexual	1996	388	5,095
	2	SSN	positive	F	31	Heterosexual	2001	455	5,098
	3	NDC	positive	F	40	Heterosexual	1999	869	6,361
	4	LNK	positive	F	38	Heterosexual	2000	915	6,935
	5	PAS	positive	M	41	Heterosexual	2003	400	10,537
	6	KPM	positive	F	33	Heterosexual	1993	707	11,387
	7	TKH	positive	M	21	Heterosexual	2003	571	11,392
	8	PYJ	positive	F	39	Heterosexual	2002	355	14,469
	9	TMM	positive	M	41	Homosexual	1993	355	14,469
	10	AKT	positive	F	27	Heterosexual	2003	502	14,506
Q1 VL>14,677 copies/ml	1	UKY	positive	F	27	Heterosexual	2002	702	14,908
	2	SRK	positive	F	32	Heterosexual	1999	444	16,128
	3	JNU	positive	F	35	Heterosexual	2003	545	21,774
	4	PLT	positive	F	24	Heterosexual	2002	452	22,767
	5	PCC	positive	F	34	Heterosexual	2003	493	46,622
	6	MWT	positive	M	27	Heterosexual	2002	306	81,550
	7	PPY	positive	M	33	Heterosexual	2003	427	92,044
	8	PCF	positive	F	30	Heterosexual	2001	576	113,695
	9	JDC	positive	F	43	Heterosexual	2000	714	115,213
	10	IYJ	positive	M	22	Heterosexual	1995	521	453,456

Q, quartile; Mode Tx, mode of transmission; M, male; F, female; VL, viral load; IVDU, Intravenous drug user

Table 9 HLA typing of HIV-1-seropositive Thais.

No	Initial name (IN)	HLA-A		HLA-B		Bw		HLA-C		DRB1		DRB3/4/5		DQB1	
1	VPT	1101	3303	5502	5801	N/A	N/A	102	302	0301	0406	N/A	N/A	0201	0302
2	PMH	24	33(03/06)	44(032/07)	27	Bw4		701	702	7	12	DRB3	DRB4	2	3
3	RML	2	11	13(01/06)	13	Bw6		3	406	15		DRB5		5	6
4	NKP	24		4002	15	Bw6		401	702	4	1101	DRB3	DRB4	301	302
5	VPL	24		1502	35	Bw6		04(01/05)	08(01/3)	12		DRB3		03(01/04)	
6	JRS	203	1101	1502	5101	N/A	N/A	801	1402	0405	1202	N/A	N/A	0301	0402
7	SPK	1101	3303	1502	5701	N/A	N/A	602	801		1202	N/A	N/A	0301	0303
8	OKC	3001	11	4001	13	Bw4	Bw6	6	0702/10	12		DRB3	DRB4	2	0301/4
9	SSK	203	1101	3909	4601	N/A	N/A	102	702	N/A	N/A	N/A	N/A	0803	1418
10	NDC	1101	3001	1302	1525	N/A	N/A	403	602		0901		DR2	0201	0303
11	KPM	30	11	13	3915	Bw4	Bw6	6	15	7	15	DR51	DR53	2	5
12	TKH	207	1101	1518	4601	N/A	N/A	102	704	0803	1401	N/A	N/A	0503	0601
13	PYJ	11		27(04/10)	46	Bw4	Bw6	1	12(02/08)	8	11	DRB3(02)		601	03(01/04)
14	AKT	206		40		Bw6		03(03/11)		14(01/05)		DRB3		5	
15	UKY	1101	1101	1301	4601	N/A	N/A	102	406	0501	0503	N/A	N/A	1405	1502
16	MWT	24	11	35(05/10)		Bw6		04(01/05)	4	12	14(04/28)	DRB3(01)	DRB3	15	03(01/04)
17	PPY	207	1101	4001	4601	N/A	N/A	102	304	0901	1101	N/A	N/A	0301	0303
18	PCF	24	11	1517	51	BW4		0701/06	1602	4	13	DRB3	DRB4	6	0301/4

N/A, data not available

1.2 High risk HIV-1-seronegative Thais and their partners

11 HIV-1-seronegative subjects who had multiple exposure to HIV-1 were recruited into this study. We confirmed their HIV status by serology. The clinical characteristics were summarised in table 10 and the averages were summarised in table 13. These subjects included 6 women and 5 men, with a mean age of 32 years old (ranging from 20 to 50 years old). The time which these individual had been exposed to HIV-1 ranged from 1 to 5 years with a mean of 2 years. The frequency of condom use to protect HIV infection ranged from 0 to 100 % of time. A written informed consent was obtained from all subjects. According to previous study, the responses were detected in highly exposed HIV-1-seronegative individual with last documented HIV exposure ranged from 2 to 8 months (96). We therefore tried to analyse the HIV-specific $CD8^+$ T cell responses in at least two time points. The last documented HIV exposure ranged from 5 days to 6 months at the first visit and ranged from 4 to 5 months at the second visit.

We hypothesised that the specificity of the T cell responses in highly exposed HIV-1-seronegative subjects differed from those in HIV-1-positive partners. We were therefore interested in studying $CD8^+$ T lymphocyte responses in all HIV-1-seropositive partners but only 9 partners agreed to participate in the study. We confirmed their HIV status by standard serology technique. The clinical characteristics were summarised in table 11. These subjects included 5 women and 6 men, with a mean age of 31 years old (ranging from 20 to 40 years old). HIV-subtype and HLA types of these subjects were not identified. The $CD4^+$ T lymphocytes ranged from 20 to 290 cells/mm^3 with median of 202 cells/mm^3. Median plasma viral load was 1,188 copies/ml (ranging from less than 50 to 62,299 copies/ml). At the time of enrollment, these subjects have been diagnosed HIV-positive for 1 to 2 years. 7 HIV-1-seropositive partners were asymptomatic and antiretrovirals naïve and 4 HIV-1-seropositive partners were on antiretroviral therapy. A written informed consent was obtained from all subjects.

Table 10 Clinical information of high risk HIV-1-seronegative Thais.

Subject no.	Initial name (IN)	HIV status	Gender	Age (year)	Risk factor	Duration of being couple	Frequency of condom use, % of time	Last exposure		Partner
								1st visit	2nd visit	
1	ARK	negative	F	28	Heterosexual	> 1 year	< 50 %	1 month ago	N/A	ALS
2	MTK	negative	F	43	Heterosexual	> 5 years	0%	6 months ago	N/A	SCK
3	ASP	negative	M	40	Heterosexual	> 3 years	0%	5 days	4 months ago	ABM
4	PWC	negative	M	20	Heterosexual	> 2 years	< 50 %	1 month ago	4 months ago	OOS
5	ANN	negative	M	24	Heterosexual	> 1 year	> 50 %	6 months ago	N/A	PDT
6	RBS	negative	F	25	Homosexual	> 5 years	0%	1 month ago	5 months ago	SSN
7	TYM	negative	M	50	Heterosexual	> 1 year	100%	1 month ago	N/A	NKM
8	TTT	negative	F	29	Heterosexual	> 2 years	0%	2 months ago	4 months ago	MTT
9	CKK	negative	F	29	Heterosexual	> 2 year	0%	1 month ago	N/A	MCP
10	NOT	negative	F	31	Heterosexual	> 1 year	< 50 %	3 months ago	N/A	SOT
11	IJR	negative	M	36	Heterosexual	> 1 year	0%	4 months ago	5 months ago	NJR

N/A, data not available; M, male; F, female

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Table 11 Clinical information of HIV-1-seropositive partners.

Subject no.	Initial name (IN)	HIV status	Gender	Age (year)	Risk factor	known seroconversion	ART	CD4⁺ T cell (cells/mm³)	Viral load (copies/ml)
1	ALS[a]	negative	M	52	Heterosexual	2004	on drug (2 weeks before study)	N/A	N/A
2	SCK[a]	negative	M	43	Heterosexual	2004	on drug (3 weeks before study)	N/A	N/A
3	ABM	negative	F	37	Heterosexual	2004	drug naïve	N/A	< 50
4	OOS	negative	F	20	Heterosexual	2004	drug naïve	290	1,188
5	PDT	negative	M	24	Heterosexual	2004	drug naïve	230	10,320
6	SSN	negative	M	28	Homosexual	2004	drug naïve	169	5,000
7	NKM	negative	F	39	Heterosexual	2005	drug naïve	267	69,299
8	MTT	negative	M	29	Heterosexual	2005	on drug (1 week before study)	20	694
9	MCP	negative	M	27	Heterosexual	2005	drug naïve	196	2,180
10	SOT	negative	F	40	Heterosexual	2005	drug naïve	135	< 50
11	NJR	negative	F	35	Heterosexual	2005	on drug (2 months before study)	207	< 50

[a] Refuse to participate in the study; N/A, data not available; m, male; F, female

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1.3 Low risk HIV-1-seronegative controls

5 healthy HIV-1-seronegative controls that were at low risk of HIV exposure were recruited for the experiment controls. The clinical characteristics were summarised in table 12. These subjects include 5 women, with mean age of 25 years old (ranging from 23 to 28 years old).

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Table 12 Clinical information of low risk HIV-1-seronegative control.

Subject no.	Initial name (IN)	HIV status	Gender	Age (year)
1	PIK	negative	F	28
2	RSS	negative	F	24
3	SVS	negative	F	25
4	SJT	negative	F	23
5	TPP	negative	F	25

F, female

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Table 13 The summary and the average of the clinical information of overall subjects.

Demographic data	HIV-1-seropositive subjects	HIV-1-seronegative subjects who has multiple exposures to HIV-1	HIV-1-seropositive partners	Low risk HIV-1-seronegative controls
Male/female	14/21	5/6	6/5	0/5
Age [Mean (SD)]	34.8 (SD 6.7)	32.3 (SD 11)	34 (SD 9.4)	25 (SD 1.9)
CD4 [median {range (SD)}]	502 {306-1,318 (SD 208)}	N/A	202 {20-290(SD 84.7)}	N/A
VL [median {range (SD)}]	6,648 {<50-453,456(SD 78,818.7)}	N/A	1,188 {<50-69,299(SD 22,538.1)}	N/A

CD4, $CD4^+$ T lymphocyte; VL, viral load; SD, standard deviation; N/A , data not available

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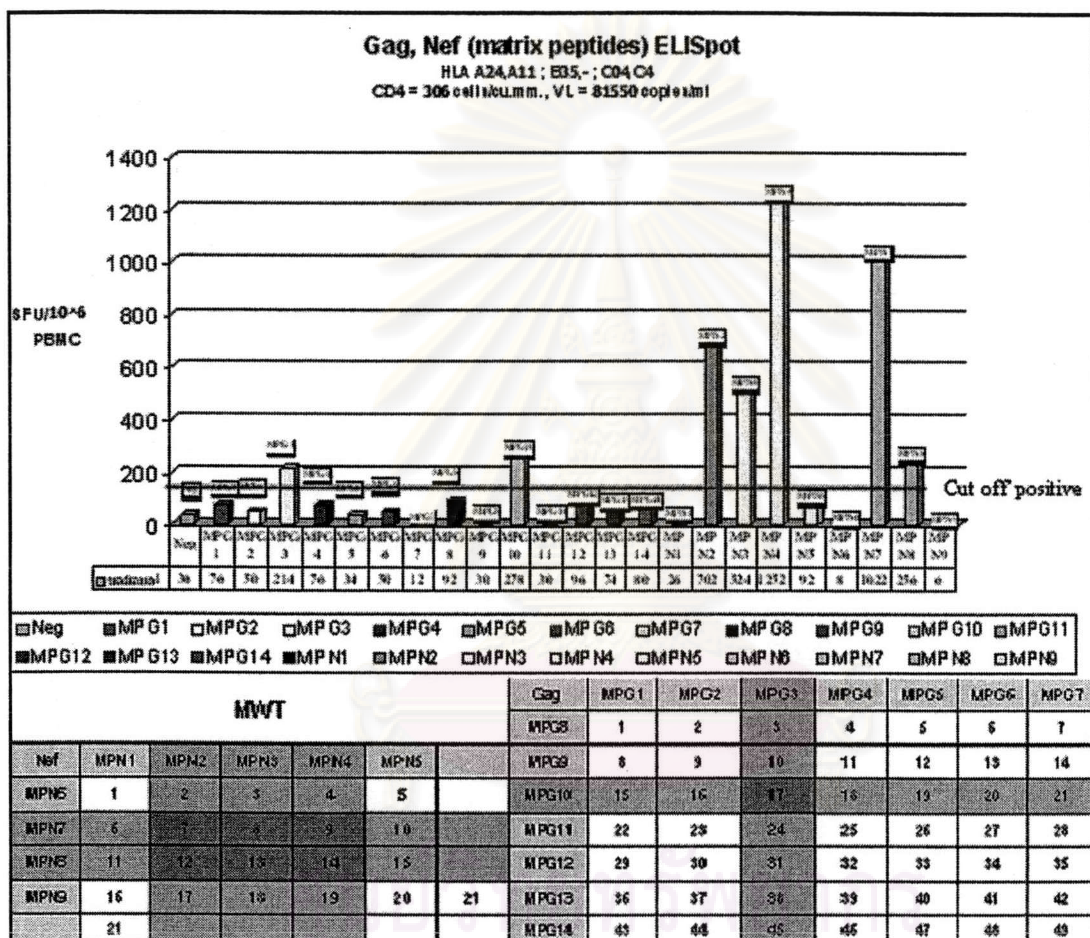
2. HIV-1-specific CD8$^+$ T lymphocyte responses

Nef plays an important role in an enhancement of HIV infection by interfering with immunity against infected cells. Gag-specific CD8$^+$ T lymphocyte responses contribute to maintenance of the asymptomatic state by effectively controlling HIV-1 replication. Investigating into Nef and Gag-specific immune responses may therefore provide useful information for HIV/AIDS pathogenesis. The analysis of theses responses requires large numbers of PBMC. The use of peptides pools for initial screening offers the possibility of minimisation of specimen usage. In this study, we therefore characterised Nef and Gag-specific CD8$^+$ T lymphocyte responses using matrix peptide-based IFN-γ ELISpot assay.

2.1 HIV-1-specific CD8$^+$ T lymphocyte responses in HIV-1-seropositive Thais

We adopted the matrix system to analyse Gag-specific T cell responses using peptide-based ELISpot assay. For example, subject MWT responded to MPG 3 and MPG 10, MWT had positive responses to Gag 17. Thus, only Gag 17 was reconfirmed individually in an ELISpot assay (Figure 15). On the other hand, Nef protein is so immunogenic that many peptides were simultaneously positive. The most frequently recognised peptides were Nef 7 to Nef 15. For example, subject MWT responded to MPN 2, MPN 3, MPN 4, MPN 7, MPN 8, and MPN 10 so there are more-than-one Nef peptides which might mediated the T cell responses, i.e. Nef 7, Nef 8, Nef 9, Nef 10, Nef 12, Nef 13, Nef 14, and Nef 15 (Figure 15). These peptides were subsequently reconfirmed individually in ELISpot assay; only Nef 7, Nef 8, Nef 9, and Nef 14 were positive responded peptides. Therefore, we used the modified matrix system whereby immunogenic Nef peptides (Nef 7 to Nef 15) were individually tested, and the remaining 12 Nef peptides were pooled into seven different two-dimension matrices. The modified matrix setup was summarised in table 14.

Figure 15 Example of Nef and Gag-specific CD8^{+} T lymphocyte responses of subject MWT in preliminary study. The dark shade indicates a positive response. The positive responses to MPN 2 to 4 and MPN 7 to 8 reflect positive responses in peptide Nef 7 to Nef 9 and Nef 12 to Nef 14. The positive responses to MPG 3 and MPG 10 reflect a positive response in peptide Gag 17.



Nef	MPN1	MPN2	MPN3	MPN4	MPN5	
MPN6	1	2	3	4	5	
MPN7	6	7	8	9	10	
MPN8	11	12	13	14	15	
MPN9	16	17	18	19	20	21
	21					

Gag	MPG1	MPG2	MPG3	MPG4	MPG5	MPG6	MPG7
MPG8	1	2	3	4	5	6	7
MPG9	8	9	10	11	12	13	14
MPG10	15	16	17	18	19	20	21
MPG11	22	23	24	25	26	27	28
MPG12	29	30	31	32	33	34	35
MPG13	36	37	38	39	40	41	42
MPG14	43	44	d5	46	47	48	49

CD4, CD4^{+} T lymphocytes (cells/million PBMC); VL, viral load; MPG, matrix pool Gag; MPN, matrix pool Nef

Table 14 The modified matrix system for 21 Nef peptides.

A Peptides matrix setup for Nef and individual Nef peptide.

	MPN1	MPN2	MPN3	MPN4	Individual	Nef	Nef	Nef	Nef
MPN5	1	2	3	4	Nef	7	8	9	10
MPN6	5	6	16	17	Nef	11	12	13	14
MPN7	18	19	20	21	Nef	15			

MPN, matrix pool Nef

B Nef peptides sequences.

Matrix	NO.	Amino acid sequences	Position	MPN6	N5	KHGAVTSSNMNNADCVWLRA	39-60
MPN1	N1	MGGKWSKSSIVGWPQVRERI	1-20		N6	NNADCVWLRAQEEEEVGFPV	49-70
	N5	KHGAVTSSNMNNADCVWLRA	39-60		N16	KLVPVDPREVEEDNK	144-158
	N18	GENNCLLHPMSQHGIEDEER	159-178		N17	DPREVEEDNKGENNCLLHPM	149-168
MPN2	N2	VGWPQVRERIKQTPPATEGV	11232	MPN7	N18	GENNCLLHPMSQHGIEDEER	159-178
	N6	NNADCVWLRAQEEEEVGFPV	49-70		N19	SQHGIEDEEREVLMWKFDSA	169-188
	N19	SQHGIEDEEREVLMWKFDSA	169-188		N20	EVLMWKFDSALARKHIAREL	179-198
MPN3	N3	KQTPPATEGVGAVSQDLDKH	21-40		N21	LARKHIARELHPEYYKDCA	189-207
	N16	KLVPVDPREVEEDNK	114-158	Individual peptide	N7	AQEEEEVGFPVRPQVPLRPM	60-79
	N20	EVLMWKFDSALARKHIAREL	179-198		N8	VRPQVPLRPMTYKGAFDLSF	70-89
MPN4	N4	GAVSQDLDKHGAVTSSNM	31-48		N9	TYKGAFDLSFFLKEKGGL	80-97
	N17	DPREVEEDNKGENNCLLHPM	149-168		N10	SFFLKEKGGLDGLIYSKRR	88-106
	N21	LARKHIARELHPEYYKDCA	189-207		N11	LDGLIYSKRRQEILDLWVY	97-115
MPN5	N1	MGGKWSKSSIVGWPQVRERI	1-20		N12	RQEILDLWVYNTQGFFPDW	106-124
	N2	VGWPQVRERIKQTPPATEGV	10-30		N13	YNTQGFFPDWQNYTPGPGIR	115-134
	N3	KQTPPATEGVGAVSQDLDKH	21-40		N14	WQNYTPGPGIRYPLCFGWCF	124-143
	N4	GAVSQDLDKHGAVTSSNM	31-48		N15	RYPLCFGWCFKLVPVDPREV	134-153

MPN, matrix pool Nef; N, Nef peptide

2.1.1 The frequencies of HIV-1-specific CD8$^+$ T lymphocyte recognition in HIV-1-seropositive Thais

Nef and Gag-specific CD8$^+$ T lymphocyte responses in HIV-1-seropositive Thais could be detected by peptide-based ELISpot assay. The individual peptides were targeted at different frequencies. Some peptides were targeted by several subjects, whilst some peptides were only recognised by one subject or not at all (Table 15, 16 and Figure 16). The three most frequently recognised peptides were located in Nef protein: Nef 9 (TYKGAFDLSFFLKEKGGL) (25 of 35 [71 %]), Nef 8 (VRP QVPLRPMTYKGAFDLSF) (15/35 [43 %]), and Nef 7 (AQEEEEVGFPVRPQVPLRPM) (8/35 [23 %]), respectively (Figure 16). For Gag peptides, the three most frequently recognised peptides were Gag 30 (EPFRDYVDRFFKTLRAEQAT) (7/35 [20%]), Gag 17 (EKALSPEVIPMF SALSEGAT) (4/35 [11.4%]), and Gag 28 (NKIVRMYSPVSILDIKQGPK) (3/35 [8.6%]), respectively (Figure 16). In the overall response, the most frequently recognised protein was Nef (30/35 [86 %]), and followed by p24 Gag (15/35 [43 %]), p 17 Gag (5/35 [14 %]), p2p7p1p6 Gag (2/35 [6 %]), respectively (Figure 17a). According to previous study, we adjusted the recognition to amino acid length of the protein and protein subunits (assessed by dividing the frequency of recognition of a protein by its length)(8, 9). The highest density of responses were in Nef (16/207 [0.07]), p24 Gag (11/220 [0.05]), p17 Gag (4/150 [0.03]), closely followed by p2p7p1p6 Gag (3/149 [0.02]), respectively (Figure 17b).

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2.1.2 Breadth and magnitude of HIV-1-specific $CD8^+$ T lymphocyte responses in HIV-1-seropositive Thais

We next investigated how many individual epitopic regions within the HIV-1 protein and subunits were targeted by each subject. The number of epitopes targeted per subject ranged from 0 to 12 peptides (median, 3). Nef protein was targeted ranged from 0 to 8 (median, 2) and Gag protein was targeted ranged from 0 to 6 (median, 2) (Figure 18a). The broadest $CD8^+$ T lymphocyte responses were demonstrated in subject IY who recognised 12 of the 70 peptides tested comprising 4 Gag peptides [Gag 15 (GQMIHQSLSPRTLNAWVKVIE), Gag 16 (TLNAWVKVIEEKALSPEVIP), Gag 17 (EKALSPEVIPMFSALSEGAT) and Gag 28 (NKIVRMYSPVSILDIKQGPK)], and 8 Nef peptides [Nef 7 (AQEEEEVGFPVRPQVPLRPM), Nef 8 (VRPQVPLRPMTYKGAFDLSF), Nef 9 (TYKGAFDLSFFLKEKGGL), Nef 13 (YNTQGFFPDWQNYTPGPGIR), Nef 15 (RYPLCFGWCFKLVPVDPREV), Nef 16 (KLVPVDPREVEEDNK), Nef 19 (SQHGIEDEEREVLMWKFDSA) and Nef 20 (EVLMWKFDSALARKHIAREL)] (Table 15 and 16).

We next examined the total magnitude of responses against Nef and Gag proteins. The magnitude of responses ranged from 0 to 17,124 SFU/million PBMC (median, 836 SFU/million PBMC). The strongest $CD8^+$ T lymphocyte responses were demonstrated in subject IY (median, 412; range, 0 to 3,436). The magnitude of responses against Nef protein ranged from 0 to 7,520 (median, 1,262 SFU/million PBMC) and the magnitude of responses against Gag protein ranged from 0 to 7,708 (median, 1,472 SFU/million PBMC) (Figure 18b).

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Table 15 HIV-1-specific CD8^{+} T lymphocyte responses to Gag in HIV-1-seropositive Thais.

NO.	Percentage of subjects			5.7	5.7	0	2.9	0	0	0	2.9	0	0	0	0	0	0	5.7	5.7	11.4	2.9	0	0	5.7	0	2.9	0	0
	IN	CD4	VL	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25
1	VMK	1318	< 50																									
2	RSW	369	< 50																									
3	VPT	621	159																									
4	SMK	792	204																									
5	CHL	920	260																									
1	PMH	431	477																									
2	NKS	624	1609																					1020				
3	RML	486	1846	654																240						580		
4	RPK	476	1846																									
5	TKK	553	1993																									
6	NKP	572	2180																									
7	VPL	360	2317																									
8	JRS	418	2409																									
9	SPT	386	2538																									
10	ACS	526	2580																									
1	OKC	388	5095																									
2	SSN	455	5098															228	164	1828								
3	NDC	869	6361		836																							
4	LNK	915	6935	802			686																					
5	PAS	400	10537								2074										2682							
6	KPM	707	11387																									
7	TKH	571	11392																									
8	PYJ	355	14469																									
9	TMM	355	14469																									
10	AKT	502	14506																					1163				
1	UKY	702	14908																									
2	SRK	444	16128																									
3	JNU	545	21774																									
4	PLT	452	22767		2186																							
5	PCC	493	46622																									
6	MWT	306	81550																	118								
7	PPY	427	92044																									
8	PCF	576	113695																									
9	JDC	714	115213																									
10	IYJ	521	453456															2224	106	3436								

No., subject number; IN, initial name; CD4, CD4^{+} T lymphocyte (copies/ml); VL, viral load; G, Gag peptide

Table 15 HIV-1-specific CD8$^+$ T lymphocyte responses to Gag in HIV-1-seropositive Thais (continued)

NO.	of subjects (%)			5.7	0	8.6	0	20	0	0	0	5.7	5.7	0	0	0	0	0	0	0	5.7	2.9	0	0	0	0	0
	IN	CD4	VL	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48	G49
1	VMK	1318	< 50																								
2	RSW	369	< 50																								
3	VPT	621	159																								
4	SMK	792	204																								
5	CHL	920	260			716		1146																			
1	PMH	431	477					128																			
2	NKS	624	1609																		746						
3	RML	486	1846					598													222	196					
4	RPK	476	1846																								
5	TKK	553	1993																								
6	NKP	572	2180																								
7	VPL	360	2317																								
8	JRS	418	2409																								
9	SPT	386	2538					104																			
10	ACS	526	2580																								
1	OKC	388	5095																								
2	SSN	455	5098																								
3	NDC	869	6361																								
4	LNK	915	6935			358																					
5	PAS	400	10537	1342																							
6	KPM	707	11387																								
7	TKH	571	11392					872																			
8	PYJ	355	14469																								
9	TMM	355	14469																								
10	AKT	502	14506					1101				522	593														
1	UKY	702	14908																								
2	SRK	444	16128									628	444														
3	JNU	545	21774																								
4	PLT	452	22767	426																							
5	PCC	493	46622																								
6	MWT	306	81550																								
7	PPY	427	92044					410																			
8	PCF	576	113695																								
9	JDC	714	115213																								
10	IYJ	521	453456			1942																					

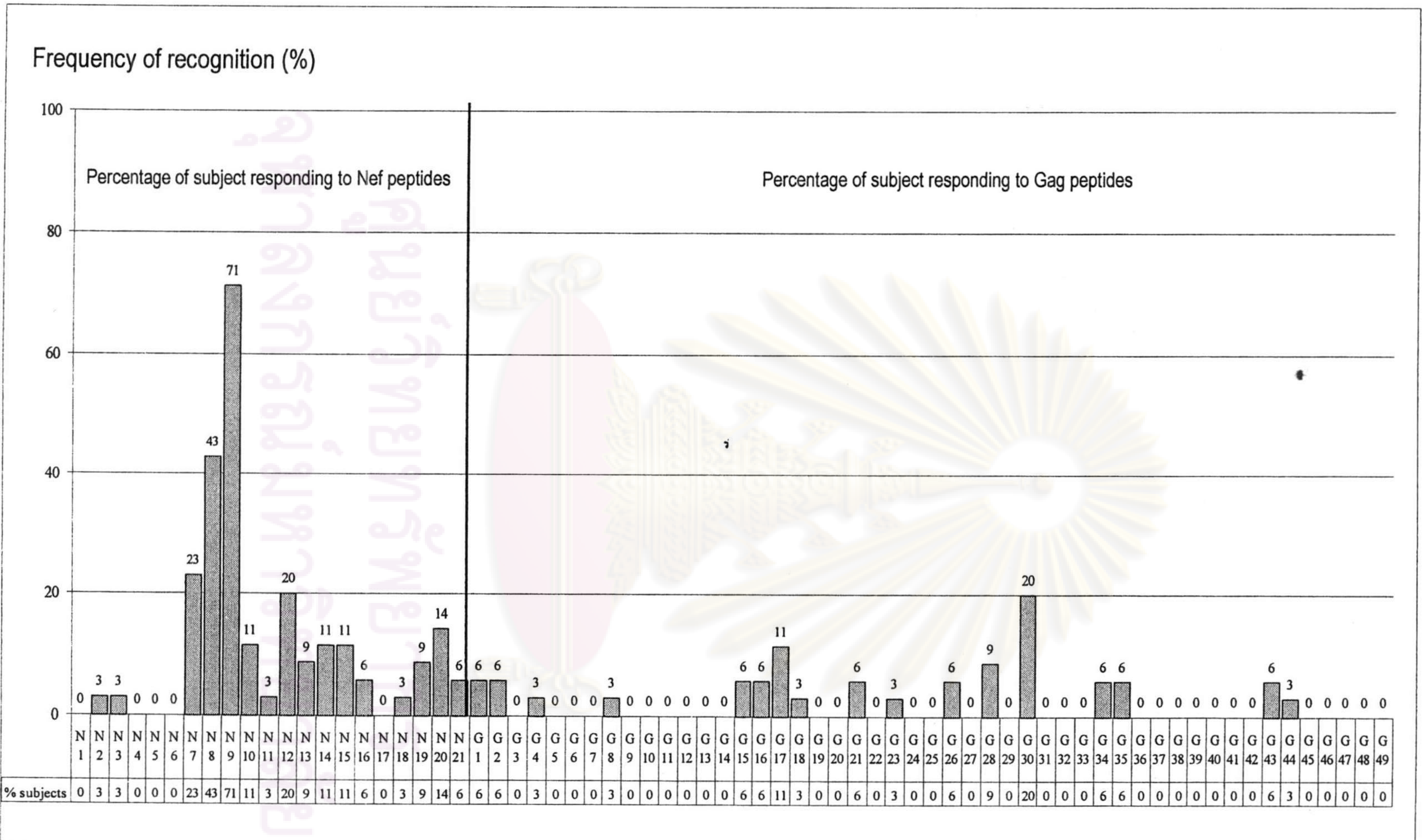
No., subject number; IN,initial name; CD4, CD4$^+$ T lymphocyte (copies/ml); VL, viral load; G, Gag peptide

Table 16 HIV-1-specific CD8^{+} T lymphocyte responses to Nef in HIV-1-seropositive Thais

NO.	of subjects (%)			0	5.7	2.9	0	0	0	22.9	45.7	71.4	11.4	5.7	20	8.6	14.3	11.4	5.7	0	2.9	8.6	14.3	5.7
	IN	CD4	VL	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21
1	VMK	1318	< 50																					
2	RSW	369	< 50													396								
3	VPT	621	159																					
4	SMK	792	204									1032			230									
5	CHL	920	260							1156	1324													
1	PMH	431	477																					
2	NKS	624	1609									334												
3	RML	486	1846		140							2044			240									
4	RPK	476	1846		132					546		500			1546									
5	TKK	553	1993									260												
6	NKP	572	2180																					
7	VPL	360	2317							208	484						1136	1114						384
8	JRS	418	2409									700						204	246					
9	SPT	386	2538							674	398	254												
10	ACS	526	2580								118	770												
1	OKC	388	5095										276		398									
2	SSN	455	5098								1102	4544		858	638	268							110	
3	NDC	869	6361									244			458									
4	LNK	915	6935									444			348							798	1112	
5	PAS	400	10537							820		1120												
6	KPM	707	11387							888	272	2400												
7	TKH	571	11392																					
8	PYJ	355	14469								170	1004											362	332
9	TMM	355	14469									152												
10	AKT	502	14506									2071	2063									634		
1	UKY	702	14908								406	212												
2	SRK	444	16128								110	572					98							
3	JNU	545	21774								622	674												
4	PLT	452	22767									2488					490							
5	PCC	493	46622								640	754												
6	MWT	306	81550							172	300	246					380							
7	PPY	427	92044			314					126		110								198			
8	PCF	576	113695								210	458		250			244	444						
9	JDC	714	115213									4246	906										400	
10	IYJ	521	453456							726	372	1104				270		262	326			174	452	

No.,subject number; IN, initial name; CD4, CD4^{+} T lymphocyte (copies/ml); VL, viral load; N, Nef peptide

Figure 16 Percentage of HIV-1 seropositive Thais recognising Nef and Gag peptides



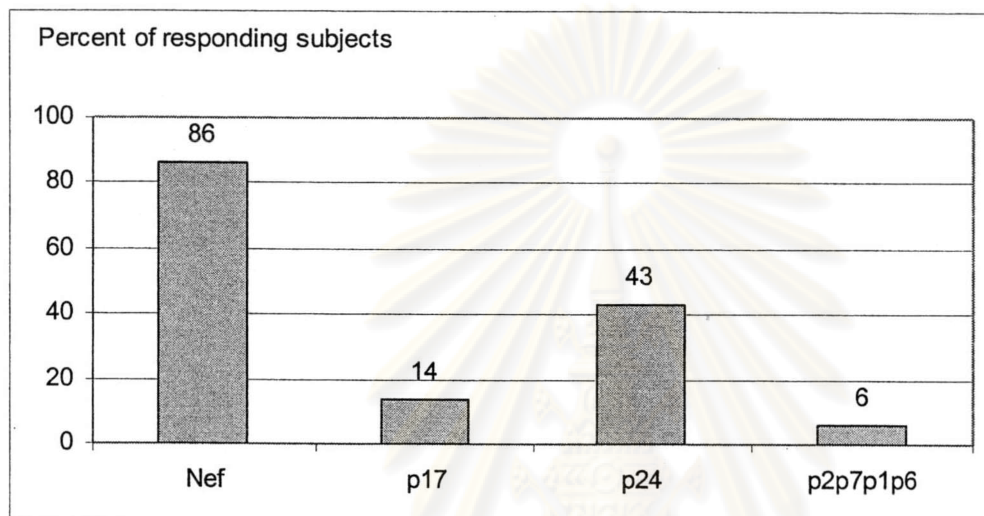
% subjects	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21
	0	3	3	0	0	0	23	43	71	11	3	20	9	11	11	6	0	3	9	14	6

% subjects	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25
	6	6	0	3	0	0	0	3	0	0	0	0	0	0	6	6	11	3	0	0	6	0	3	0	0

% subjects	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48	G49
	6	0	9	0	20	0	0	0	6	6	0	0	0	0	0	0	0	6	3	0	0	0	0	0

Figure 17 Frequency of recognition of the individual protein and protein subunits in HIV-1-seropositive Thais. Panel A shows the percentage of individuals with responses to the individual protein and protein subunits. Panel B shows percentage of subjects recognising each protein and protein subunits divided by length of the peptide.

A Percentage of subjects recognising Nef protein and Gag protein subunits.



B Frequency of recognition adjusted for protein length.

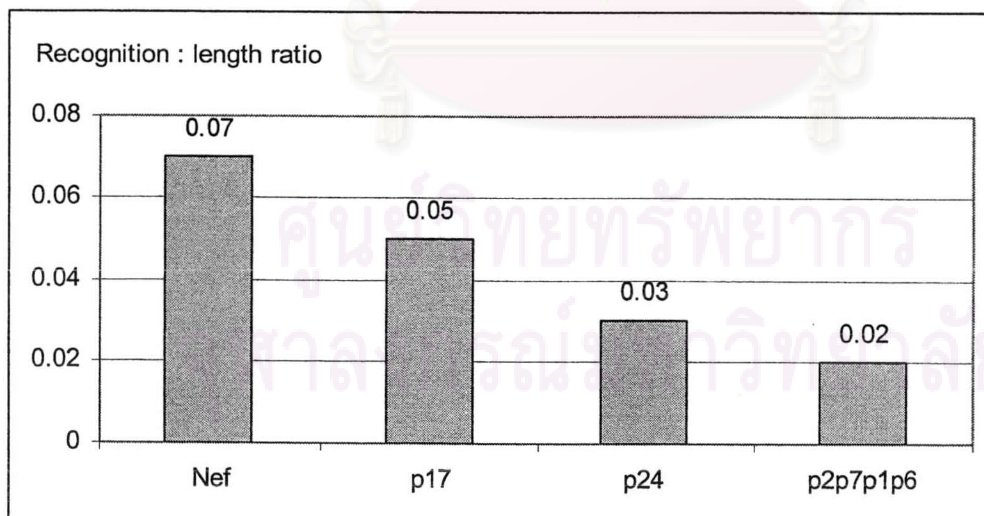
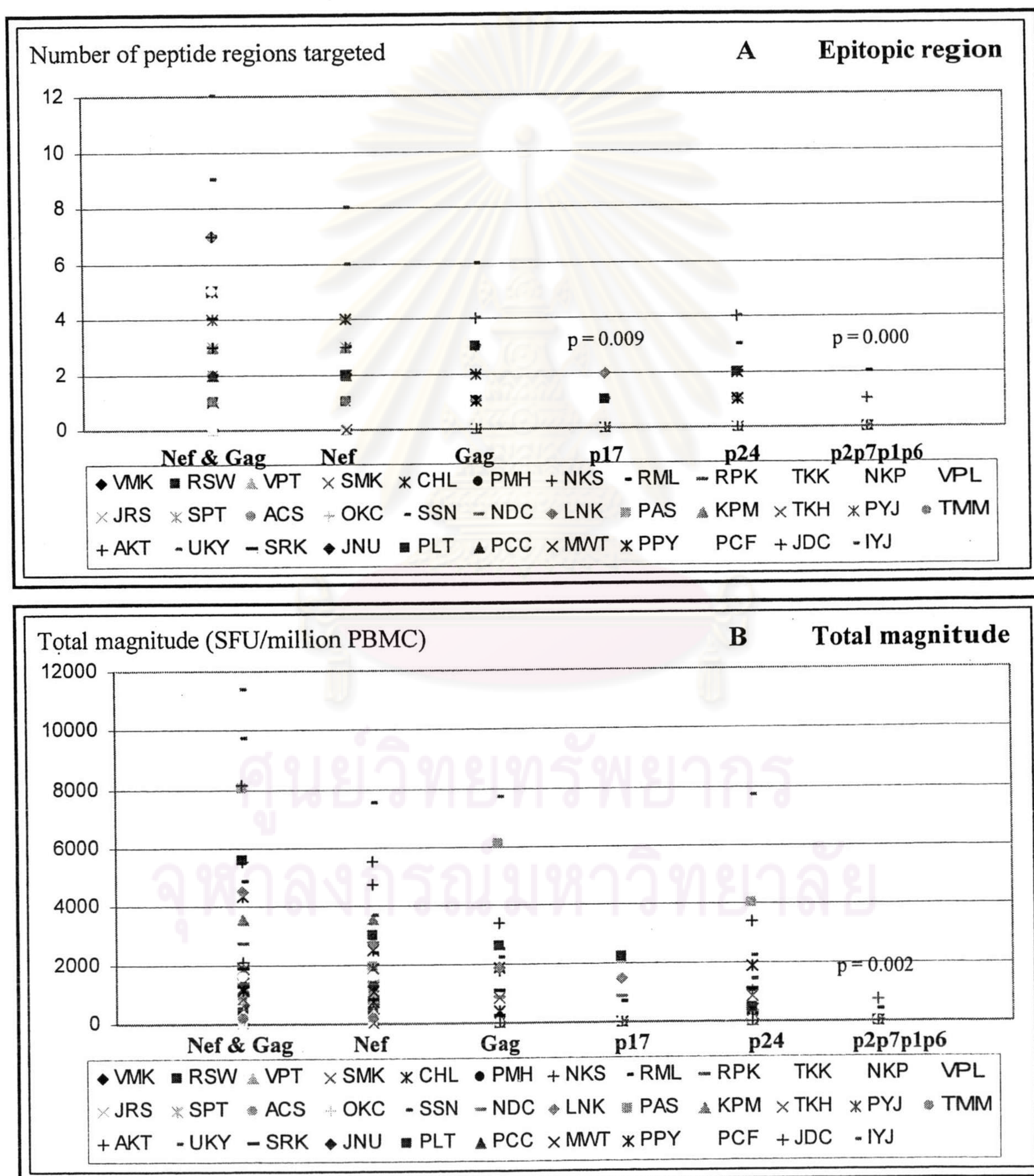


Figure 18 Comparison of breadth and magnitude between proteins and protein subunits in HIV-1-seropositive Thais. Panel A summerises the number of epitopic regions targeted per individual, whilst panel B illustrated the total magnitude of HIV-1-specific $CD8^+$ T lymphocyte responses to Nef and Gag proteins per individual. P values were calculated using Mann-Whitney Test.



2.1.3 Correlation between viral load and Nef and Gag-specific $CD8^+$ T lymphocyte responses in HIV-1-seropositive Thais

A proposed correlation between HIV viral load and HIV-1-specific $CD8^+$ T lymphocyte responses remain controversial. Whilst some previous studies demonstrated inverse correlation between the HIV viral load and HIV-1-specific $CD8^+$ T lymphocyte responses, others found no such correlation, or even positive correlation. In this study, we investigated the potential association between plasma viral load and three entities: the frequencies of HIV-1-specific $CD8^+$ T lymphocyte recognition, the breadth of HIV-1-specific $CD8^+$ T lymphocyte responses, and the magnitude of HIV-1-specific $CD8^+$ T lymphocyte responses.

The results indicated that there were no significant correlations between viral load and either breadth (R^2 = 0.2742; P = 0.5236) and magnitude (R^2 = 0.1454; P = 0.3814) of the total HIV-1-specific $CD8^+$ T lymphocyte responses (Figure 19). Furthermore, there were no significant correlations between viral load and either breadth (R^2 = 0.3440; P = 0.5865) or magnitude (R^2 = 0.1126; P = 0.3356) of Nef-specific $CD8^+$ T lymphocyte responses (Figure 20). Likewise, there were no significant correlations between viral load and either breadth (R^2 = 0.0528; P = 0.2299) or magnitude (R^2 = 0.0911; P = 0.3019) of Gag-specific $CD8^+$ T lymphocyte responses (Figure 21).

Nef 9 (TYKGAFDLSFFLKEKGGL) seemed to be the most immunodominant peptides. This peptide was most frequently recognised and mediated the highest magnitude of responses. In order to see whether this peptide might mediate protective HIV-specific immunity, we established the correlation between $CD8^+$ T lymphocyte responses against Nef 9 and HIV viral load. The results indicated that there were no significant correlations between viral load and the magnitude of $CD8^+$ T lymphocyte responses against Nef 9 (R^2 = 0.1107; P = 0.3327) (Figure 22).

Figure 19 Correlation between viral load and breadth or magnitude of the total HIV-1-specific $CD8^+$ T lymphocyte responses in HIV-1-seropositive Thais. The top panel shows the relationship between viral load in plasma on the *y* axis and the total number of peptides recognised per subject on *x* axis. The bottom panel shows the relationship between viral load in plasma on the *y* axis and the total HIV-1-specific $CD8^+$ T lymphocyte responses on *x* axis. P values shown in each panel were calculated using Pearson correlations.

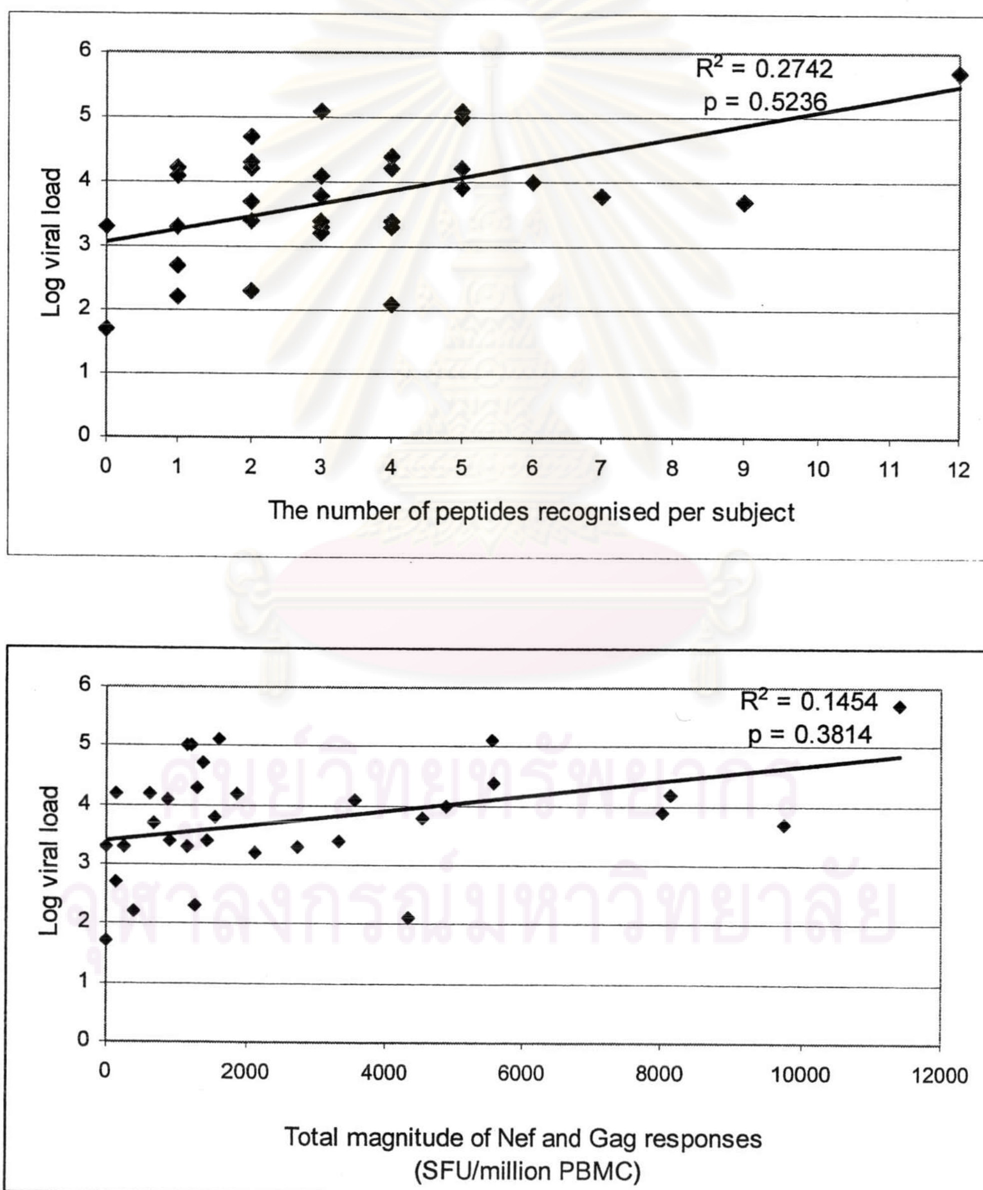


Figure 20 Correlation between viral load and breadth or magnitude of the total Nef-specific $CD8^+$ T lymphocyte responses in HIV-1-seropositive Thais. The top panel shows the correlation between viral load in plasma on the *y* axis to the total number of Nef peptides recognised per subject on *x* axis. The bottom panel shows the correlation between viral load in plasma on the *y* axis to the total Nef-specific $CD8^+$ T lymphocyte responses on *x* axis. P values shown in each panel were calculated using Pearson correlations.

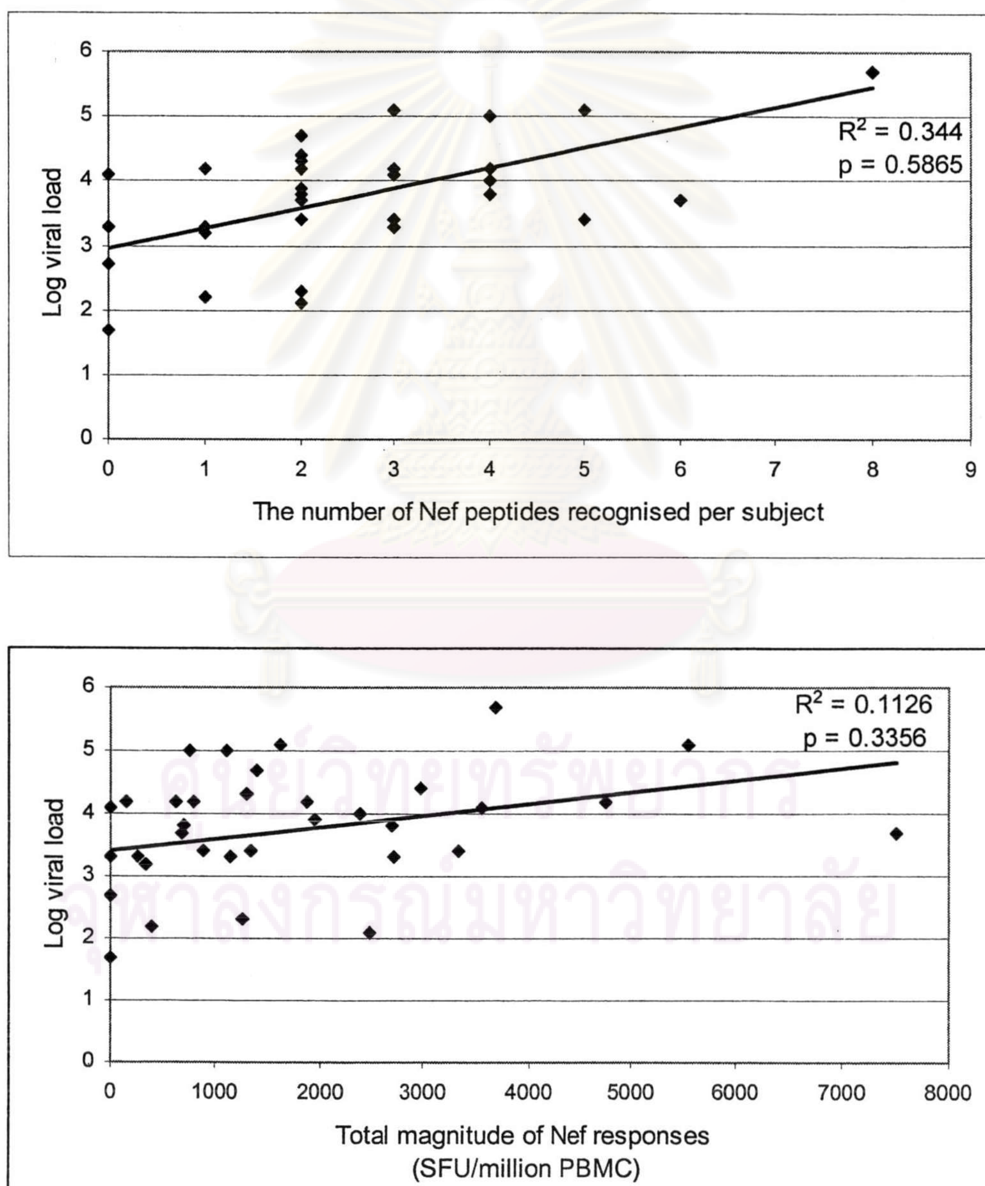


Figure 21 Correlation between viral load and breadth or magnitude of the total Gag-specific $CD8^+$ T lymphocyte responses in HIV-1-seropositive Thais. The top panel shows the relationship between viral load in plasma on the *y* axis is to the total number of Gag peptides recognised per subject on *x* axis. The bottom panel shows the relationship between viral load in plasma on the *y* axis to the total Gag-specific $CD8^+$ T lymphocyte responses on *x* axis. P values shown in each panel were calculated using Pearson correlations.

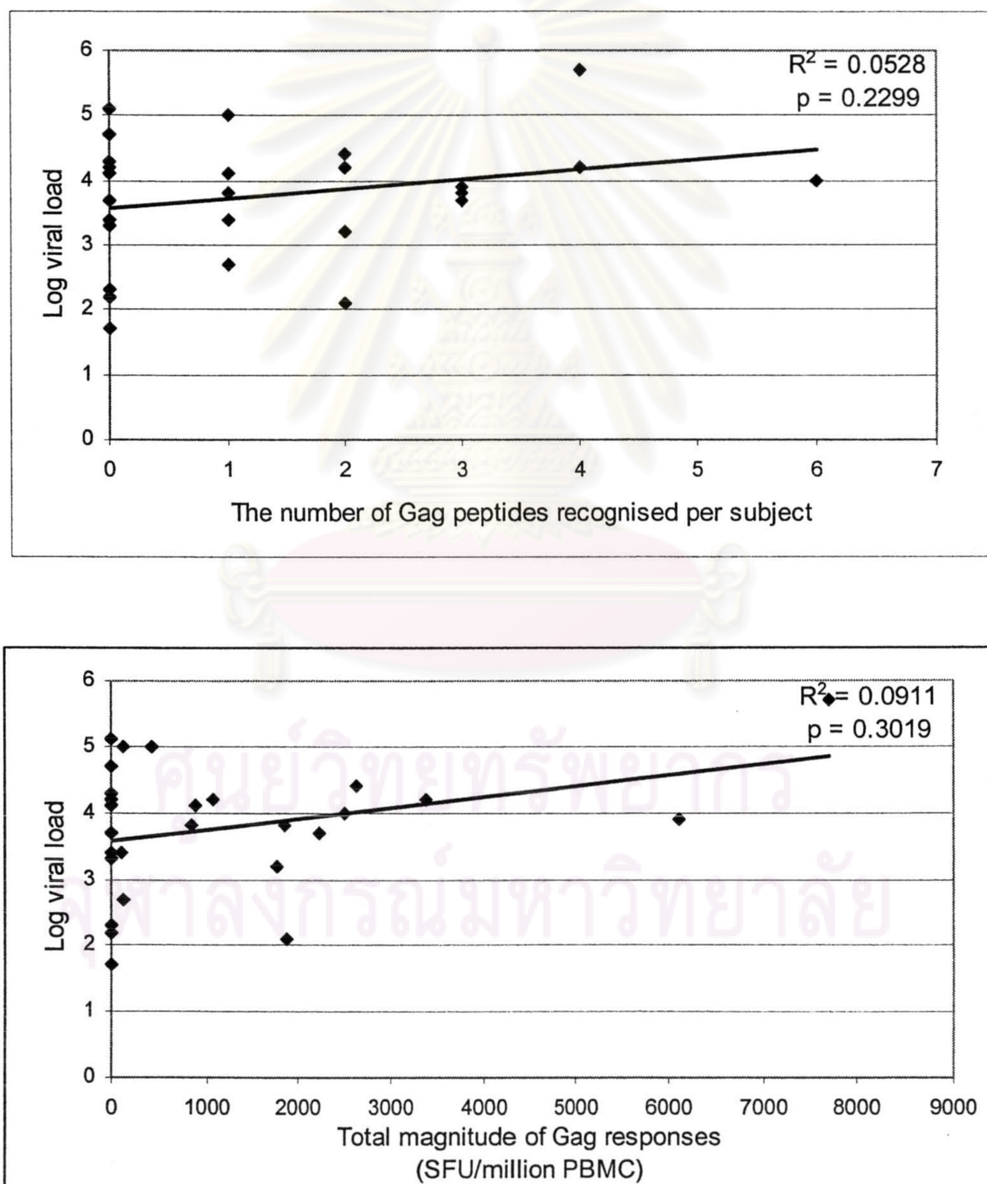
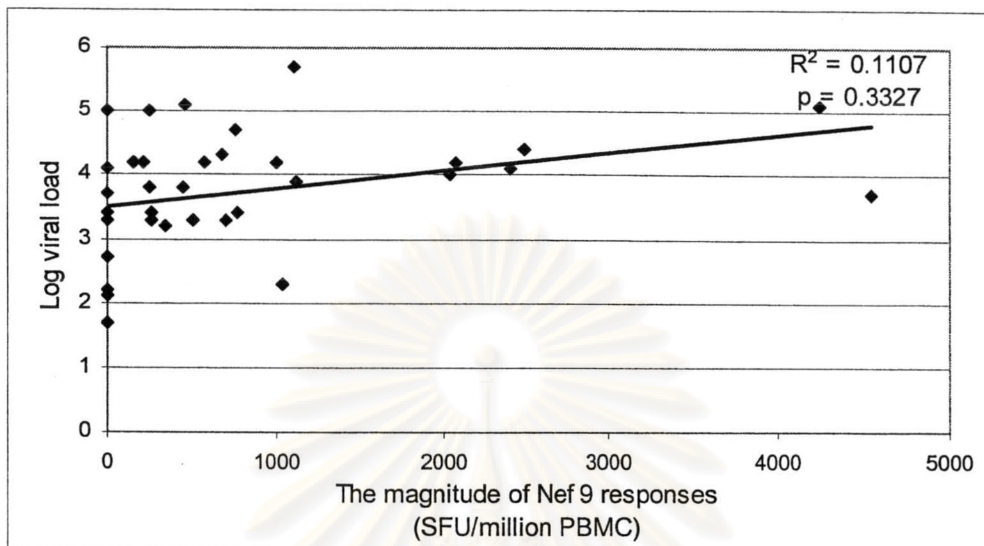


Figure 22 Correlation between viral load and the magnitude of $CD8^+$ T lymphocyte responses against Nef 9. P values were calculated using Pearson correlations.



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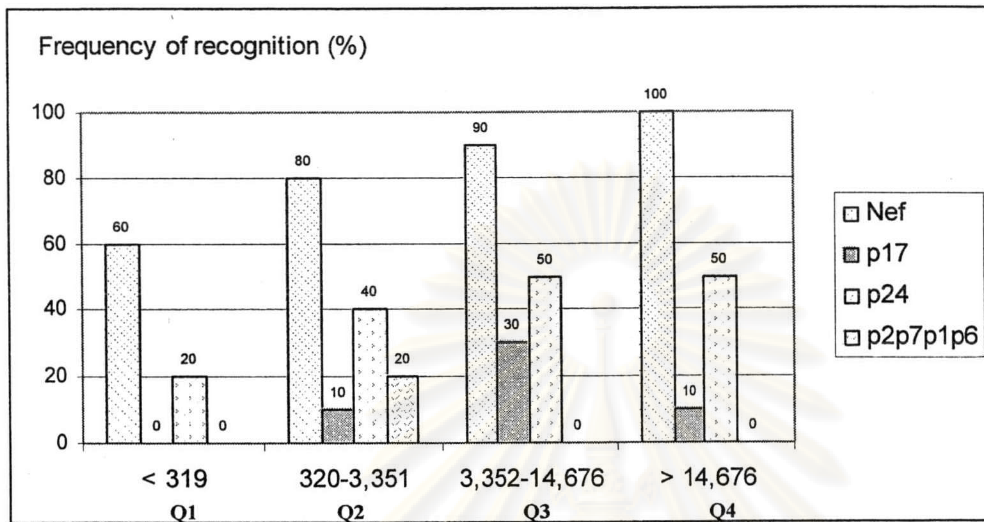
2.1.4 The frequencies of HIV-1-specific CD8$^+$ T lymphocyte recognition at different viral load quartiles

HIV-specific CTL play an important role in the control of the viral replication (1, 4, 97). We therefore hypothesised that HIV-1-specific CD8$^+$ T lymphocyte responses differ among HIV-1-infected individual with different level of HIV RNA. We found that Nef protein and p24 subunit were recognised by the subjects in all viral load quartiles whilst p17 subunit was recognised by the subjects who had viral load more than 320 copies/ml (second to the fourth quartile) and p2p7p1p6 subunits were recognised by only the subjects who had viral load between 320-3,351 copies/ml (second quartile). There were no significant different of the frequencies of recognition between viral load quartiles (Figure 23).

Figure 23 Comparison of frequency of recognition between viral load quartiles. P values were calculated using Mann-Whitney Test.



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2.1.5 Breadth and magnitude of HIV-1-specific $CD8^+$ T lymphocyte response at different viral load quartiles

We next investigated how many individual epitopic regions within Nef and Gag were targeted by subjects in each viral load quartile. The individuals with the highest number of responsive peptides targeted were in the fourth quartile, with median of 4.5 (range, 2 to 12), followed by the third quartile, with median 3.5 (range, 2 to 9), the second quartile, with median 2.5 (range, 0 to 7), and the first quartile, with median 0 (range, 0 to 4), respectively (Figure 24). We next assessed the breadth of peptides in an individual protein. There were no significant differences of the breadth of the total HIV-1-specific $CD8^+$ T lymphocyte responses, Nef-specific $CD8^+$ T lymphocyte responses, and Gag-specific $CD8^+$ T lymphocyte responses (Figure 25-26).

We next examined the total magnitude of responses against Nef and Gag proteins by each viral load quartiles. The individuals with the strongest responses were in the third quartile, with median of 2,714 (range, 152 to 9,740), followed by the fourth quartile, with median 1,500 (range, 618 to 11,394), the second quartile, with median 1,288 (range, 0 to 4,874), and the first quartile, with median 396 (range, 0 to 4,342), respectively (Figure 27). We next assessed the total magnitude in an individual protein. There were no significant differences of the total magnitude of HIV-1-specific $CD8^+$ T lymphocyte responses, Nef-specific $CD8^+$ T lymphocyte responses, Gag-specific $CD8^+$ T lymphocyte responses and Nef 9-specific $CD8^+$ T lymphocyte responses among individuals in different viral load quartiles (Figure 28-30).

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Figure 24 Comparison of breadth of the total HIV-1-specific $CD8^+$ T lymphocyte responses in HIV-infected subjects with different HIV-RNA level. P values were calculated using Mann-Whitney Test.

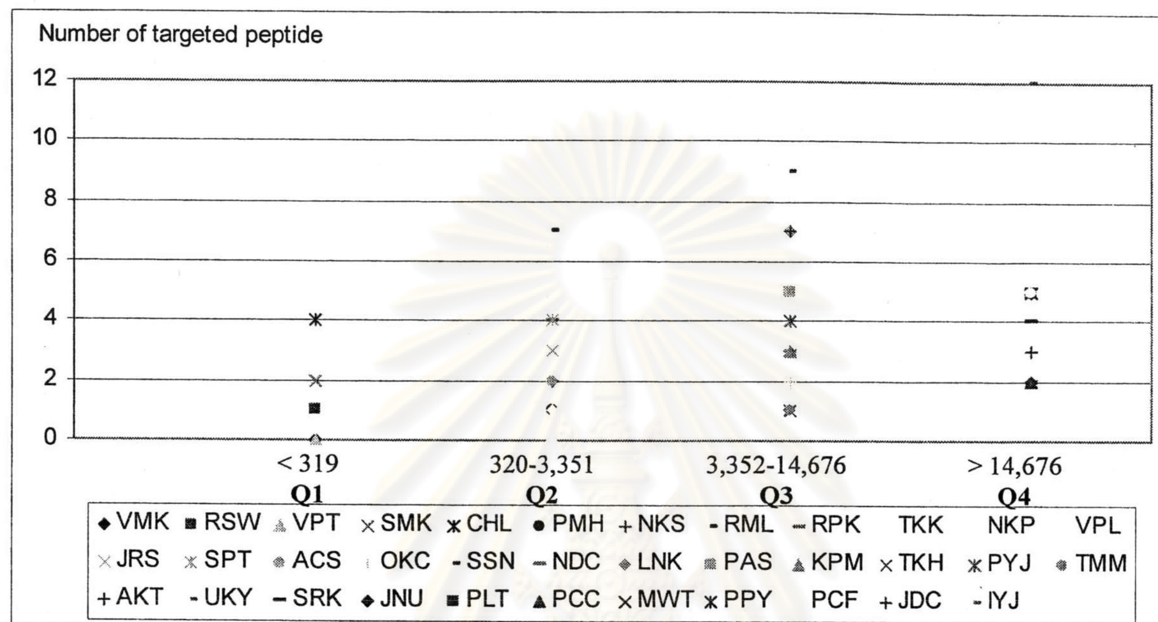

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Figure 25 Comparison of breadth of the total Nef-specific CD8$^+$ T lymphocyte responses in HIV-infected subjects with different HIV-RNA level. P values were calculated using Mann-Whitney Test.

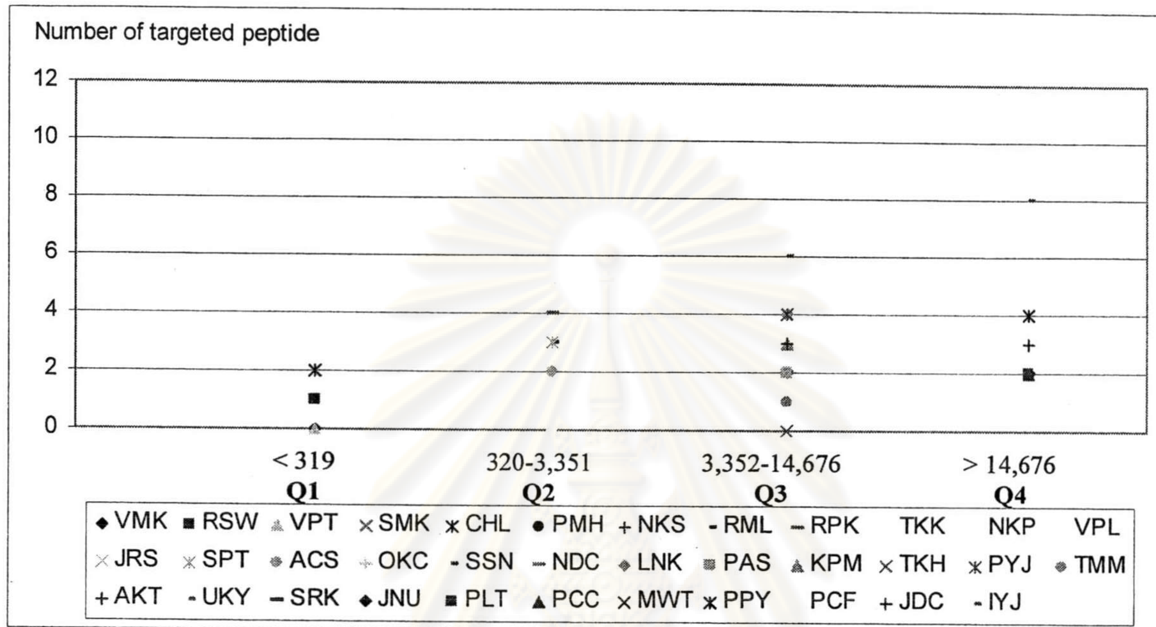

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Figure 26 Comparison of breadth of the total Gag-specific CD8^{+} T lymphocyte responses in HIV-infected subjects with different HIV-RNA level. P values were calculated using Mann-Whitney Test.

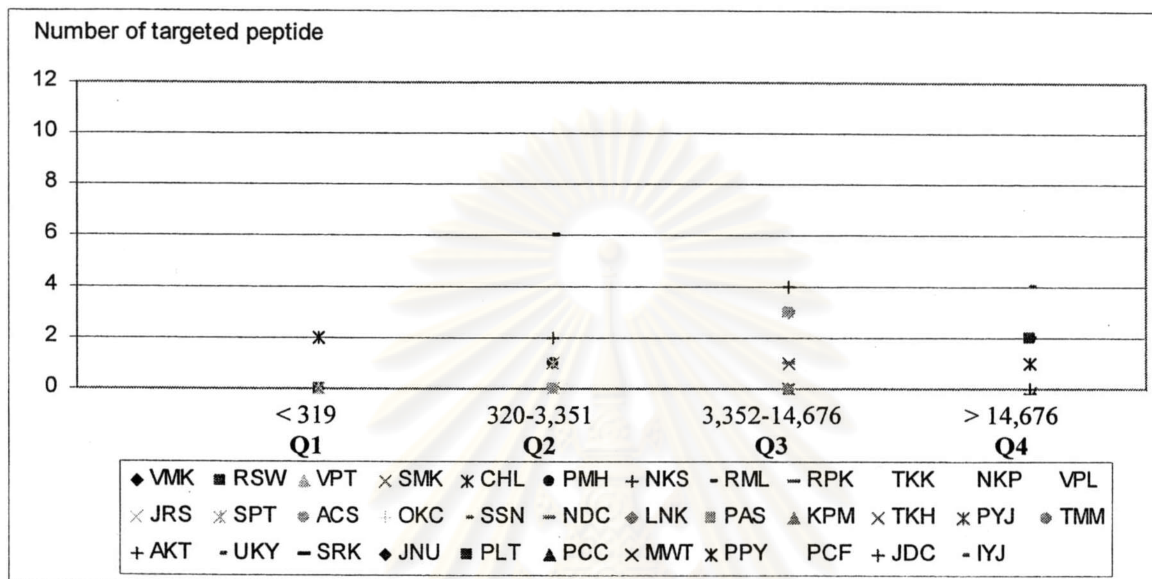

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Figure 27 Comparison of magnitude of the total HIV-1-specific $CD8^+$ T lymphocyte responses in HIV-infected subjects with different HIV-RNA level. P values were calculated using Mann-Whitney Test.

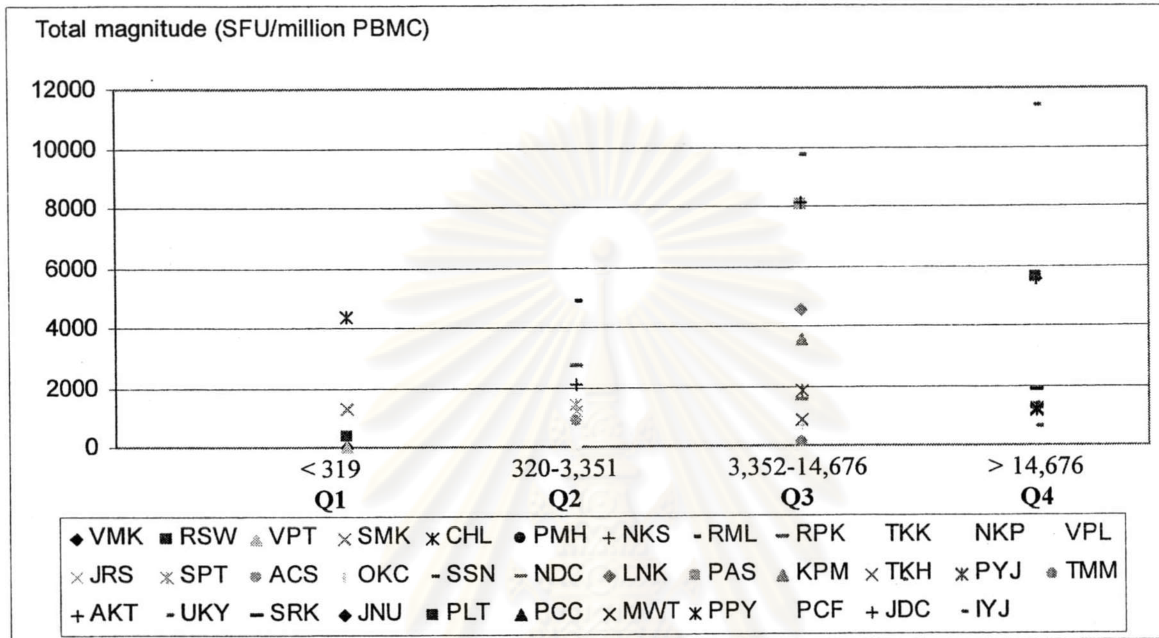

Figure 28 Comparison of magnitude of the total Nef-specific CD8^{+} T lymphocyte responses in HIV-infected subjects with different HIV-RNA level. P values were calculated using Mann-Whitney Test.

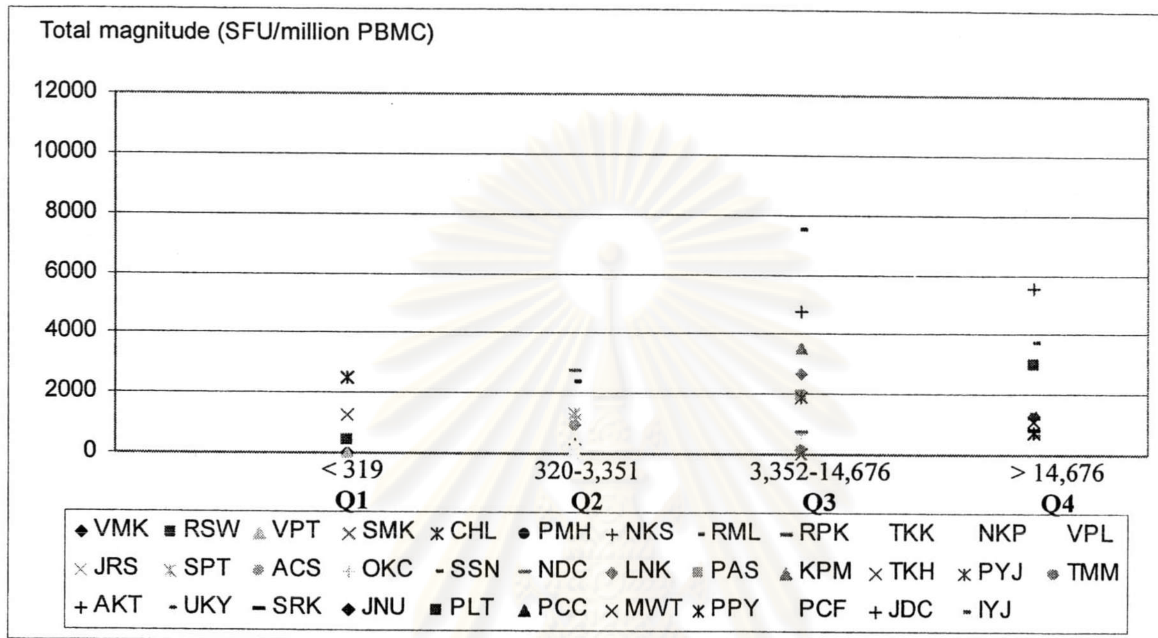

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Figure 29 Comparison of magnitude of the total Gag-specific CD8^{+} T lymphocyte responses in HIV-infected subjects with different HIV-RNA level. P values were calculated using Mann-Whitney Test.

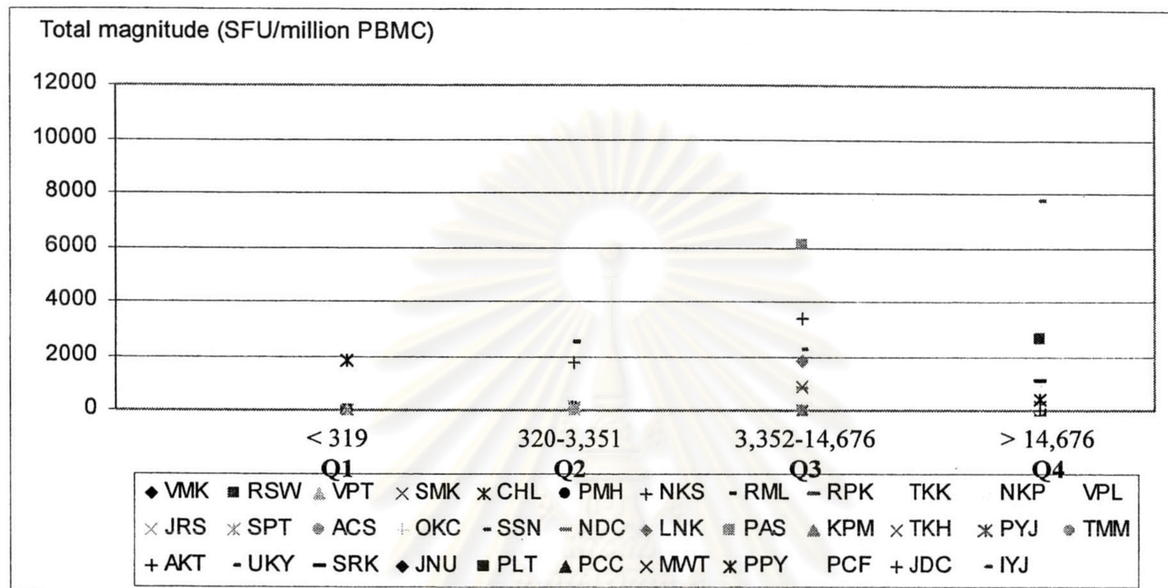

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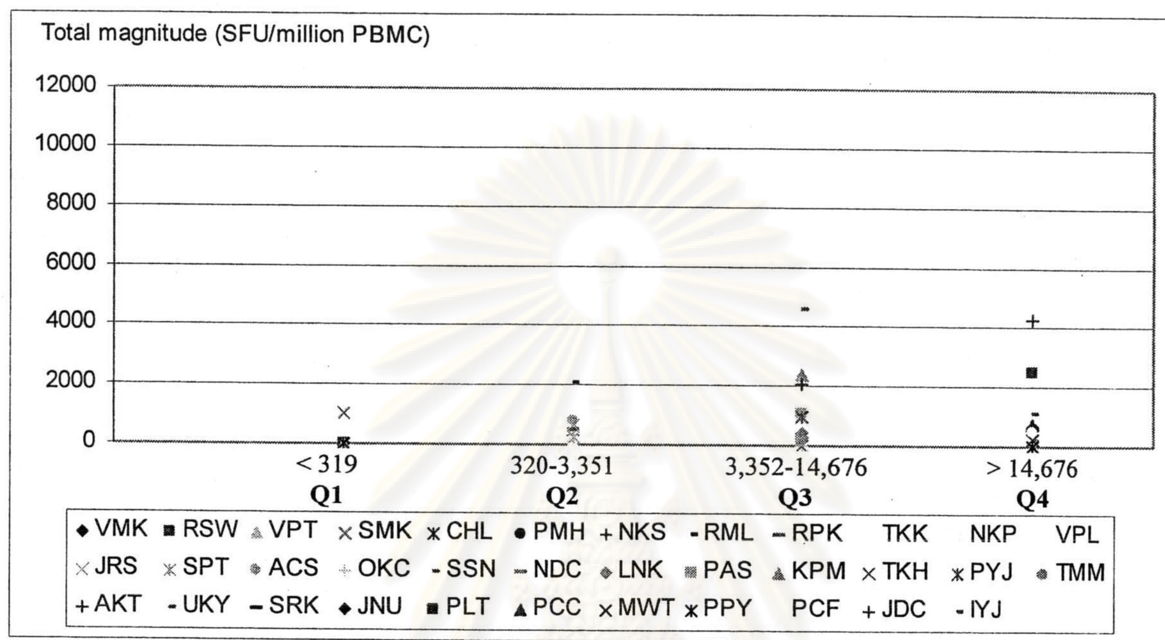
Figure 30 Comparison of magnitude of $CD8^+$ T lymphocyte responses against Nef 9 in HIV-infected subjects with different HIV-RNA level. P values were calculated using Mann-Whitney Test.



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2.2 HIV-1-specific $CD8^+$ T lymphocyte responses in high risk HIV-1-seronegative Thais and their partners

HIV-1-specific $CD8^+$ T lymphocyte responses were demonstrated in several highly-exposed, persistently seronegative (HEPS) cohorts over the past decade. However, since it remained unclear about the phenomenon of resistance in these subjects, we were interested to study HIV-1-specific $CD8^+$ T lymphocyte responses in HIV-1-seronegative Thais who had multiple exposed to HIV-1.

Unexpectedly, no Nef or Gag-specific $CD8^+$ T lymphocyte responses were detected by *ex vivo* peptide-based ELISpot assay in almost all highly exposed HIV-1-seronegative subjects at both visits, except for subject ASP who responded to Nef 7 (AQEEEEVGFPVRPQVPLRPM) (1,114 SFU/million PBMC), Nef 8 (VRPQVPL RPMTYKGAFDLSF) (334 SFU/million PBMC), and Nef 9 (TYKGAFDLSFFLKEKGGL) (1,852 SFU/million PBMC) at the second visit (4 months after last exposure). The HIV-1-seropositive partner of this subject (subject ABM) responded to Nef 9 (TYKGAFDLSFFLKEKGGL) (602 SFU/million PBMC), Nef 14 (WQNYTPGPG IRYPLCFGWCF) (288 SFU/million PBMC), and Nef 15 (RYPLCFGWCFKLVPVDPREV) (306 SFU/million PBMC) (Table 17 and 18).

However, Nef and Gag-specific $CD8^+$ T lymphocyte responses in all HIV-1-seropositive partners could be detected by peptide-based ELISpot assays. The individual peptides were targeted at different frequencies. Some peptides were targeted by several subjects, whilst some peptides were only recognised by one subject or not at all (Table 17, 18 and Figure 31). The three most frequently recognised peptides were located in Nef protein, Nef 9 (TYKGAFDLSFFLKEKGGL) (5/11 [45 %]), Nef 14 (WQNYTP GPGIRYPLCFGWCF) (3/11 [27 %]), and Nef 8 (VRPQVPLRPMTYKGA FDLSF) (2/11 [18 %]), respectively. In the subgroup analysis, the most frequently recognised protein subunit were Nef (5/9 [55 %]), followed by p24 Gag (1/9 [11 %]), and p 17 Gag (1/9 [11 %]) (Figure 32a). In addition, the highest density of responses (assessed by dividing the frequency of recognition of a protein by its length) was in Nef (5/207 [0.02]), and followed by p17 Gag (1/150 [0.007]), p24 Gag (1/220 [0.005]), respectively (Figure 32b).

We next investigated how many individual epitopic regions within Nef and Gag proteins were targeted by each partner. HIV-1-seropositive partners recognised 9 peptides of the 70 peptides tested (13%). The number of peptides recognised per subject ranged from 0 to 7 peptides. The broadest $CD8^{+}$ T lymphocyte responses were demonstrated in subject PDT who recognised 7 of the 70 peptides tested comprising 4 Gag peptides, Gag 1 (MGARASVLSGGKLDAWE), Gag 17 (EKALSPEVIPMFSALSEGAT), Gag 23 (GPVAPGQMREPRGSDIAG) and Gag 30 (EPFRDYVDRFFKTLRAEQAT) and, 3 Nef peptides, Nef 8 (VRPQVPLRPMTYKGAFDLSF), Nef 9 (TYKGAFDLSFFLKEKGGL), and Nef 12 (RQEILDLWVYNTQGFFPDW) (Figure 33a).

We next examined the total magnitude of responses against Nef and Gag proteins. The magnitude of responses ranged from 0 to 4,418 SFU/million PBMC (median, 740 SFU/million PBMC). The strongest $CD8^{+}$ T lymphocyte responses were demonstrated in subject PDT (median, 276 SFU/million PBMC; range 146 to 1,264) (Figure 33b).

Table 17 HIV-1-specific $CD8^{+}$ T lymphocyte responses to Gag in high risk HIV-1-seronegative Thais and their partners.

No	IN	HIV	CD4	VL	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25
1	ARK	neg	N/A	N/A																									
	ALS	pos	N/A	N/A																									
2	MTK	neg	N/A	N/A																									
	SCK	pos	N/A	N/A																									
3	ASP	neg	N/A	N/A																									
	ABM	pos	N/A	< 50																									
4	PWC	neg	N/A	N/A																									
	OOS	pos	290	1,188																									
5	ANN	neg	N/A	N/A																									
	PDT	pos	230	10320	276																244						1164		
6	RBS	neg	N/A	N/A																									
	SSN	pos	169	5923																									
7	TYM	neg	N/A	N/A																									
	NKM	pos	267	69299																									
8	TTT	neg	N/A	N/A																									
	MTT	pos	20	694																									
9	CKK	neg	N/A	N/A																									
	MCP	pos	196	2,180																									
10	NOT	neg	N/A	N/A																									
	SOT	pos	135	<50																									
11	IJR	neg	N/A	N/A																									
	NJR	pos	207	< 50																									

Table 17 HIV-1-specific $CD8^+$ T lymphocyte responses to Gag in high risk HIV-1-seronegative Thais and their partners (continued).

No	IN	HIV	CD4	VL	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48	G49
1	ARK	neg	N/A	N/A																								
	ALS	pos	N/A	N/A																								
2	MTK	neg	N/A	N/A																								
	SCK	pos	N/A	N/A																								
3	ASP	neg	N/A	N/A																								
	ABM	pos	N/A	< 50																								
4	PWC	neg	N/A	N/A																								
	OOS	pos	290	1,188																								
5	ANN	neg	N/A	N/A																								
	PDT	pos	230	10320					1,164																			
6	RBS	neg	N/A	N/A																								
	SSN	pos	169	5923																								
7	TYM	neg	N/A	N/A																								
	NKM	pos	267	69299																								
8	TTT	neg	N/A	N/A																								
	MTT	pos	20	694																								
9	CKK	neg	N/A	N/A																								
	MCP	pos																										
10	NOT	neg	N/A	N/A																								
	SOT	pos	135	<50																								
11	IJR	neg	N/A	N/A																								
	NJR	pos	207	< 50																								

Table 18 HIV-1-specific $CD8^+$ T lymphocyte responses to Nef in high risk HIV-1-seronegative Thais and their partners.

No	IN	HIV	CD4	VL	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21
1	ARK	neg	N/A	N/A																					
	ALS	pos	N/A	N/A																					
2	MTK	neg	N/A	N/A																					
	SCK	pos	N/A	N/A																					
3	ASP	neg	N/A	N/A							1,114	334	1,852												
	ABM	pos	N/A	< 50									602					288	306						
4	PWC	neg	N/A	N/A								68			54										
	OOS	pos	290	1,188								402	1,996												
5	ANN	neg	N/A	N/A																					
	PDT	pos	230	10320								160	1,264			146									
6	RBS	neg	N/A	N/A								56		68											
	SSN	pos	169	5923														556							
7	TYM	neg	N/A	N/A																					
	NKM	pos	267	69299							446		240												
8	TTT	neg	N/A	N/A														62							
	MTT	pos	20	694									632					108							
9	CKK	neg	N/A	N/A																					
	MCP	pos																							
10	NOT	neg	N/A	N/A																					
	SOT	pos	135	<50																					
11	IJR	neg	N/A	N/A																					
	NJR	pos	207	< 50																					

Figure 31 Percentage of HIV-1-seropositive partners recognising Nef and Gag.

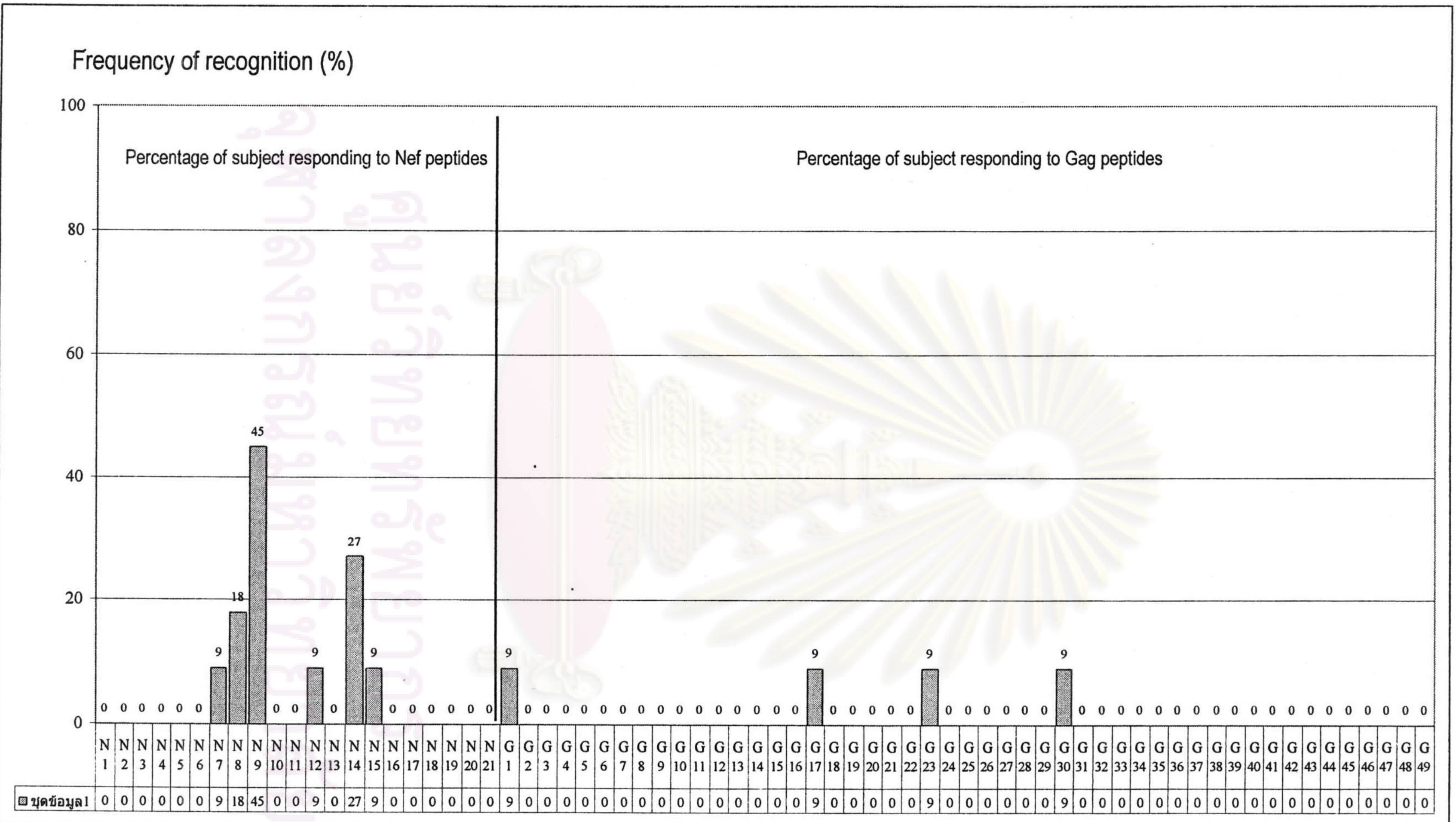
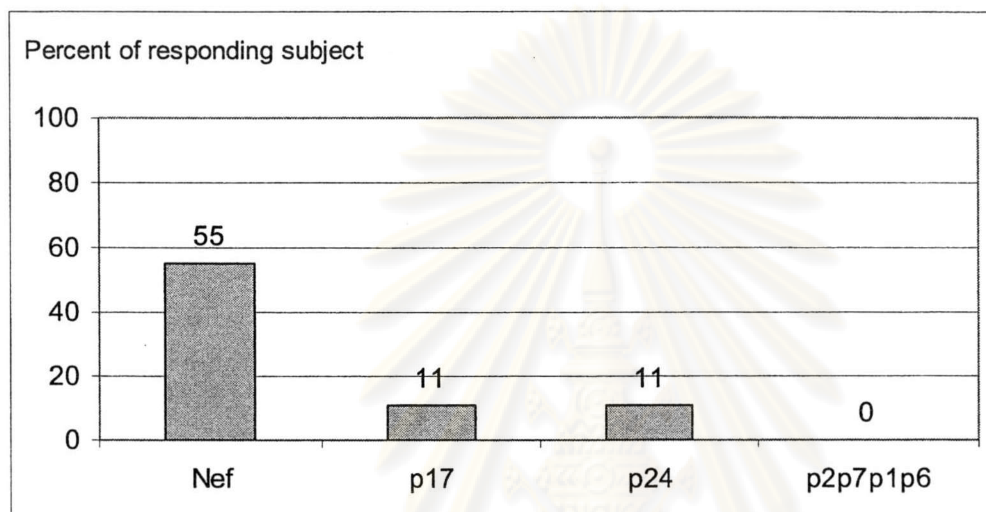


Figure 32 Frequency of recognition of the individual protein and protein subunits in HIV-1-seropositive partners. Panel A shows the percentage of individuals with responses to the individual protein and protein subunits. Panel B shows percentage of subjects recognising each protein and protein subunits divided by its length in amino acid.

A Percentage of subjects recognising Nef protein and Gag protein subunits



B Frequency of recognition adjusted for protein length

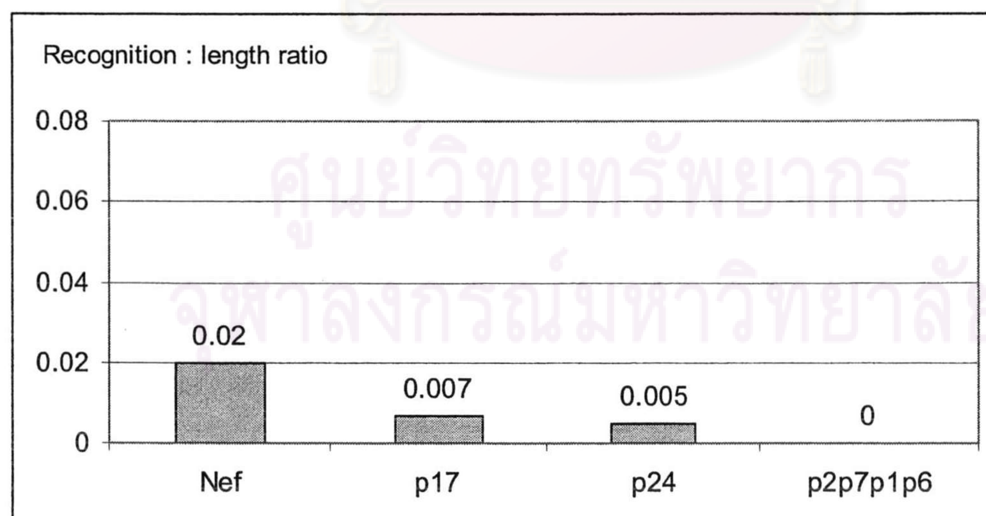


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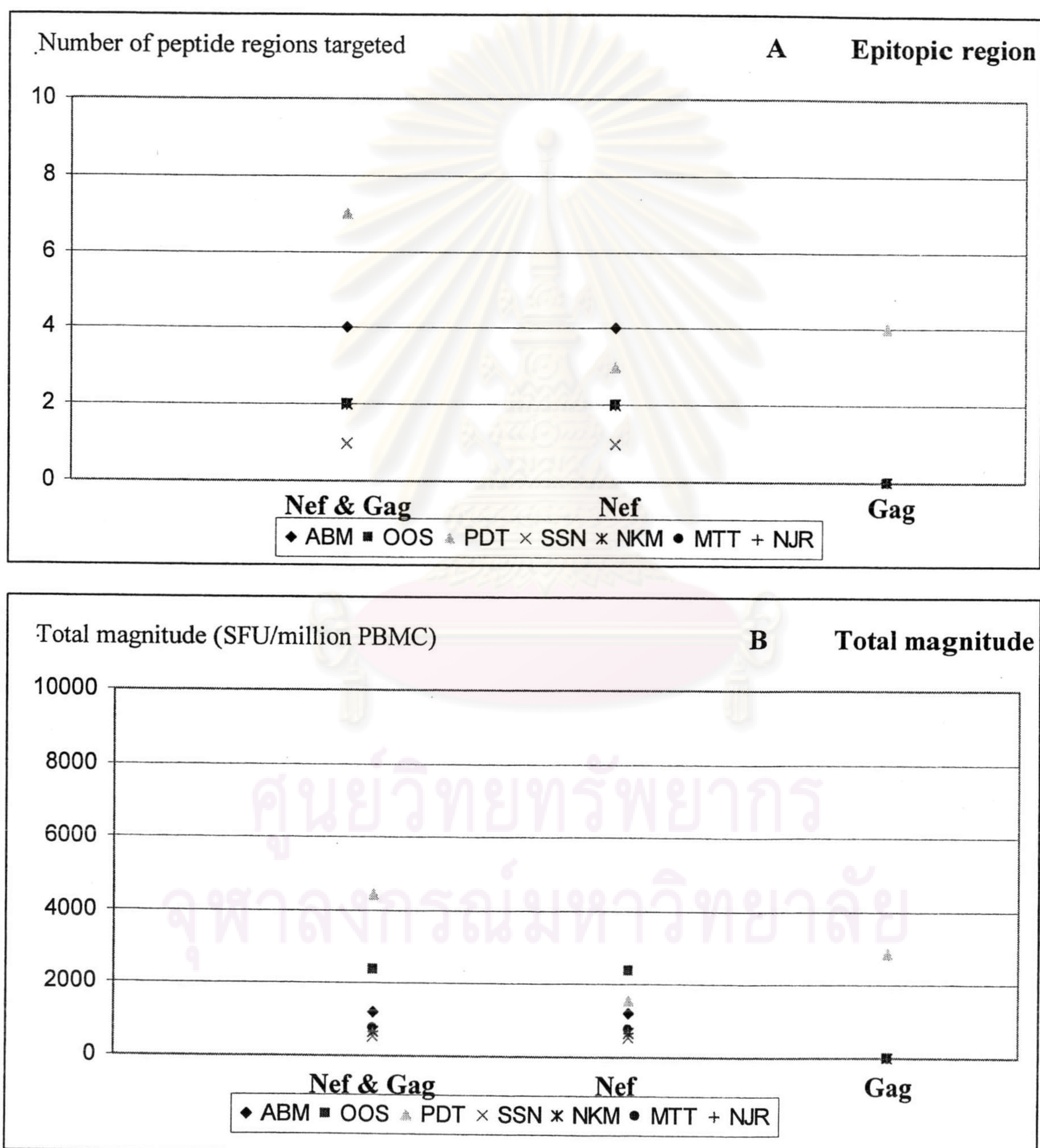
Figure 33 Comparison of breadth and magnitude between Nef and Gag proteins in HIV-1-seropositive partners. Panel A summerises the number of epitopic regions targeted per individual, whilst panel B indicates the total magnitude of HIV-1-specific CD8$^+$ T lymphocyte responses to Nef and Gag proteins per individual. P values were calculated using Mann-Whitney Test.



2.2.1 HIV-1-specific $CD8^+$ T lymphocyte responses in high risk HIV-1-seronegative Thais after augmentation

The fact that $CD8^+$ T lymphocyte responses were directed against structural (Gag) and non-structural (Nef) HIV proteins strongly suggested that HIV must have replicated in these subjects sufficiently to prime a cell-mediated immune response to expand both memory and effector CTL populations, albeit at low frequency. In order to increase sensitivity of an ELISpot assay, we augmented the frequency of the CTL by the culturing the PBMC with pooled overlapping peptides for 10 days before the peptide-based ELISpot assay was performed as previously described.

We generated CTL lines of ASP, PWC, from frozen PBMC, and CTL line of NJR from fresh PBMC; all PBMC were obtained at the second visit. The HIV-1-specific $CD8^+$ T lymphocyte responses against Nef 7 (800 SFU/million PBMC), Nef 8 (1,080 SFU/million PBMC), and Nef 9 (1,800 SFU/million PBMC) remain found in subject ASP (Figure 34) whilst subject PWC and NJR still had no responses against any peptides (data not shown). The frequencies of responses against Nef 7 and Nef 9 in cultured ELISpot assay were similar to that in *ex vivo* ELISpot assay. Whilst the frequencies of responses against Nef 8 in cultured ELISpot assay were stronger than that in *ex vivo* ELISpot assay.

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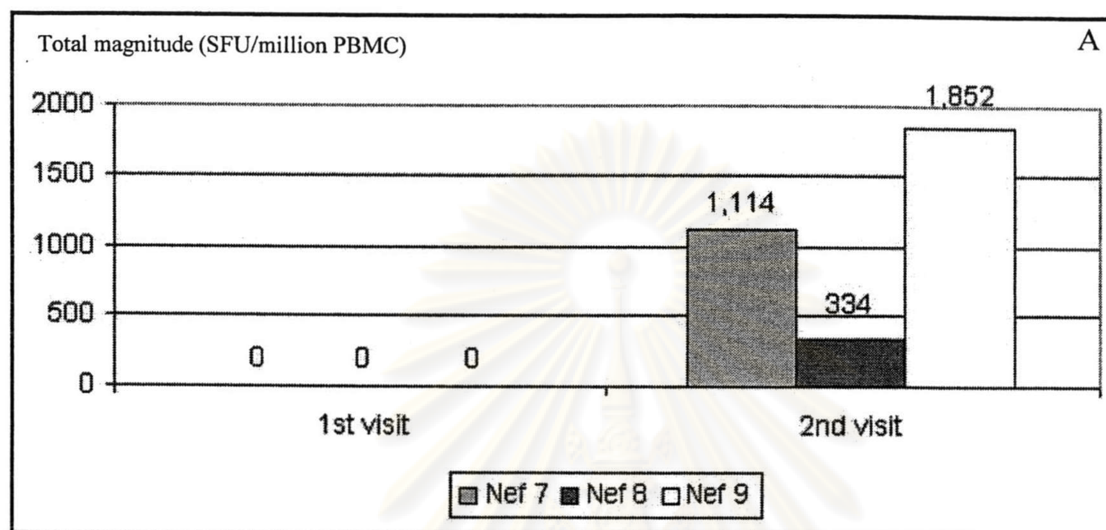
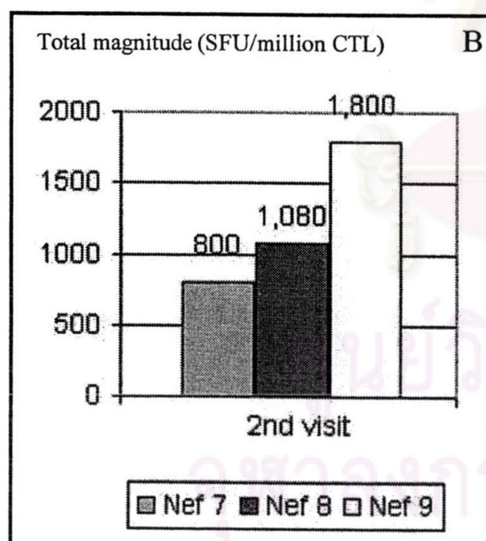
Figure 34 HIV-1-specific $CD8^+$ T lymphocyte responses to Nef and Gag proteins in subject ASP. The ELISpot responses at the first and the second visit were shown in panel A. The cultured ELISpot responses which obtained at the second visit were shown in panel B.





2.3 HIV-1-specific $CD8^+$ T lymphocyte responses in low risk HIV-1-seronegative controls

In our study, we investigated the role of HIV-1- specific $CD8^+$ T lymphocyte responses in HIV-1-seropositive subjects and in highly exposed HIV-1-seronegative subjects. In order to show the specificity of this ELISpòt assay, we recruited 5 low risk HIV-1 seronegative subjects as the control. We found that Nef and Gag-specific $CD8^+$ T lymphocyte responses were not detected in all HIV-1-seronegative controls (Figure 35).

Figure 35 HIV-1-specific $CD8^+$ T lymphocyte responses in low-risk HIV-1-seronegative controls

SFU/10^6 PBMC

1000
800
600
400
200
0

PIK
SVS
RSS
SJT
TPP

	Neg	MPN1	MPN2	MPN3	MPN4	MPN5	MPN6	MPN7	Nef7	Nef8	Nef9	Nef10	Nef11	Nef12	Nef13	Nef14
PIK	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SVS	12	0	0	6	4	4	6	4	12	10	0	4	0	6	0	0
RSS	22	0	12	1	0	1	0	6	0	5	5	0	0	12	0	0
SJT	17	0	6	0	2	0	0	0	0	2	0	2	4	2	2	0
TPP	14	4	0	0.	2	2	6	0	0	12	6	0	0	6	8	4

	Nef15	MPG1	MPG2	MPG3	MPG4	MPG5	MPG6	MPG7	MPG8	MPG9	MPG10	MPG11	MPG12	MPG13	MPG14
PIK	0	12	0	0	2	2	0	0	0	0	0	0	0	0	0
SVS	0	12	8	0	8	14	10	2	0	8	16	0	0	0	0
RSS	0	0	1	1	1	15	0	0	4	0	6	18	0	6	4
SJT	4	6	0	0	0	0	0	0	0	4	0	0	2	0	0
TPP	0	6	0	2	0	2	12	4	0	0	8	2	0	6	0

3. HIV-1-specific T cell responses following $CD8^+$ T lymphocyte depletion

In our study, peptide-based ELISpot assay was used to enumerate HIV-1-specific $CD8^+$ T lymphocyte secreting IFN-γ upon stimulation. However, IFN-γ could also be secreted from $CD4^+$ T lymphocyte and NK cells. We therefore depleted $CD8^+$ T lymphocyte by Immunomagnetic beads (Dynal®beads) to prove that these responses were mediated solely or mainly by $CD8^+$ T lymphocyte. We selected HIV-1-seropositive subjects (CHL, SSN, PAS, JRS, and AKT) who had T lymphocyte responses against HIV specific peptides by peptide-based ELISpot assays. We found that the magnitude of responses was significantly reduced. Indeed, the fact that more than 80% of spots were abrogated by $CD8^+$ depletion confirmed these responses were mediated by $CD8^+$ T lymphocyte (Table 19).

Table 19 HIV-1-specific T lymphocyte responses after depletion of $CD8^+$ T lymphocyte by immunomagnetic beads.

NO	IN	Peptides no.	Sequence	Magnitude of HIV-specific T cell responses (SFU/10^6 PBMC)		% reduction
				Pre depletion	Post depletion	
1	CHL	G30	EPFRDYVDRFFKTLRAEQAT	602	116	80.73
		N7	AQEEEEVGFPVRPQVPLRPM	872	64	92.66
		N8	VRPQVPLRPMTYKGAFDLSF	1532	64	95.82
2	SSN	N13	YNTQGFFPDWQNYTPGPGIR	106	20	81.13
		N14	WQNYTPGPGIRYPLCFGWCF	576	154	73.26
3	PAS	G8	GTEELRSLYNTVATLYCVHQ	2412	52	97.84
		G18	MFSALSEGATPQDLNMMLNI	2834	120	95.77
		G26	TGNPPIPVGDIYKRWMILGL	1368	20	98.54
		N7	AQEEEEVGFPVRPQVPLRPM	1232	52	95.78
		N9	TYKGAFDLSFFLKEKGGL	2768	192	93.06
4	JRS	N9	TYKGAFDLSFFLKEKGGL	700	25	96.43
		N15	RYPLCFGWCFKLVPVDPREV	204	7	96.57
		N16	KLVPVDPREVEEDNK	246	10	95.93
5	AKT	G21	LKDTINEEAAEWDRLHPVHA	1163	149	87.19
		G30	EPFRDYVDRFFKTLRAEQAT	1101	32	97.09
		G34	CKSILRALGAGATLEEMMTA	522	22	95.79
		G35	ATLEEMMTACQGVGGPGHKA	593	20	96.63
		N9	TYKGAFDLSFFLKEKGGL	2071	204	90.15
		N10	SFFLKEKGGLDGLIYSKRR	2063	106	94.86
		N19	SQHGIEDEEREVLMWKFDSA	634	28	95.58

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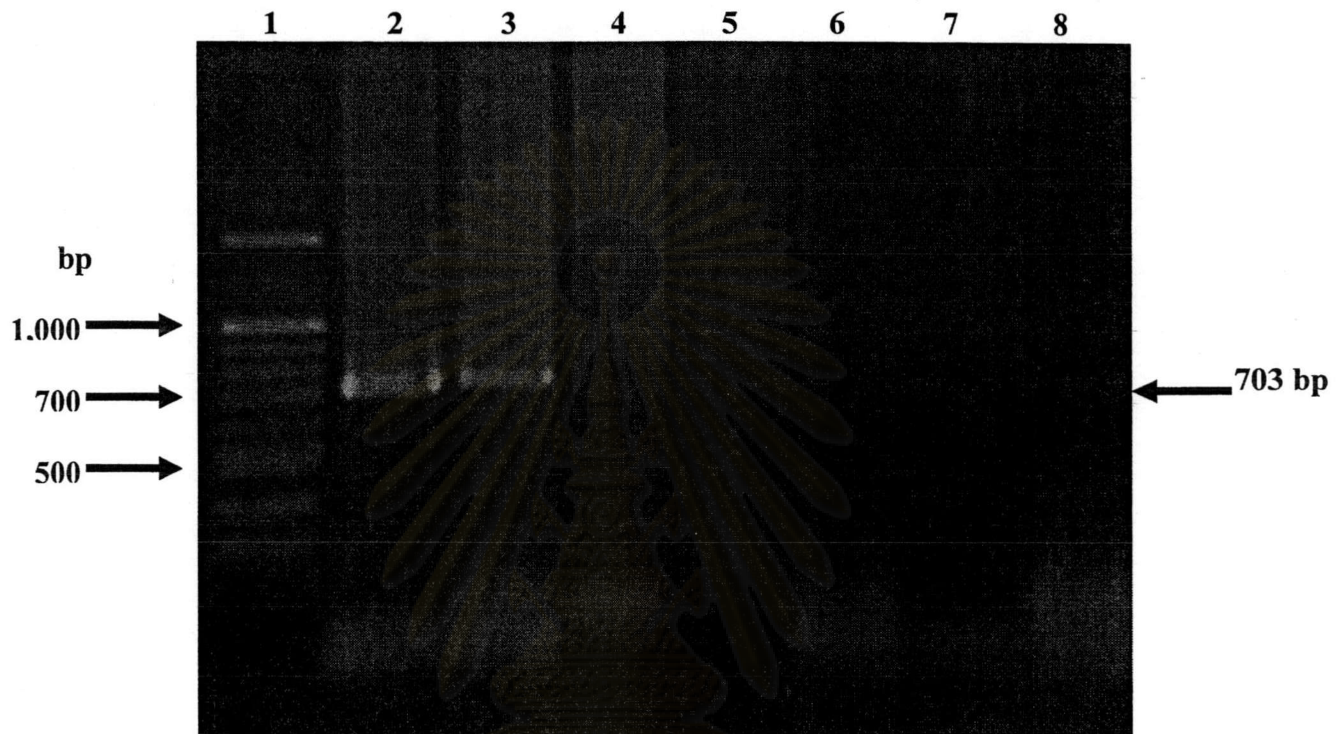
4. Cloning and sequencing of *nef*

This study confirms other previous works which demonstrated that Nef is the most immunodominant protein. However, some subjects had no detectable responses against this immunodominant protein. We hypothesised that the unusual lack of responses observed in this study was due to mutational escape in the subjects. In order to prove this hypothesis, we selected five subjects who had absence of responses against Nef protein, and analysed the *nef* sequences of the HIV quasispecies by DNA cloning and sequencing.

4.1 Sensitivity of PCR assay

To determine sensitivity of PCR assay, proviral DNA from the subject IY was diluted in 10-fold dilution and amplified by using the nested PCR assay. At least 100 ng of *nef* could be detected, as shown in Figure 36.

Figure 36 Sensitivity of the nested PCR assay. *nef* was amplified from proviral DNA of subject IY. Lane 1: 100 bp molecular marker, lane 2: 200 ng, lane 3: 100 ng, lane 4: 10 ng , lane 5: 1 ng, lane 6 : 100 pg, lane 7: 10 pg, lane 8: negative control (distilled water). The amplified *nef* product was 703 bp.



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4.2 Amplification of *nef*

To amplify *nef* we used nested PCR method to enhance both sensitivity and specificity of the PCR. The nested PCR was done to amplify a 703-bp fragment containing the entire *nef* gene, using the primers previously described by S. Reungdehsuwan, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. These primers has specific to HIV clade A/E. The outer and inner forward primers are located at *env*, whilst the outer and inner reverse primers are located at LTR (Figure 37).

nef of subject NKP and PMH could successfully be amplified whilst *nef* of subject TKH, VMK, and VPT could not be amplified (Figure 38). To determine whether failure of the *nef* amplification in these subjects was due to insufficient amount of DNA or DNA damage or the difference of nucleotide sequences at the primer binding sites from autologous virus, we used semi-nested PCR method to amplify *nef* from subject TKH, VMK, and VPT and β-globin gene in parallel. We used outer forward and inner reverse primer or inner forward and outer reverse primer in first round of amplification. The results showed that *nef* of subject TKH, VMK, and VPT could not be amplified, whilst β-globin products were amplified in all subjects (Figure 39). This experiment confirmed that the unsuccessful amplification of *nef* from the subject TKH, VMK, and VPT was not due to the amount or the quality of DNA. However, TKH *nef* was later sucessfully amplified using different primer set (Thanks to Dr. S. Sirivichayakul and Dr. S. Lorenzen, Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand). These primers has specific to HIV clade A/E. The outer and inner forward primers are located at env, whilst the outer and inner reverse primers are located at LTR (Figure 40).

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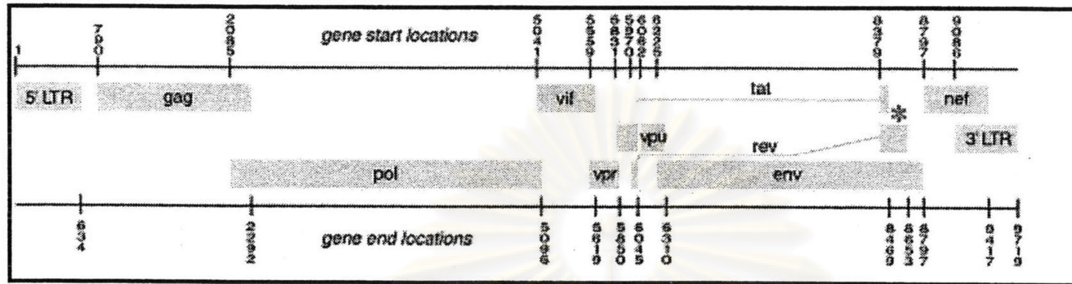
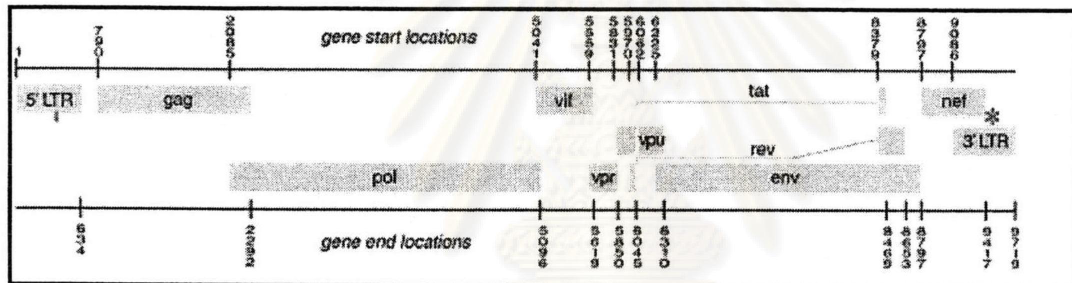
Figure 37 The position of outer and inner primers in HIV-1 subtype A/E reference strain (U54771). *nef* is located in 8371st through 8985th position residue away from the N-terminal of HIV genome. The asterisks represent the position of each primer used to amplify *nef* in this study.

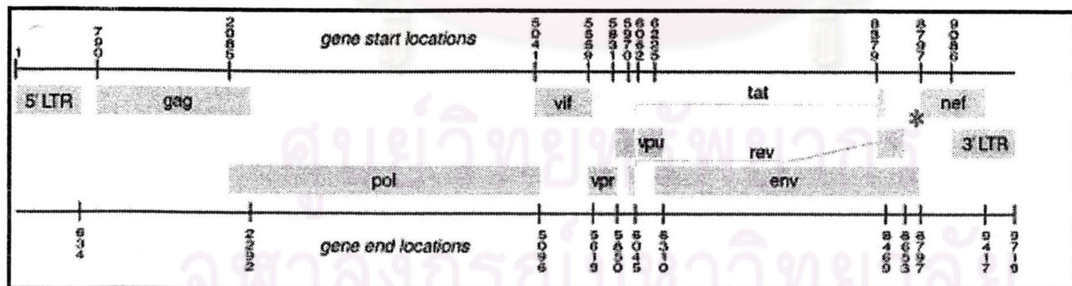
A Outer forward primer is located in 8120th through 8145th position residue.



B Outer reverse primer is located in 9045th through 9066th position residue.



C Inner forward primer is located in 8328th through 8352th position residue.



D Inner reverse primer is located in 9011th through 9030th position residue.

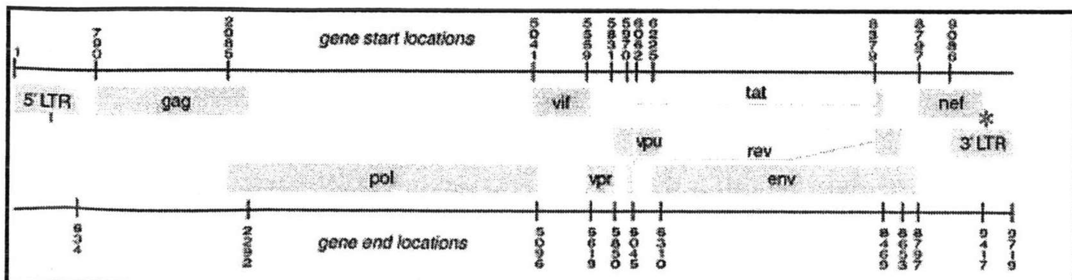


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Figure 38 Gel electrophoresis of amplified *nef* products from proviral DNA of subject NKP, PMH, TKH, VMK, and VPT. Lane 1: 1 kb molecular marker, lane 2: NKP, lane 3: PMH, lane 4: TKH, lane 5: VMK, lane 6: VPT, lane 7: DNA of healthy HIV-seronegative individual (negative control), lane 8: distilled water (negative control). The amplified *nef* product was 703 bp.

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Figure 39 Gel electrophoresis of amplified *nef* products from proviral DNA of subject TKH, VMK, and VPT. The top panel shown lane 1: 1 kb molecular marker; lane 2, 3, and 6: the PCR products of TKH, VMK, and VPT which were amplified by *nef*-specific primers by using outer forward and inner reverse primer in first round PCR; lane 4, 5, and 7: the PCR products of TKH, VMK, and VPT which were amplified by *nef*-specific primers by using inner forward and outer reverse primer in first round PCR; lane 8: distilled water (negative control). The bottom panel shown lane 1: 1 kb molecular marker; lane 2 to 4: the PCR products of TKH, VMK, and VPT which were amplified by nef-specific primers; lane 5 to 7: the PCR products of TKH, VMK, and VPT which were amplified by β-globin-specific primers; lane 8: distilled water (negative control). The amplified *nef* product was 703 bp.

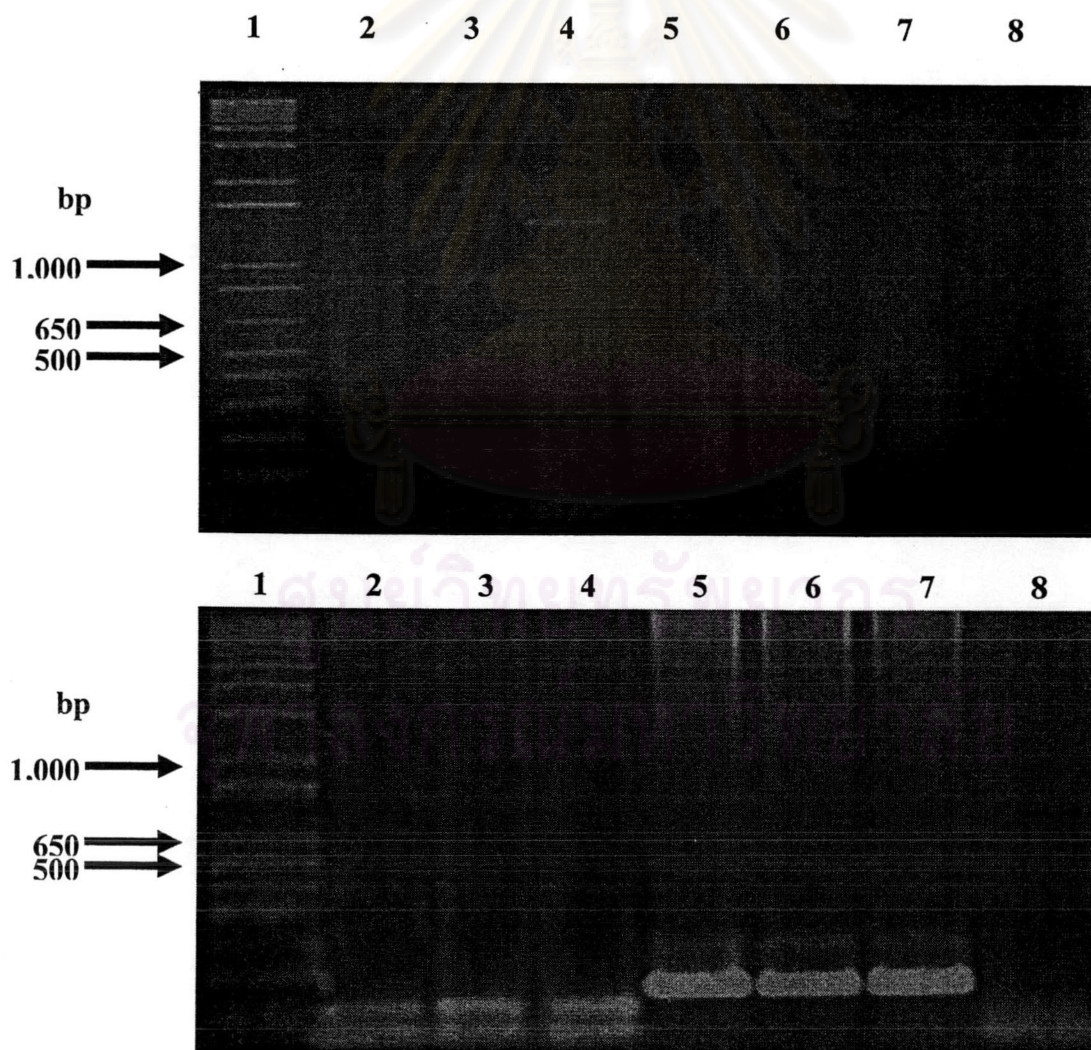
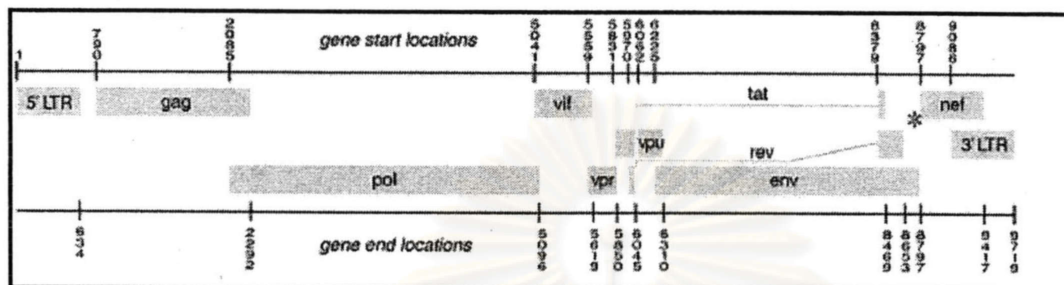
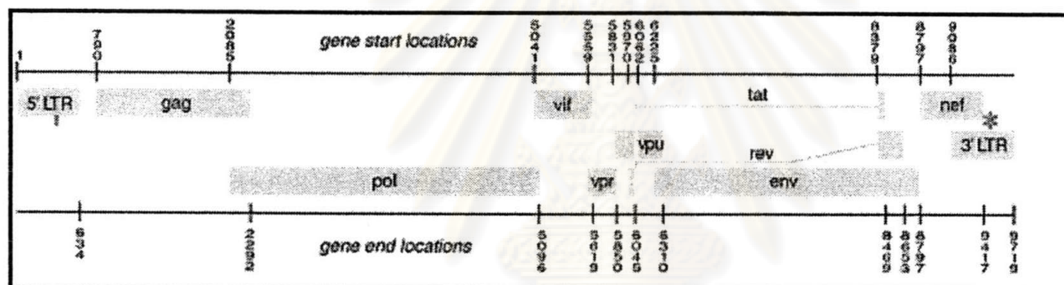


Figure 40 The position of outer and inner primers in HIV-1 subtype A/E reference strain (U54771). *nef* is located in 8371st through 8985th position residue away from the N-terminal of HIV genome. The asterisks represent the position of each primer used to amplify *nef* in this study.

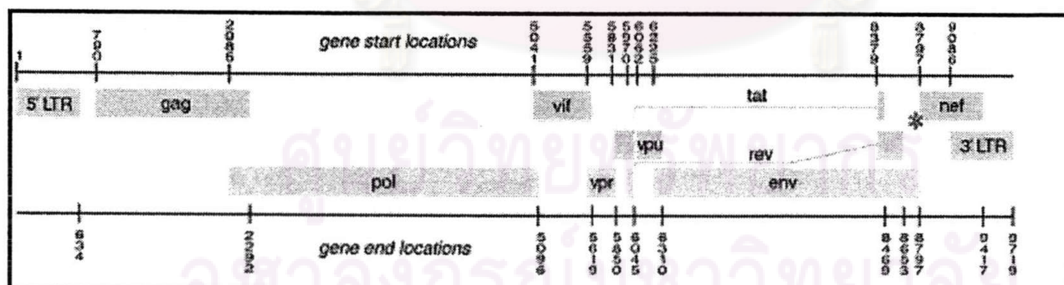
A Outer forward primer is located in 8311th through 8332th position residue.



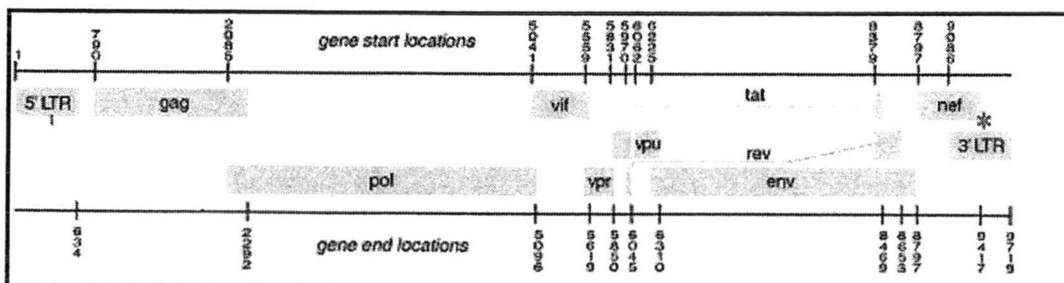
B Outer reverse primer is located in 9049th through 9069th position residue.



C Inner forward primer is located in 8328th through 8349th position residue.



D Inner reverse primer is located in 9010th through 9030th position residue.



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4.3 Cloning of *nef*

The high mutation rate of HIV can lead to emergence of quasispecies and escape mutations. Either epitope mutations or mutation at flanking region could account for abrogation of CTL recognition. Since the sequence acquired by direct sequencing method might represent only the most prevalent strain of HIV quasispecies, we decided to use cloning and sequencing method to acquire sequences of both major and minor strains. To determine whether the unusual response of subject NKP, PMH, and TKH were due to epitope or flanking region mutation, we cloned and sequenced *nef* of these subjects.

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4.4 Detection of *nef* inserts

The *nef*-inserted plasmids were cloned into competent cells (*E. coli* strain DH5α). After cloning, *nef* of each clone was amplified to confirm that these clones had *nef* in the plasmid (Figure 41). The molecular weight of amplified *nef* product was 703 bp.

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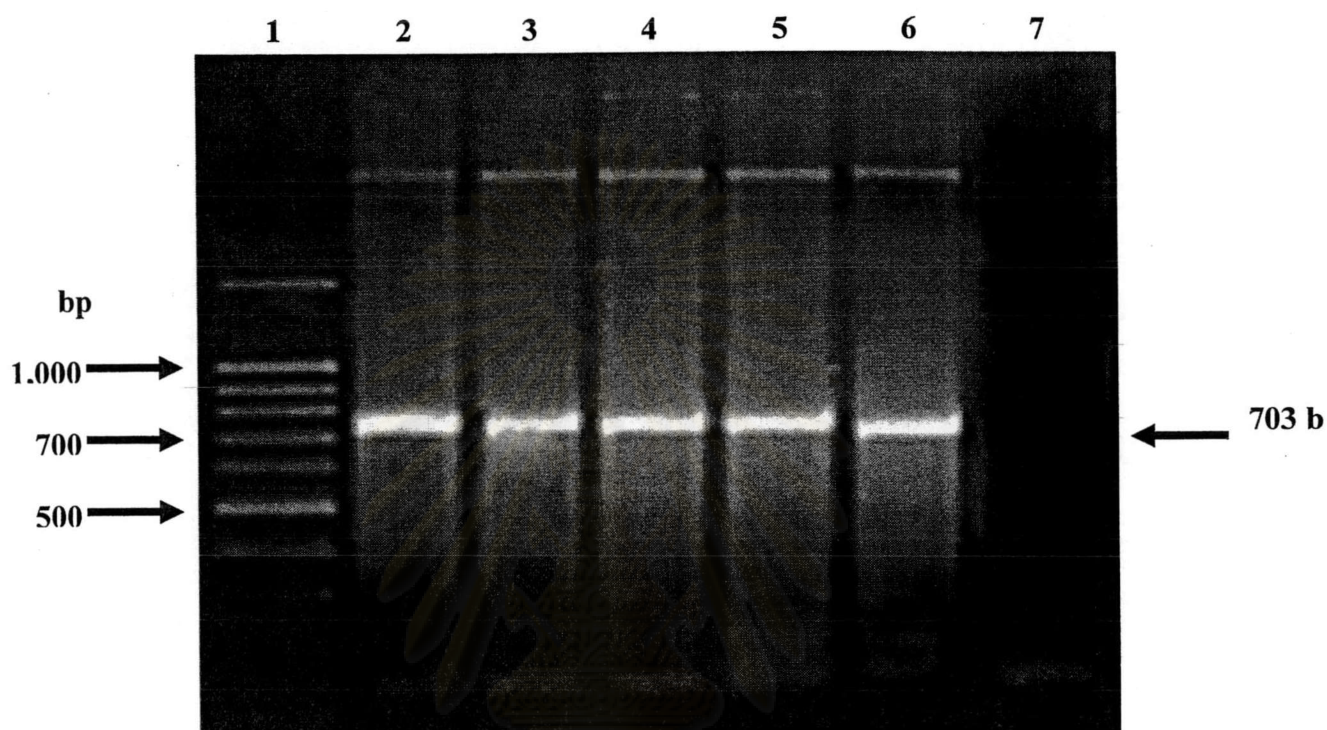
Figure 41 Gel electrophoresis of amplified *nef* products from the *nef*-inserted clones analysed by PCR assay. This figure showed the amplified products from the subject TKH. Lane 1: 100 bp molecular marker, lane 2 to 7: the amplified PCR product from 5 clones of subject TKH, lane 7: negative control (distilled water). The amplified *nef* product was 703 bp.



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4.5 Sequencing of *nef*

Amino acid sequences within the epitope and the sequences of flanking regions from each subject were compared with subtype A/E reference strain (U54771). The five clones of the purified plasmids of subject NKP, PMH, and TKH were selected to determine the nucleotide and amino acid sequences. We found that autologous isolates from these subjects differed in sequence from that of the synthetic peptide tested (Figure 42).

The DNA sequences of theses subjects had either non-synonymous or synonymous mutations. The non-synonymous mutation had either amino acid substitution or amino acid insertion. In a case of subject NKP and PMH, there are DE amino acid insertions which are located in 68th and 69th position residue away from the N-terminal of Nef protein. In a case of subject TKH, there are PAPD and AA amino acid insertions which are located in 29th to 32th and 53th and 54th position residue away from the N-terminal of Nef protein, respectively.

On the basis of epitope motifs, there are the anchored amino acid residues in epitopes that allow binding with specific HLA class I molecules for presentation on the cells surface and recognition by CTL. There are two binding anchor residues essential for binding the epitope to MHC class I molecule: the second position residue (P2) and the carboxy-terminus (C-terminus). We used "Motif Scan" from LosAlamos website analysing possible epitopes (based on HLA anchor motifs or previously described epitopes) for the subject NKP, PMH, and TKH.

There were 13 potential epitopes for subject NKP (HLA-A24, B*4002, B15) predicted by Motif Scan: 9 epitopes restricted with HLA-A24 and 4 epitopes restricted with HLA-B*4002. Since the HLA-B15 motifs have not been described, the potential HLA-B15-restricted epitopes were not taken into the analysis (Table 20). There were 4/13 potential epitopes the responses of which could have been abrogated by escape mutation: 2 epitopes restricted with HLA-A24 and 2 epitopes restricted with HLA-B*4002 (Figuer 43). These 4 epitopes were mutated at the dominant anchored residues. There were T**F**KGAFDL and T**F**KGAFDLSF which their amino acid at position 2 (P2) was changed from Y to F (5/5 clones). There were KEKGGLDG**M** and KEKGGLDGM**V** which their amino acid at C-terminus (C-ter) was changed from I to M (5/5 clones) and I to V (5/5 clones), respectively.

However, there were 3 Nef-responders (subject VPL, MWT, and PCF) who their HLA are HLA-A24. All 4 subjects had responses against Nef 8 (VRPQVPLRPMTYKGAFDLSF) and/or Nef 9 (TYKGAFDLSFFLKEKGGL). Subject VPL responed against Nef 8. Subject MWT and PCF responded against both Nef 8 and Nef 9.

There were 16 potential epitopes for subject PMH (HLA-A24, A33(03/06), B44(032/07), B27) predicted by Motif Scan: 9 epitopes restricted with HLA-A24, 3 epitopes restricted with HLA-B44, and 4 epitope restricted with HLA-B27. Since the HLA-A33 motifs have not been described, the potential HLA-A33-restricted epitopes were not taken into the analysis (Table 21). There were 8/16 potential epitopes the responses of which could have been abrogated by escape mutation: 4 epitopes restricted with HLA-A24, 2 epitopes restricted with HLA-B27, and 1 epitope restricted with HLA-B44 (Figure 44). These 8 epitopes were mutated at the dominant anchored residues. There were T**F**KAAFDL, T**F**KAAFDLSF, V**H**NTQGFFPDW, V**H**NTQGFF, P**K**EVEEDNK, and E**K**EVLMWK which their amino acid at position 2 (P2) was changed from Y to F (5/5 clones), Y to H (5/5 clones), R to K (5/5 clones), respectively. There were QEILDIWV**H** which their amino acid at C-terminus (C-ter) was changed from Y to H (5/5 clones).

However, there were 3 Nef-responders (subject VPL, MWT, and PCF) who their HLA are HLA-A24. All 3 subjects had responses against Nef 8 (VRPQVPLRPMTYKGAFDLSF), Nef 9 (TYKGAFDLSFFLKEKGGL), and Nef 11 (LDGLIYSKRRQEILDLWVY). Subject VPL responded against Nef 8. Subject MWT responded against Nef 8 and Nef 9. Subject PCF responded against Nef 8, Nef 9, and Nef 11.

There were 5 potential epitopes for subject TKH (HLA-A*0207, A*1101, B*1518, B*4601) predicted by Motif Scan: all 5 epitopes restricted with HLA-A*0207. Morover, there are only 3 potential epitopes restricted with HLA-A*1101 which have been described: QVPLRPMTYK, GAFDLSFFLK and GAFDLSFFLKEK. Since the HLA-B*1518 and B*4601 motifs have not been described, the potential HLA-B*1518 and B*4601-restricted epitopes were not taken into the analysis (Table 22). There were 2/8 potential epitopes the responses of which could have been abrogated by escape mutation (Figure 45). These epitopes were mutated at the dominant anchored residues. There were DLDKHGA**I** and PLCFGWCFK**L** which their amino acid at C-terminus (C-

ter) was changed from V to I (5/5 clones) and L to I (3/5 clones), respectively. Despite all 3 HLA-A*1101-restricted epitopes have no mutation at the dominant anchored residues, there were mutated at other amino acid positions (P3, P4, and P7). P4 was important in binding to T cell receptor and P3 and P7 were important in binding to HLA molecule.

However, there were 4 Nef-responders (subject PPY, JRS, NDC, and UKY) who their HLA are HLA-A*1101. Subject PPY had responses against Nef 8 (VRPQ**V**PLRPMTY**K**GAFDLSF). Subject JRS and NDC had responses against Nef 9 (TYKG**A**FDLSFFL**K**E**K**GGL). Subject UKY responded against Nef 8 and Nef 9.

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Figure 42 Alignment of Nef amino acid sequences. A representative Nef amino acid sequence which was tested in ELISpot assay was designated as peptides. A representative Nef amino acid sequence of subtype A/E reference strain was designated as U54771. The autologous isolates from the subjects differed in sequences from that of the synthetic peptide tested were show in the dark shade. The insertion or deletion mutations were show in the square. The numbers indicate synthetic peptide tested number.

Figure 42 Alignment of Nef amino acid sequences (continued).

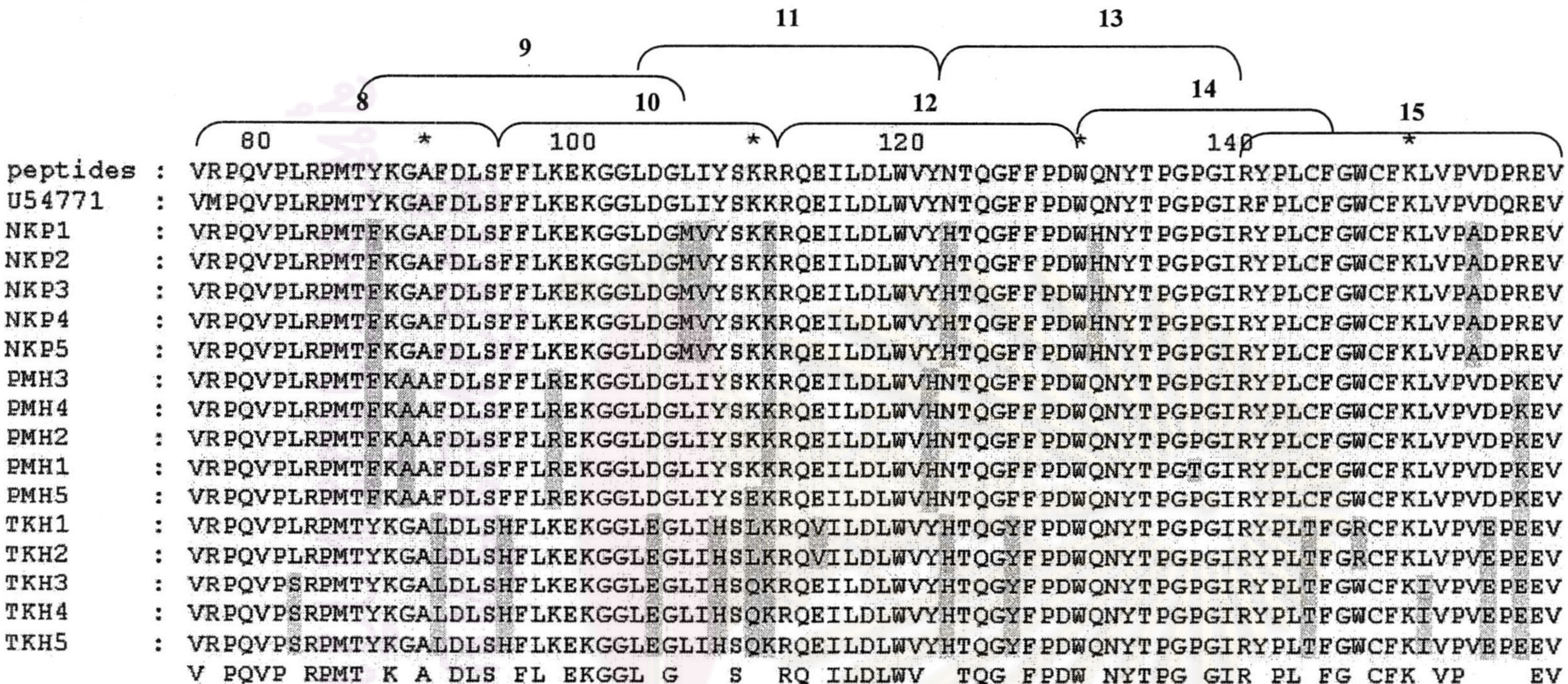

Figure 42 Alignment of Nef amino acid sequences (continued).

```
                        17                    19                  21
                  16              18                  20
            *     160          *     180          *     200        *
peptides : KLVPVDPREVEEDNKGENNCLLHPMSQHGIEDEEREVLMWKFDSALARKHIARELHPEYYKDCA- : 205
U54771   : KLVPVDQREVEEDNKGENNCLLHPMSQHGIEDEEREVLMWKFDSALARKHVAREQHPEYYKDC*- : 204
NKP1     : KLVPADPREVEEDTKEENNCLLHPANQHGLEDEDREVLIWKFDSALARRHIAREQRPEFYKDC*- : 206
NKP2     : KLVPADPREVEEDTKEENNCLLHPANQHGLEDEDREVLIWKFDSALARRHIAREQRPEFYKDC*- : 206
NKP3     : KLVPADPREVEEDTKEENNCLLHPANQHGLEDEDREVLIWKFDSALARRHIAREQRPEFYKDC*- : 206
NKP4     : KLVPADPREVEEDTKEENNCLLHPANQHGLEDEDREVLIWKFDSALARRHIAREQRPEFYKDC*- : 206
NKP5     : KLVPADPREVEEDTKEENNCLLHPANQHGLEDEDREVLIWKFDSALARRHIAREQRPEFYKDC*- : 206
PMH3     : KLVPVDPKEVEEDNKGENTCLLHPMSPHGMEDEEKEVLMWKFDSALARKHIARELHPEFYKDC*- : 206
PMH4     : KLVPVDPKEVEEDNKGENTCLLHPMSPHGMEDEEKEVLMWKFDSALARKHIARELHPEFYKDC*- : 206
PMH2     : KLVPVDPKEVEEDNKGENTCLLHPMSPHGMEDEEKEVLMWKFDSALARKHIARELHPEFYKDC*- : 206
PMH1     : KLVPVDPKEVEEDNKGENTCLLHPMSPHGMEDEEKEVLMWKFDSALARKHIARELHPEFYKDC*- : 206
PMH5     : KLVPVDPKEVEEDNKGENTCLLHPMSPHGMEDEEKEVLMWKFDSALARKHIARELHPEFYKDC*- : 206
TKH1     : KLVPVEPEEVEEANEGENNSLLHPISLHGMEDPEKEVLKWKFDSRLARKHMARELHPEFYKDC*- : 210
TKH2     : KLVPVEPEEVEEANEGENNSLLHPISLHGMEDPEKEVLKWKFDSRLARKHMARELHPEFYKDC*- : 210
TKH3     : KIVPVEPEEVEEANEGENNSLLHPISLHGMEDPEKEVLKWKFDSRLARKHMARELHPEFYKDC*- : 210
TKH4     : KIVPVEPEEVEEANEGENNSLLHPISLHGMEDPEKEVLKWKFDSRLARKHMARELHPEFYKDC*- : 210
TKH5     : KIVPVEPEEVEEANEGENNSLLHPISLHGMEDPEKEVLKWKFDSRLARKHMARELHPEFYKDC*- : 210
           K VP   EVEE    EN  LLHP    HG ED   EVL WKFDS LAR H ARE  PE YKDC
```

Table 20 Possible epitopes in Nef amino acid sequences of the subject NKP based on anchor residues. Table A shows known anchor residue motifs associated with the HLA-A24 and B*4002. X character represents any amino acid at the given position, whilst square brackets list the amino acids required at that position. Table B shows the list of potential HLA-A24 and B*4002-restricted epitopes in Nef.

A Anchor motifs examines

HLA	Anchor Residue Motifs	HLA	Anchor Residue Motifs
A24	X[Y]XXXXX[I/L/F/W]	B*4002	X[E]XXXXX[I/L]
A24	X[Y]XXXXXX[I/L/F/W	B*4002	X[E]XXXXXX[I/L]
A24	X[Y]XXXXXXX[I/L/F/W]		

B Possible epitopes based on Anchor residues

No	Position in query peptide	AA sequence	HLA
1	(107-115)	IYSKRRQEI	A24
2	(86-93)	TYKGAFDL	A24
3	(120-127)	VYNTQGFF	A24
4	(132-139)	NYTPGPGI	A24
5	(86-95)	TYKGAFDLSF	A24
6	(107-116)	IYSKRRQEIL	A24
7	(130-139)	RYPLCFGWCF	A24
8	(120-130)	VYNTQGFFPDW	A24
9	(140-147)	RYPLCFGW	A24
10	(27-41)	TEGVGAVSQDL	B*4002
11	(98-106)	KEKGGLDGL	B*4002
12	(98-107)	KEKGGLDGLI	B*4002
13	(179-187)	IEDEEREVL	B*4002

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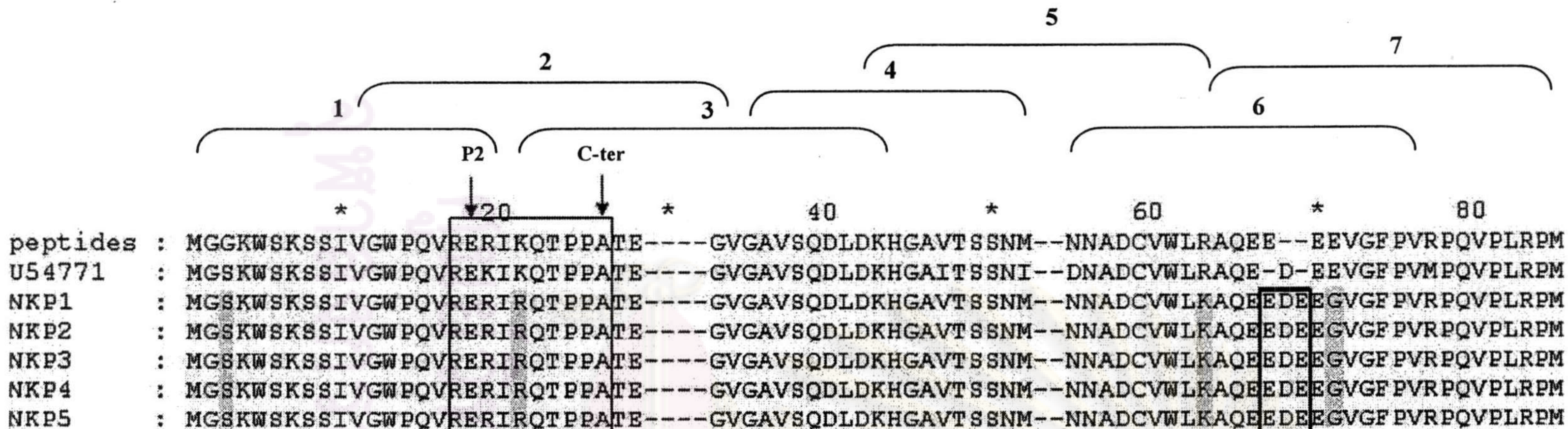
Figure 43 Possible HLA-A24 and B*4002-restricted epitopes in Nef amino acid sequences of the subject NKP based on anchor residues.



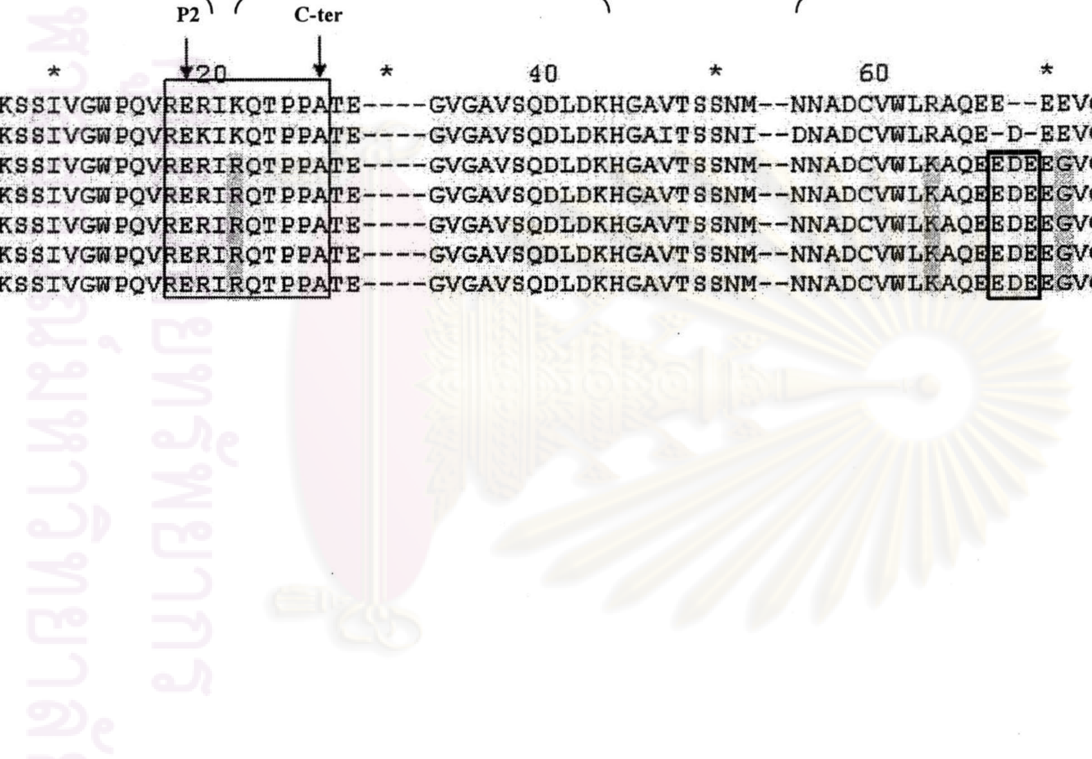


Figure 43 Possible HLA-A24 and B*4002-restricted epitopes in Nef amino acid sequences of the subject NKP based on anchor residues (continued).

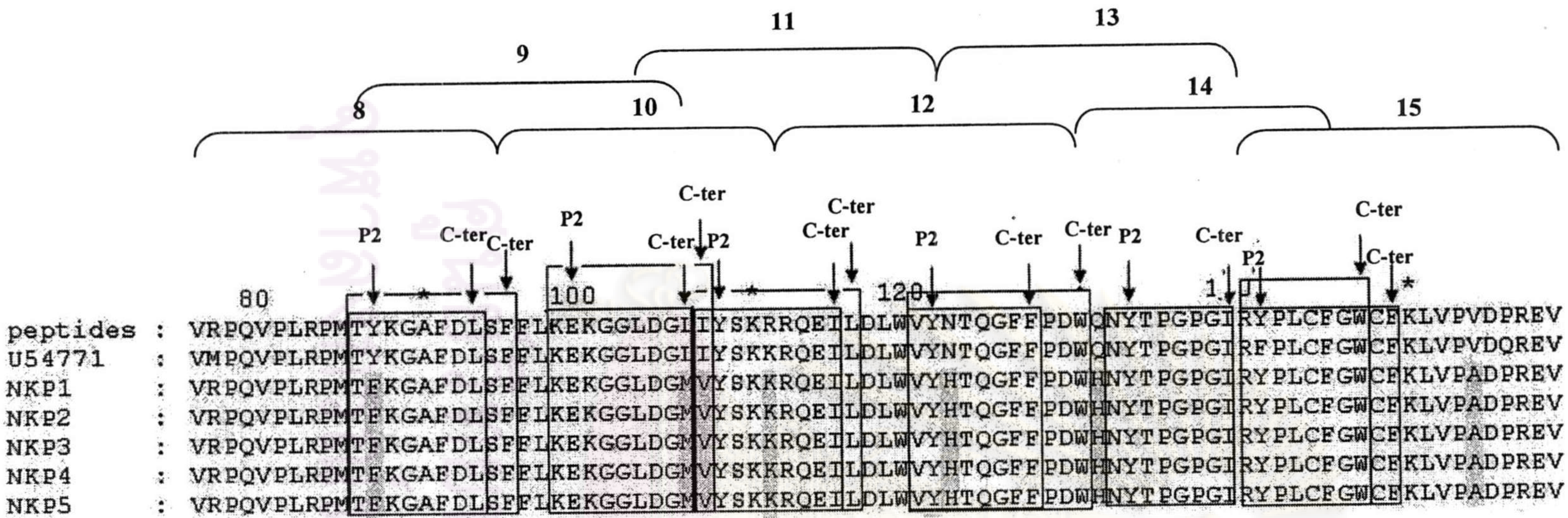


Figure 43 Possible HLA-A24 and B*4002-restricted epitopes in Nef amino acid sequences of the subject NKP based on anchor residues (continued).

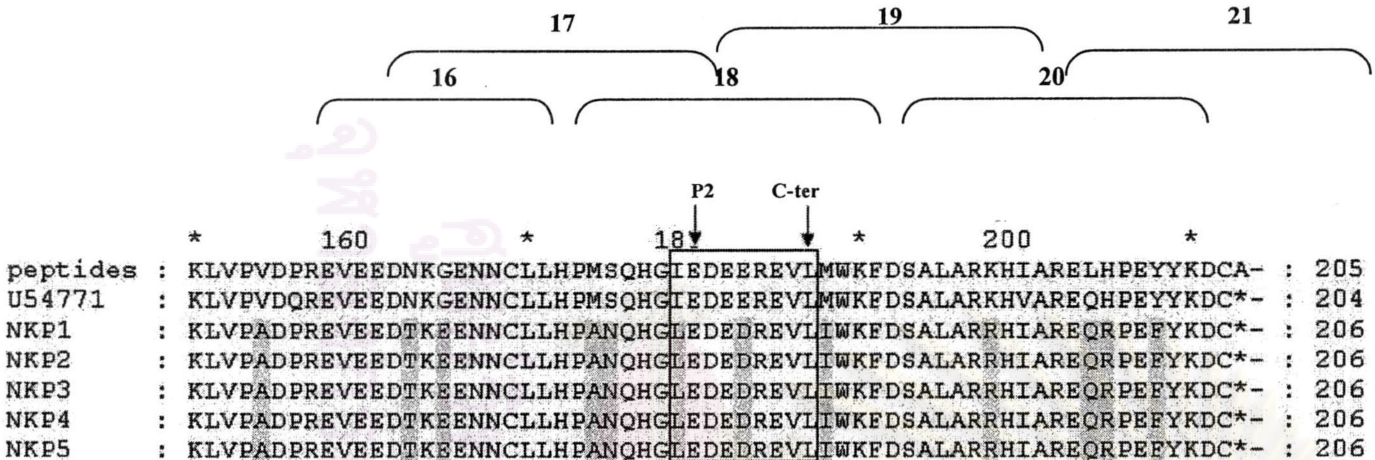


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Table 21 Possible epitopes in Nef amino acid sequences of the subject PMH based on anchor residues. Table A shows known anchor residue motifs associated with the HLA submitted. X represents any amino acid at the given position, whilst square brackets list the amino acids required at that position. Table B shows the list of potential HLA-A24, B44, and B27-restricted epitopes in Nef.

A Anchor motifs examines

HLA	Anchor Residue Motifs	HLA	Anchor Residue Motifs
A24	X[Y]XXXXX[I/L/F/W]	B44	X[E/F]XXXXXXX[Y]
A24	X[Y]XXXXXX[I/L/F/W]	B27	X[R]XXXXX[K/L]
A24	X[Y]XXXXXXX[I/L/F/W]	B27	X[R]XXXXXX[K/L]
B44	X[E/F]XXXXX[Y]	B27	X[R]XXXXXXX[K/L]
B44	X[E/FXXXXXX[Y]		

B Possible epitopes based on Anchor residues

No	Position in query peptide	AA sequence	HLA
1	(86-93)	TYKGAFDL	A24
2	(86-95)	TYKGAFDLSF	A24
3	(107-115)	IYSKRRQEI	A24
4	(107-116)	IYSKRRQEIL	A24
5	(120-130)	VYNTQGFFPDW	A24
6	(120-127)	VYNTQGFF	A24
7	(132-139)	NYTPGPGI	A24
8	(140-147)	RYPLCFGW	A24
9	(140-149)	RYPLCFGWCF	A24
10	(98-108)	KEKGGLDGLIY	B44
11	(114-121)	EIIDLWVY	B44
12	(126-133)	FFPDWQNY	B44
13	(111-118)	RRQEILDL	B27
14	(110-118)	KRRQEILDL	B27
15	(156-164)	PREVEEDNK	B27
16	(183-190)	EREVLMWK	B27

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Figure 44 Possible HLA-A24, B44, and B27-restricted epitopes in Nef amino acid sequences of the subject PMH based on anchor residues.

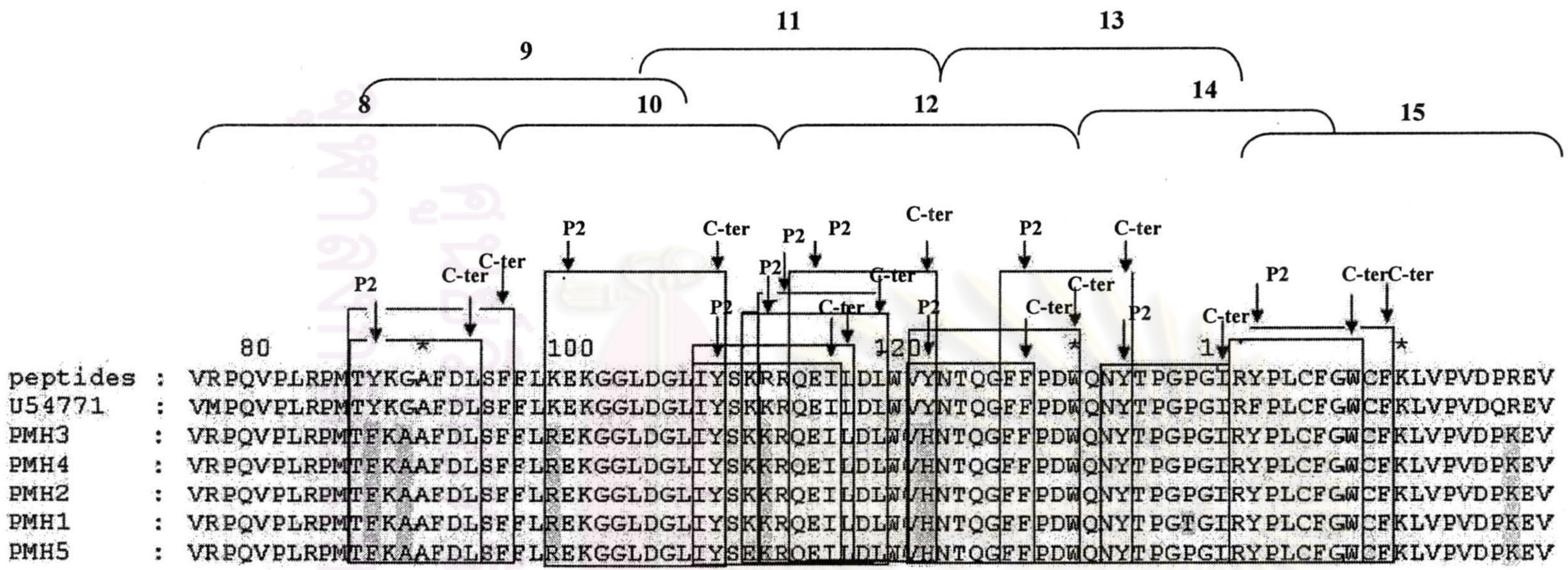

Figure 44 Possible HLA-A24, B44, and B27-restricted epitopes in Nef amino acid sequences of the subject PMH based on anchor residues (continued).

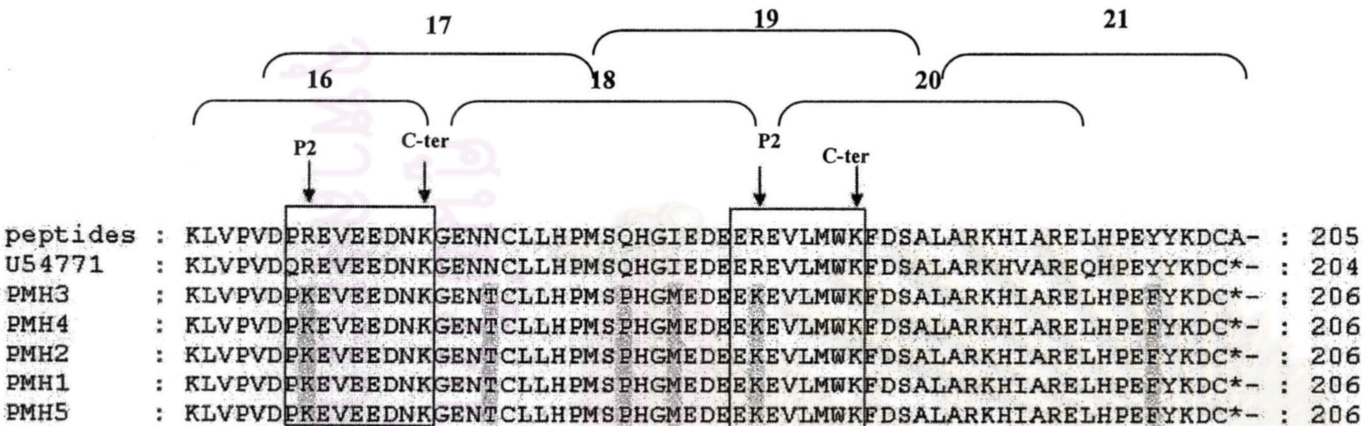

Table 22 Possible epitopes in Nef amino acid sequences of the subject TKH based on anchor residues. Table A shows known anchor residue motifs associated with the HLA-A*0207, and A*1101. X character represents any amino acid at the given position, whilst square brackets list the amino acids required at that position. Table B shows the list of potential HLA-A*0207, A*1101-restricted epitopes in Nef

A Anchor motifs examines

HLA	Anchor Residue Motifs	HLA	Anchor Residue Motifs
A*0207	X[L/D]XXXX[V/L]	A*1101	X[V/A]XXXXX[K]
A*0207	X[L/D]XXXXX[V/L]	A*1101	X[V/A]XXXXXX[K]
A*0207	X[L/D]XXXXXX[V/L]	A*1101	X[V/A]XXXXXXX[K]

B Possible epitopes based on Anchor residues

No	Position in query peptide	AA sequence	HLA
1	(40-47)	DLDKHGAV	A*0207
2	(61-72)	WLRAQEEEEV	A*0207
3	(142-151)	PLCFGWCFKL	A*0207
4	(142-152)	PLCFGWCFKLV	A*0207
5	(186-115)	VLMWKFDSAL	A*0207
6	(79-88)	QVPLRPMTYK	A*1101
7	(89-98)	GAFDLSFFLK	A*1101
8	(89-100)	GAFDLSFFLKEK	A*1101

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Figure 45 Possible HLA-A*1101 and A*0207-restricted epitopes in Nef amino acid sequences of the subject TKH based on anchor residues.

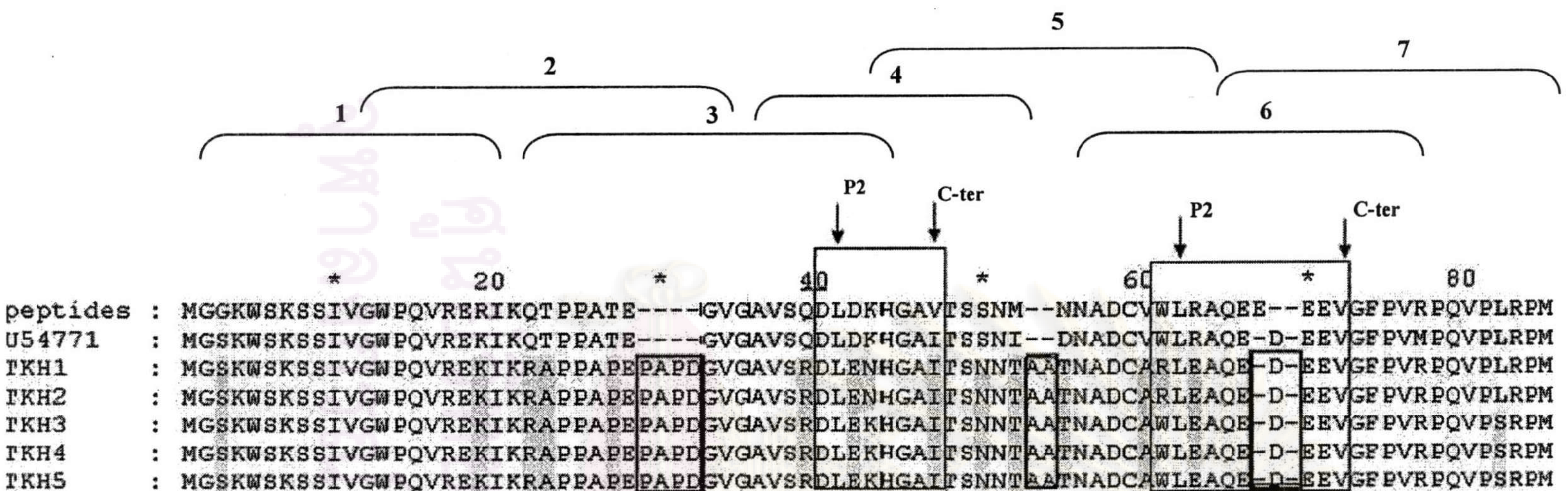
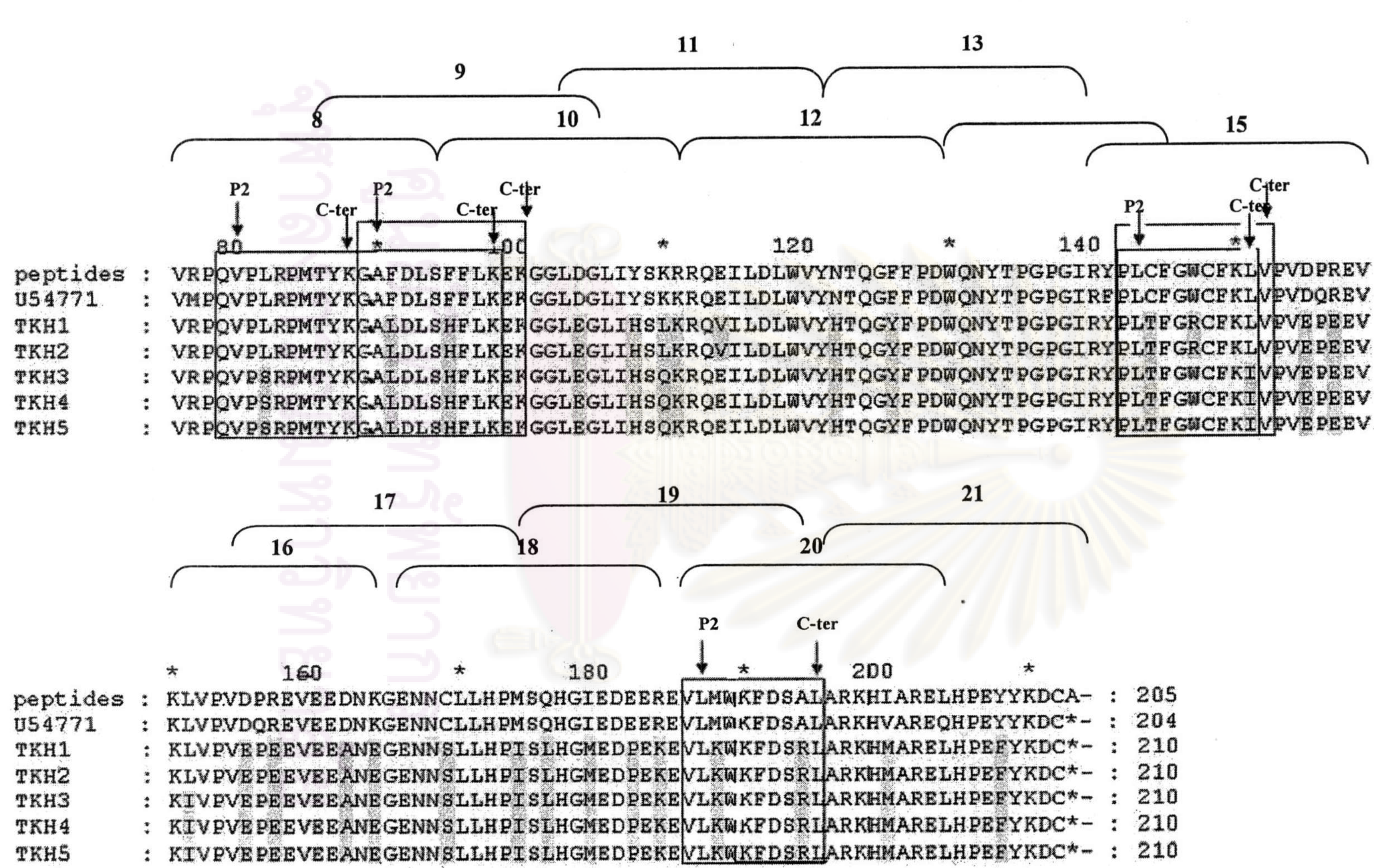


Figure 45 Possible HLA-A*1101 and B*0207-restricted epitopes in Nef amino acid sequences of the subject TKH based on anchor residues (continued).



5. Cloning and sequencing of *gag*

The most common of subtype of HIV-1 in HIV-1-infected Thais is subtype A/E which is a mosaic of a clade A virus and clade E virus, with the gag gene (and other regions) of subtype E viral genome falling within clade A. We therefore used Gag peptides spanning HIV-1 subtype A strain 92UG037 in peptide-based IFN-γ ELISpot assay. Unexpectedly, Gag responses were recognised at low frequency compared to what reported in a number of studies whereby comprehensive epitope analysises Gag was the most frequency recognised protein (8, 9). We therefore hypothesised that the unusual responses observed in this study was due to amino acid variation of Gag peptides differed from autologous virus. In order to prove this hypothesis, we interested to analyse the *gag* sequences of the HIV quasispecies by DNA cloning and sequencing of the subject VMK, VPT, and NKP who had absence of responses against both Gag and Nef protein and had low viral load. The viral load of VMK was lower than 50 copies/ml, the viral load of VPT was 159 copies/ml, and the viral load of NKP was 2,180 copies/ml.

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5.1 Sensitivity of *gag*

To determine the sensitivity of PCR assay, serial 10-fold dilutions of proviral DNA from subject IY was amplified. At least 100 ng of *gag* could be detected, as shown in Figure 46.

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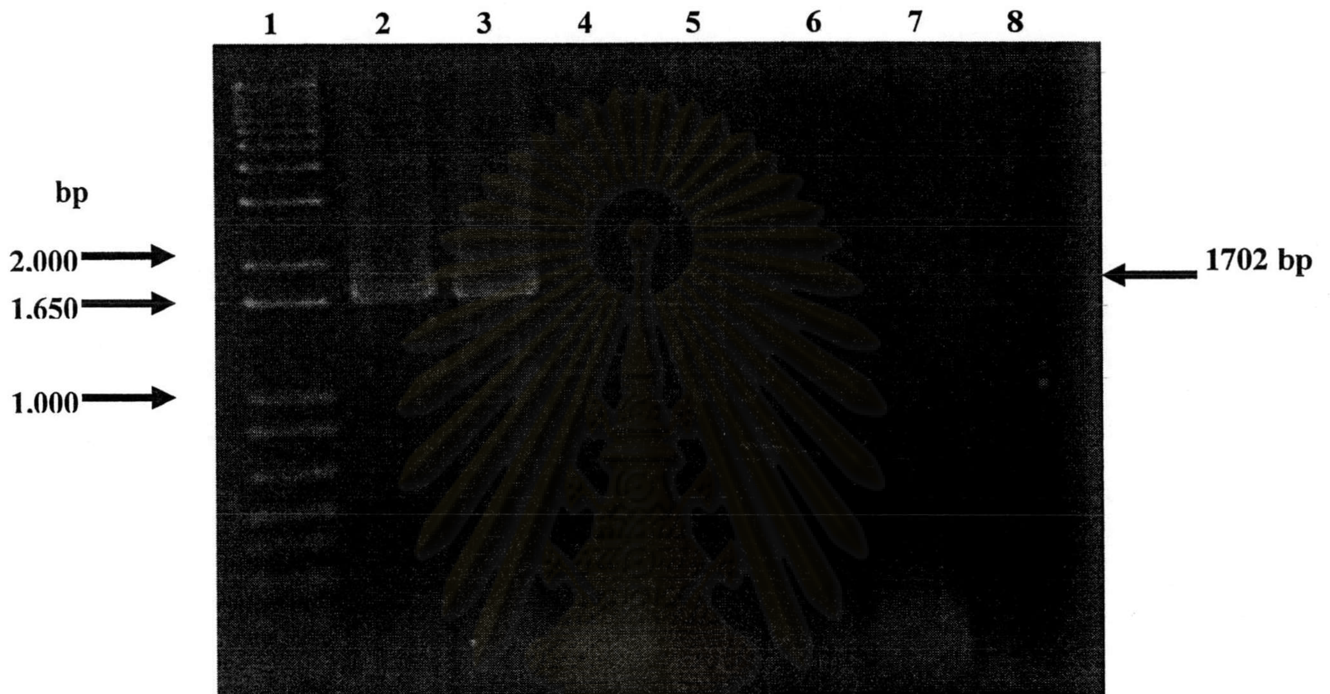
Figure 46 Sensitivity of the semi-nested PCR assay. *gag* was amplified from proviral DNA of subject IY. Lane 1: 1 kb molecular marker, lane 2: 200 ng, lane 3: 100 ng, lane 4: 10 ng , lane 5: 1 ng, lane 6 : 100 pg, lane 7: 10 pg, lane 8: negative control (distilled water). The amplified *gag* product was 1702 bp.



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5.2 Amplification of *gag*

To amplify *gag* we used semi-nested PCR method to enhance both sensitivity and specificity of The PCR. The semi-nested PCR was performed to amplify a 1702-bp fragment containing the entire *gag* gene using the primers previously described by Associate professor Dr. Arunee Thitithanyanont, Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. These primers have specific to HIV clade A/E. The outer and inner forward primers are located at non-coding region, whilst the outer and inner reverse primers are located at *pol* gene (Figure 47).

gag of subject VMK and NKP could be successfully amplified whilst *gag* of subject VPT could not be amplified (Figure 48). To determine whether failure of *gag* amplification in these subjects was due to insufficient amount of DNA or DNA damage or the difference of nucleotide sequences at the primer binding sites from autologous virus, we used semi-nested PCR method to amplify *gag* from subject VPT and β-globin gene in parallel. We used outer forward and inner reverse primer or inner forward and outer reverse primer in first round of amplification (data not shown). The results showed that *gag* of subject VPT could not be amplified, whilst β-globin products were amplified (Figure 49). This experiment confirmed that the unsuccessful amplification of *gag* from the subject VPT was not due to the amount or the quality of DNA.

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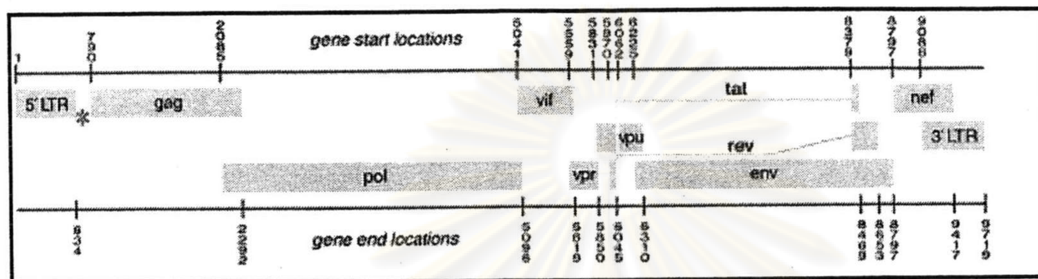
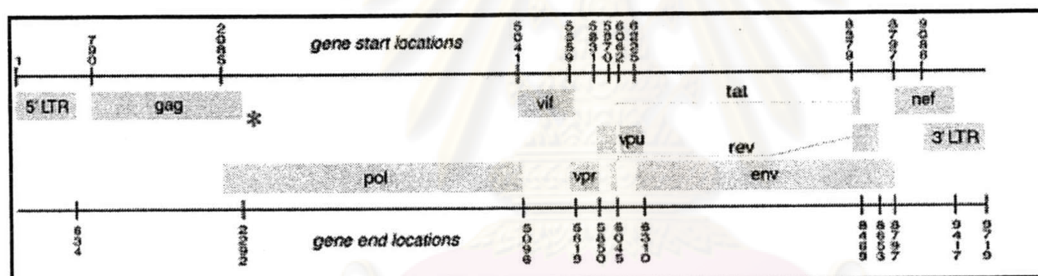
Figure 47 The position of outer and inner primers in HIV-1 subtype A/E reference strain (U54771). *gag* is located in 790st through 2492th position residue away from the N-terminal of HIV genome. The astherisks represent the position of each primer used to amplify *gag* in this study.

A Outer forward primer is located in 684th through 707th position residue.



B Outer and inner reverse primer is located in 2382th through 2407th position residue.



C Inner forward primer is located in 705th through 728th position residue.

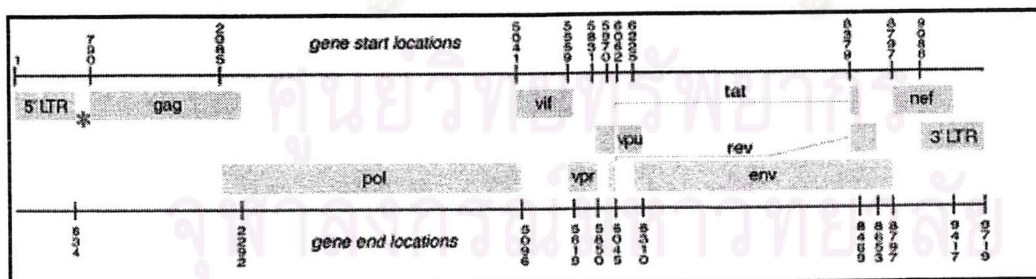


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Figure 48 Gel electrophoresis of amplified *gag* products from proviral DNA of subject NKP, VMK, and VPT. Lane 1: 100 bp molecular marker, lane 2: NKP, lane 3: VMK, lane 4: VPT, lane 5: DNA of healthy HIV-seronegative individual (negative control), lane 6: distilled water (negative control). The amplified *gag* product was 1702 bp.

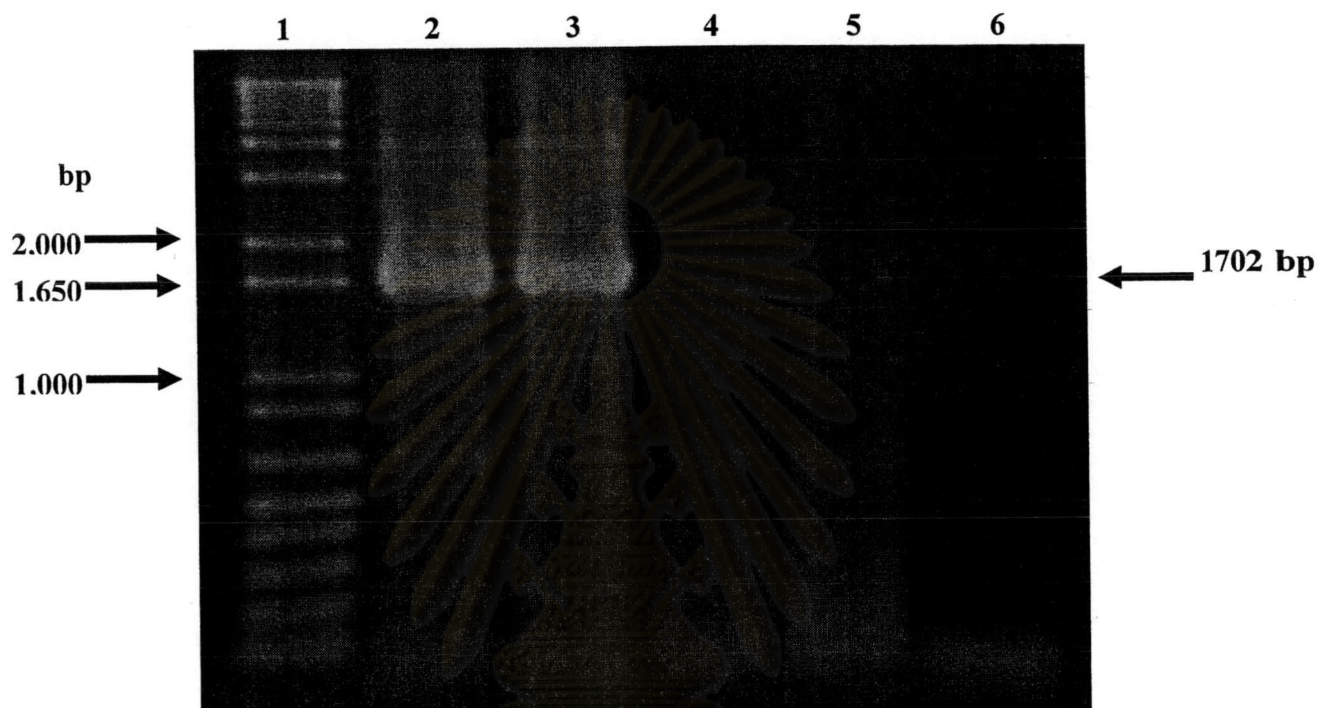

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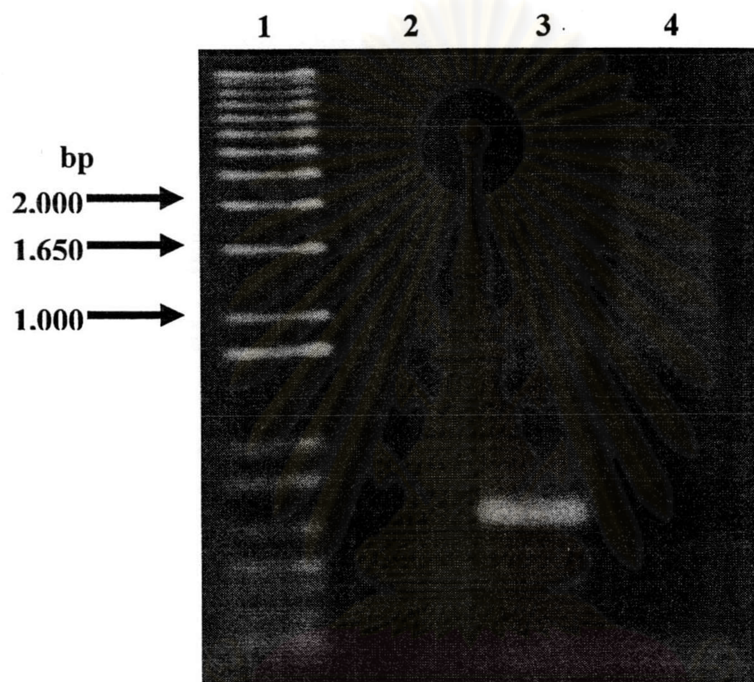
Figure 49 Gel electrophoresis of amplified *gag* products from proviral DNA of subject VPT. Lane 1: 1 kb molecular marker; lane 2: the PCR product of subject VPT which were amplified by *gag*-specific promer; lane 3 the PCR product of subject VPT which were amplified by β-globin-specific primers; lane 4: distilled water (negative control). The amplified *gag* product was 1702 bp.



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5.3 Cloning of *gag*

The high mutation rate of HIV can lead to emergence of quasispecies and escape mutations. The amino acid variation of autologous virus may differ from Gag peptides which were used in the ELISpot assay. Since the sequence acquired by direct sequencing method might represent only the most prevalent strain of HIV quasispecies, we decided to use cloning and sequencing method to acquire sequences of both major and minor strains. To determine whether the unusual response of subject NKP and VMK were due to epitope or flanking region mutation, we cloned and sequenced *gag* of these subjects.

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5.4 Detection of *gag* inserts

The *gag*-inserted plasmids were cloned into competent cells (E. coli strain DH5α). After cloning, *gag* of each clone was amplified to confirm that these clones had *gag* in the plasmid (figure 50). The molecular weight of amplified *gag* product was 1702 bp.

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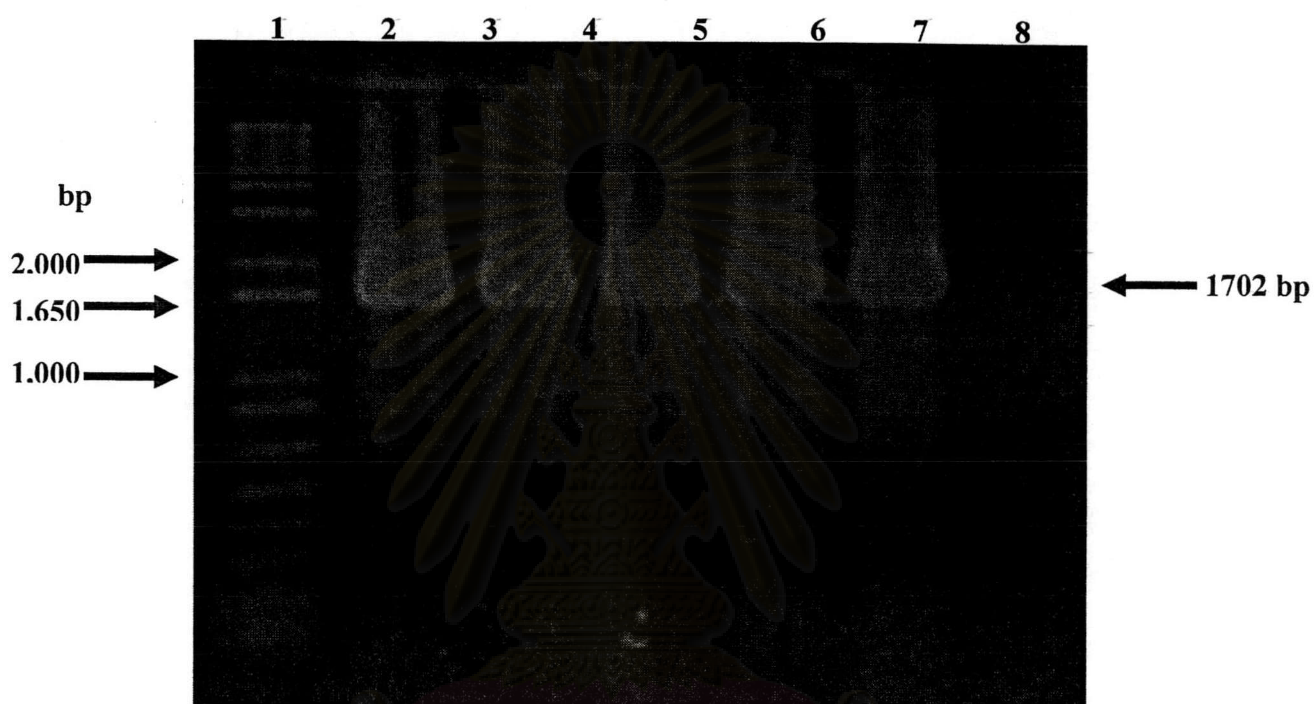
Figure 50 Gel electrophoresis of amplified *gag* products from the *gag*-inserted clones analysed by PCR assay. This figure showed the amplified product from the subject VMK. Lane 1: 100 bp molecular marker, lane 2 to 7: the amplified PCR product from 5 clones of subject VMK, lane 7: negative control (distilled water). The amplified *gag* product was 1702 bp.



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5.5 Sequencing of *gag*

Amino acid sequences within the epitope and the sequences of flanking regions from each subject were compared with subtype A/E reference strain (U54771). The five clones of the purified plasmids of subject NKP, and VMK were selected to determine the nucleotide and amino acid sequences. We found that autologous isolates from these subjects differed in sequence from that of the synthetic peptide tested (Figure 51).

The DNA sequences of these subjects had either non-synonymous or synonymous mutations. The non-synonymous mutation had either amino acid substitution or amino acid insertion or deletion. In a case of subject NKP there are HER amino acid insertion which is located in 428th to 430th position residue and A, SPP, RDQ amino acid deletions which are located in 462th, 478th to 480th and 497th to 499th position residue away from the N-terminal of Gag protein, respectively. In a case of subject VMK, there are A amino acid deletion which is located in 462th position residue away from the N-terminal of Gag protein. Moreover, there was nonsence mutation which is any change in DNA that causes a termination codon to replace a codon representing an amino acid. This stop codon has occurred in 505th position residue away from the N-terminal of Gag protein in 2/5 clones of subject VMK and all 5 clones in subject NKP.

On the basis of epitope motifs, there are the anchored amino acid residues in epitopes that allow binding with specific HLA class I molecules for presentation on the cells surface and recognition by CTL. There are two binding anchor residues essential for binding the epitope to MHC class I molecule: the second position residue (P2) and the carboxy-terminus (C-terminus). We used "Motif Scan" from LosAlamos website analysing possible epitopes (based on HLA anchor motifs or previously described epitopes) for the subject NKP, and VMK.

There were 23 potential epitopes for subject NKP (HLA-A24, B*4002, B15) predicted by Motif Scan: 7 epitopes restricted with HLA-A24 and 17 epitopes restricted with HLA-B*4002. Since the HLA-B15 motifs have not been described, the potential HLA-B15-restricted epitopes were not taken into the analysis (Table 23). There were 4/23 potential epitopes the responses of which could have been abrogated by escape mutation: 1 epitope restricted with HLA-A24 and 3 epitopes restricted with HLA-B*4002 (Figuer 52). These 5

epitopes were mutated at the dominant anchored residues. There were L**F**NTVATL, I**G**QLQSTL, and L**D**VKDTKEAL which their amino acid at position 2 (P2) was changed from Y to F (5/5 clones), E to G (5/5 clones), and E to D (5/5 clones), respectively. There were EEAAEWDR**V** and NEEAAEWDR**V** which their amino acid at C-terminus (C-ter) was changed from L to V (5/5 clones).

However, there were 3 subjects (VPL, MWT, and PCF) who their HLA are HLA-A24, had no responses against Gag 8 (GTEELRSLYNTVATLYCVHQ).

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Figure 51 Alignment of Gag amino acid sequences. A representative Gag amino acid sequence which was tested in ELISpot assay was designated as peptides. A representative Gag amino acid sequence of subtype A/E reference strain was designated as U54771. The autologous isolates from the subjects differing in sequences from that of the synthetic peptide tested were shown in the dark shade. The insertion or deletion mutations were show in the square. The numbers indicate synthetic peptide tested number.

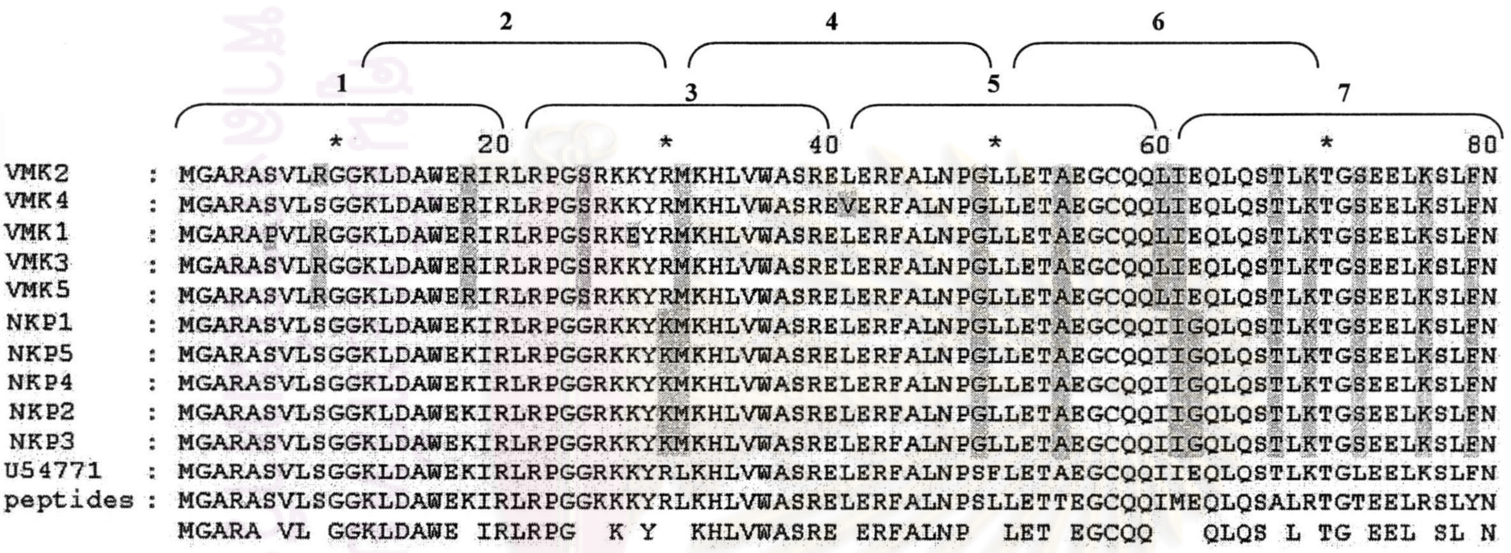

Figure 51 Alignment of Gag amino acid sequences (continued).

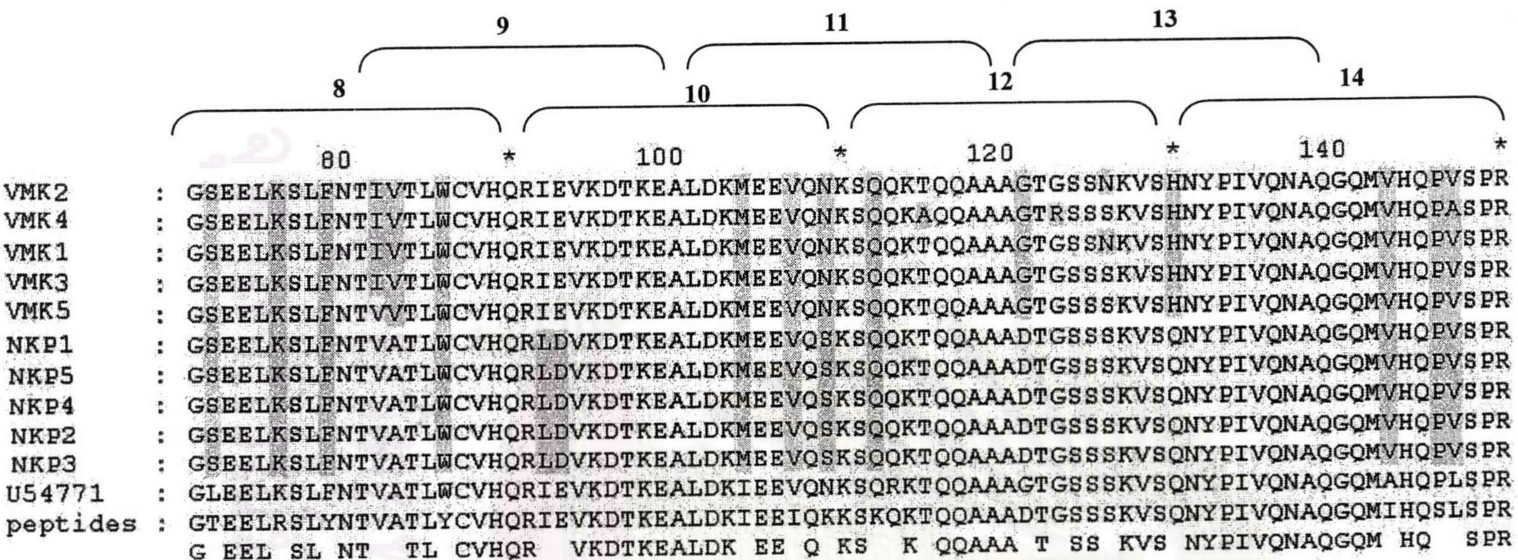

Figure 51 Alignment of Gag amino acid sequences (continued).

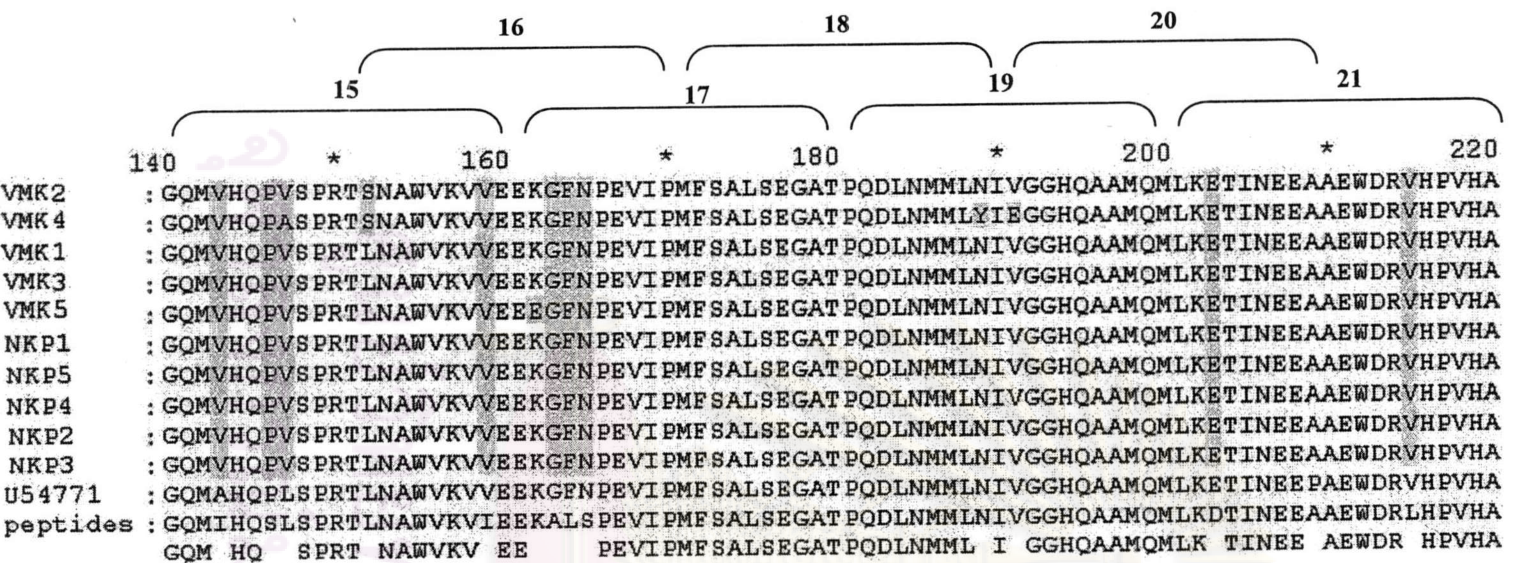

Figure 51 Alignment of Gag amino acid sequences (continued).

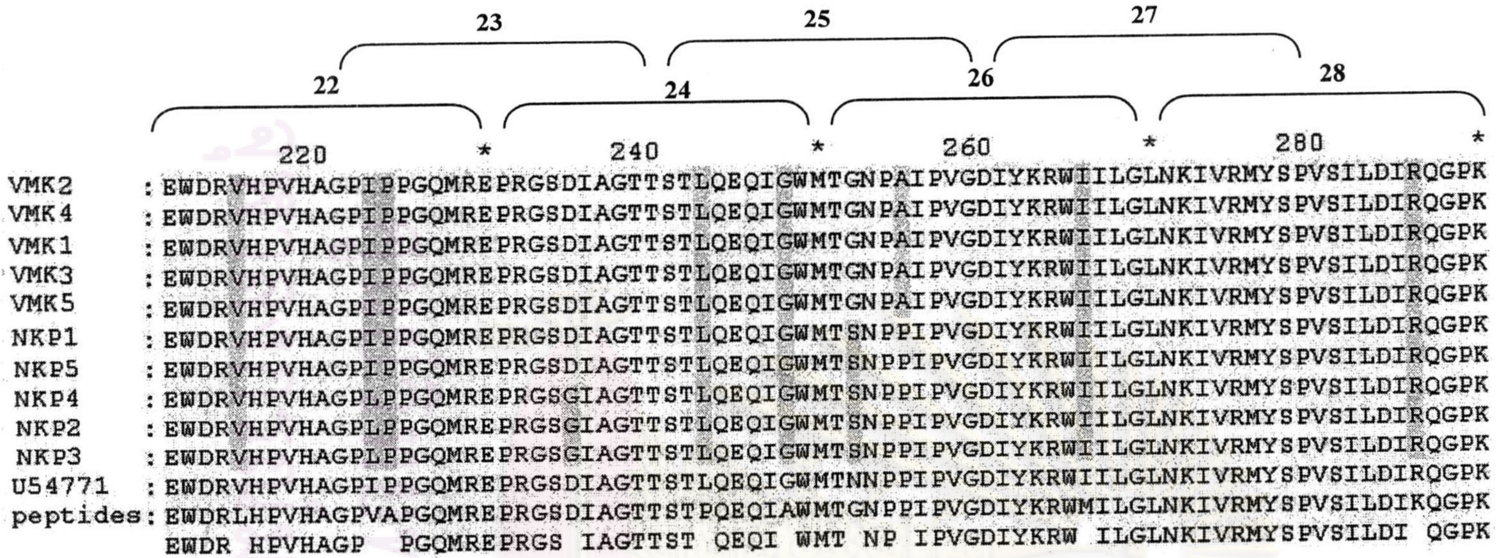

Figure 51 Alignment of Gag amino acid sequences (continued).

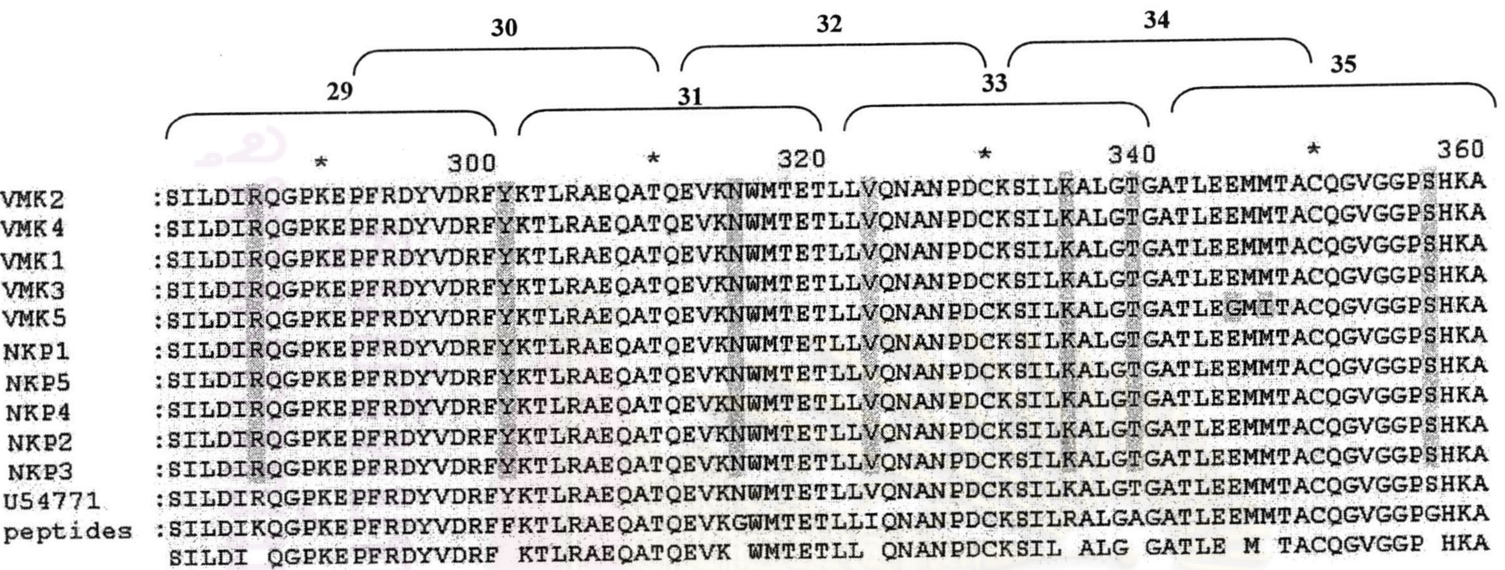

Figure 51 Alignment of Gag amino acid sequences (continued).

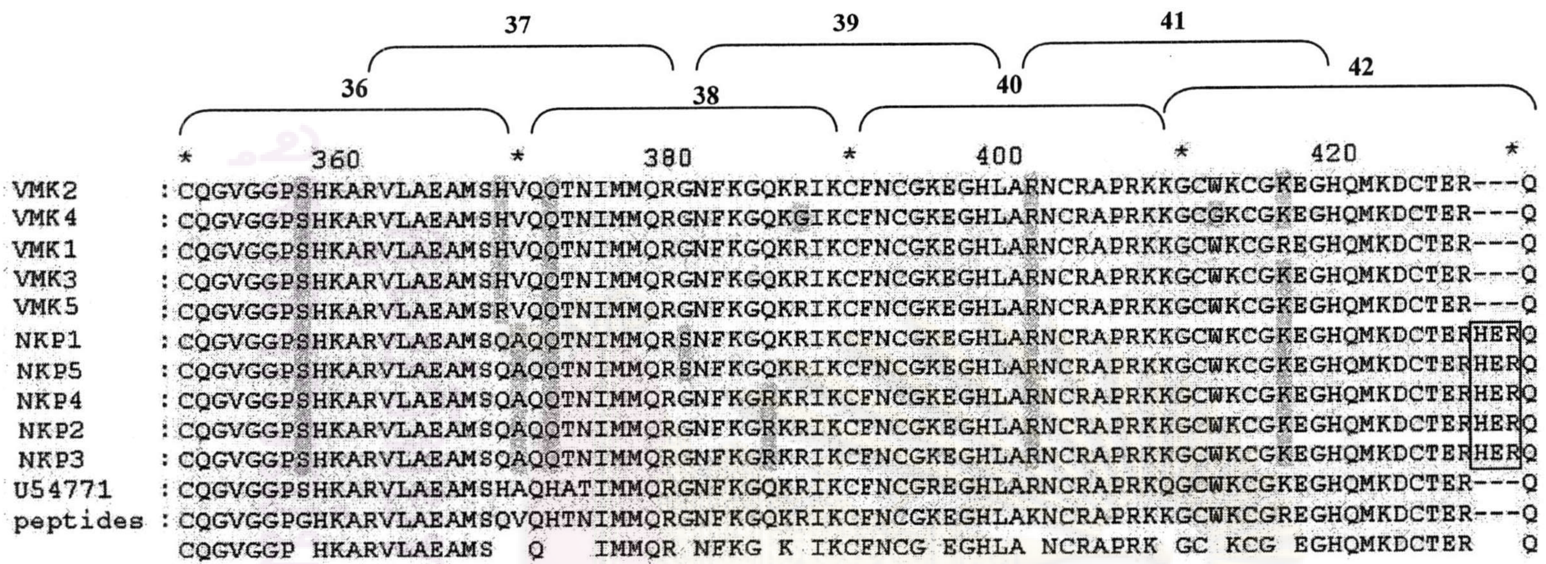

Figure 51 Alignment of Gag amino acid sequences (continued).

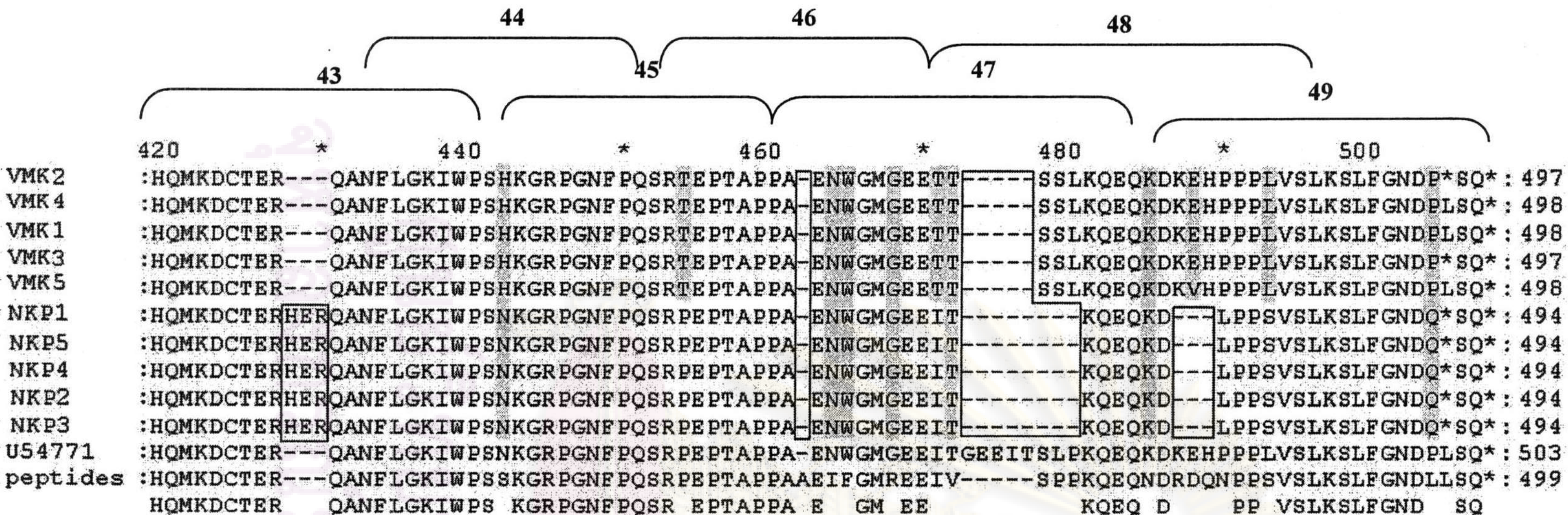

Table 23 Possible epitopes in Gag amino acid sequences of the subject NKP based on anchor residues. Table A shows known anchor residue motifs associated with the HLA-A24 and B*4002. X character represents any amino acid at the given position, whilst square brackets list the amino acids required at that position. Table B shows the list of potential HLA-A24-restricted epitopes in Gag.

A Anchor motifs examines

HLA	Anchor Residue Motifs	HLA	Anchor Residue Motifs
A24	X[Y]XXXXX[I/L/F/W]	B*4002	X[E]XXXXX[I/L]
A24	X[Y]XXXXXX[I/L/F/W]	B*4002	X[E]XXXXXX[I/L]
A24	X[Y]XXXXXXX[I/L/F/W]	B*4002	X[E]XXXXXXX[I/L]

B Possible epitopes based on Anchor residues

No	Position in query peptide	AA sequence	HLA
1	(28-36)	KYRLKHLVW	A24
2	(78-85)	LYNTVATL	A24
3	(261-268)	IYKRWMIL	A24
4	(261-270)	IYKRWMILGL	A24
5	(275-283)	MYSPVSIL	A24
6	(275-285)	MYSPVSILDI	A24
7	(295-304)	DYVDRFFKTL	A24
8	(39-50)	RELERFALNPGL	B*4002
9	(41-51)	LERFALNPSLL	B*4002
10	(41-50)	LERFALNPSLL	B*4002
11	(61-68)	MEQLQSAL	B*4002
12	(72-82)	EELRSLYNTV	B*4002
13	(71-82)	TEELRSLYNTV	B*4002
14	(92-101)	IEVKDTKEAL	B*4002
15	(160-169)	EEKALSPEVI	B*4002
16	(159-169)	VEEKGFNPEV	B*4002
17	(176-184)	SEGATPQDL	B*4002
18	(207-215)	EEAAEWDRL	B*4002
19	(206-215)	NEEAAEWDRL	B*4002
20	(209-236)	REPRGSDI	B*4002
21	(311-321)	QEVKGWMTETL	B*4002
22	(311-322)	QEVKGWMTETLL	B*4002
23	(364-375)	AEAMSQVQHTNI	B*4002
24	(425-438)	TERQANFLGKI	B*4002

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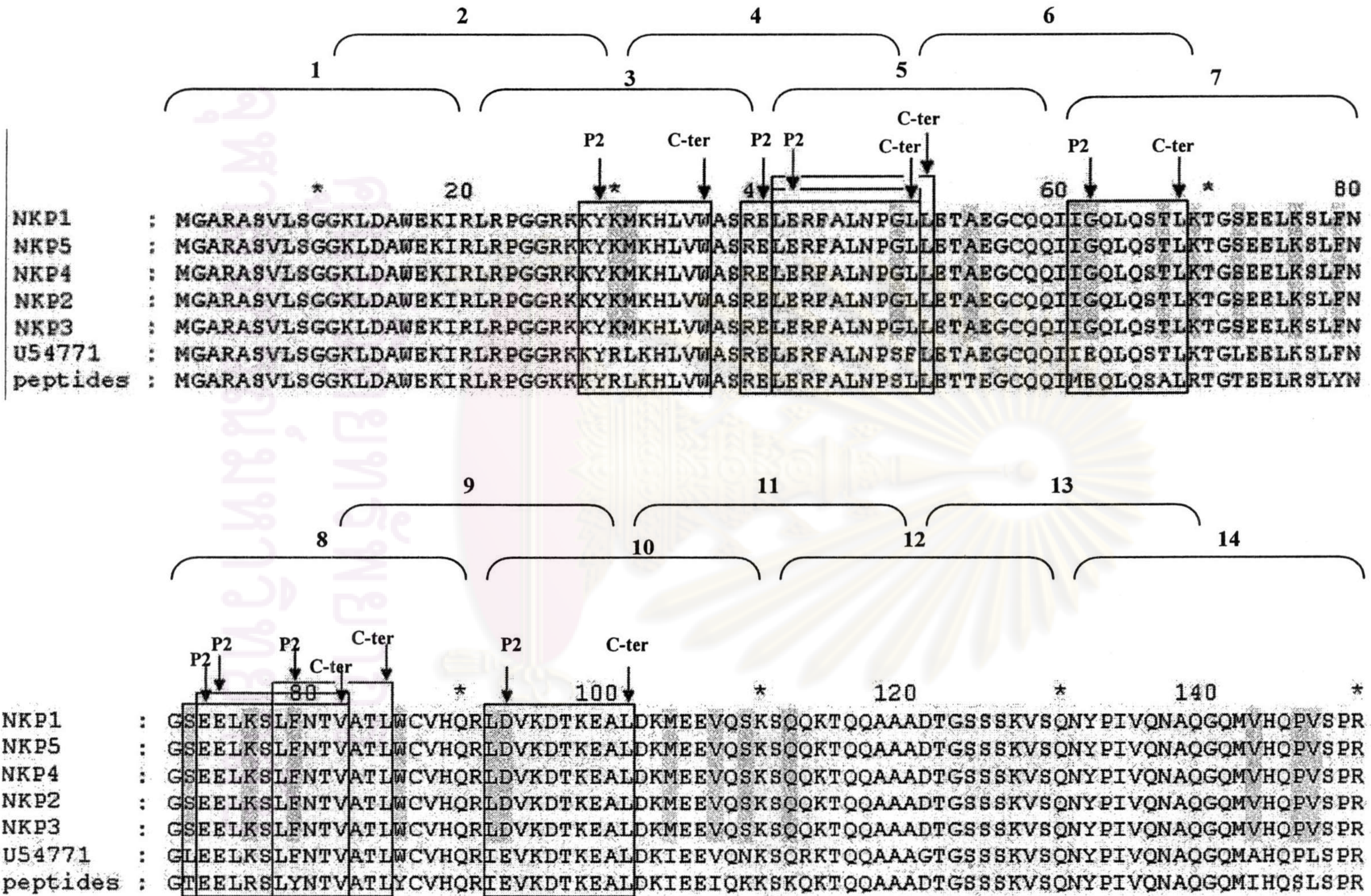
Figure 52 Possible HLA-A24 and B*4002-restricted epitopes in Gag amino acid sequences of the subject NKP based on anchor residues.



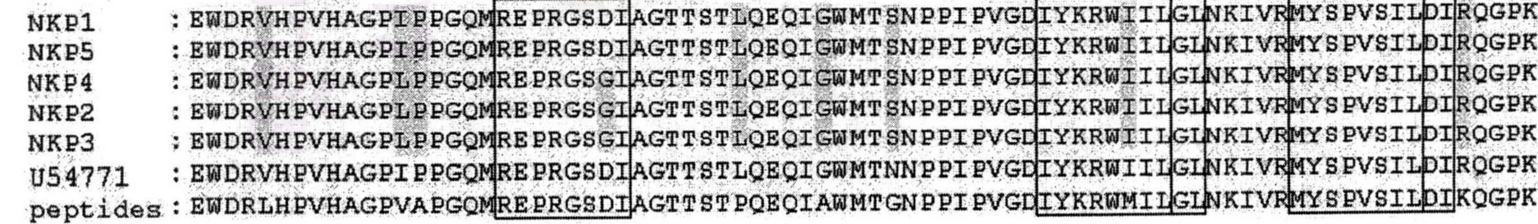


Figure 52 Possible HLA-A24 and B*4002-restricted epitopes in Gag amino acid sequences of the subject NKP based on Anchor residues (continued).

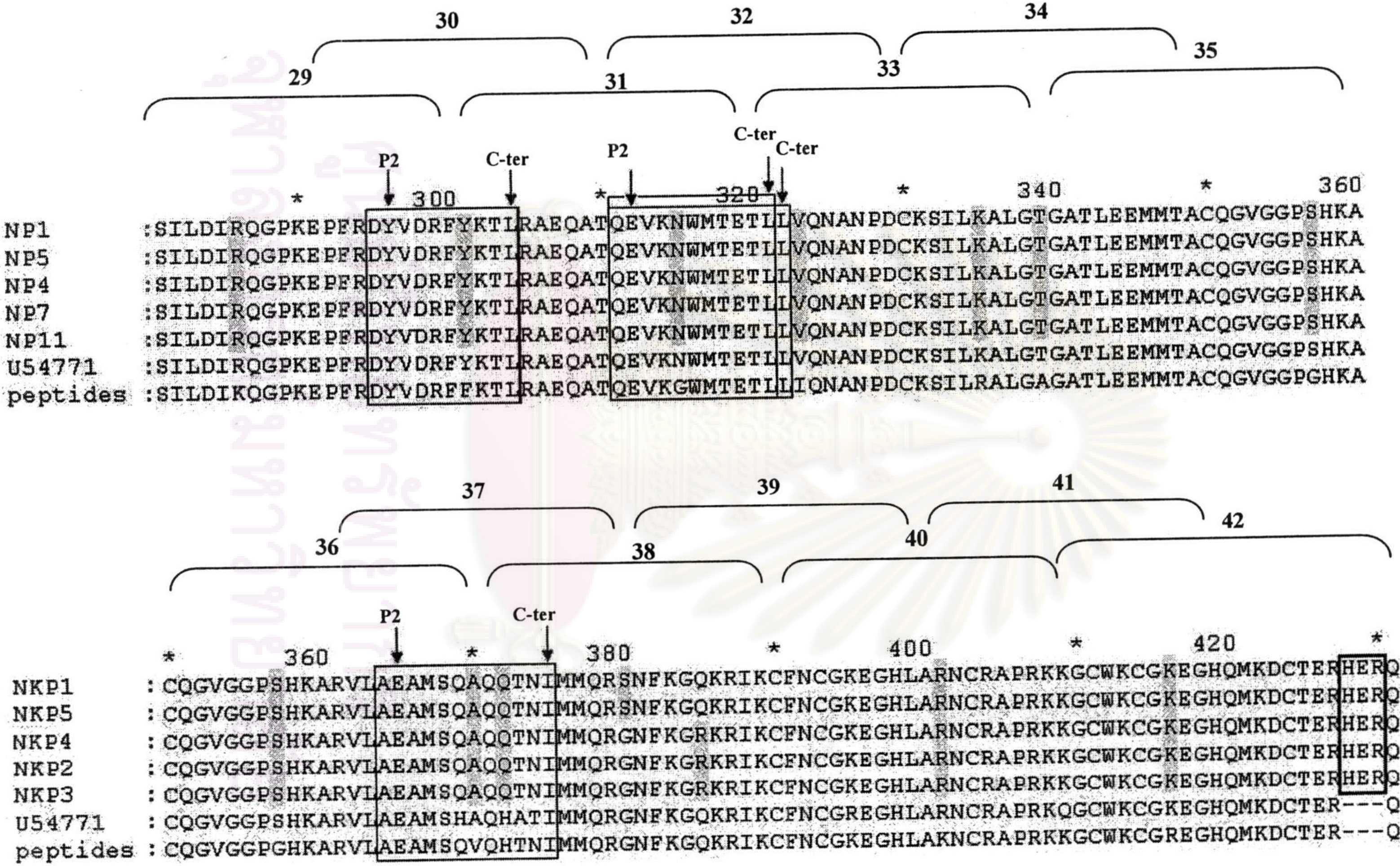


Figure 52 Possible HLA-A24 and B*4002-restricted epitopes in Gag amino acid sequences of the subject NKP based on Anchor residues (continued).

Figure 52 Possible HLA-A24 and B*4002-restricted epitopes in Gag amino acid sequences of the subject NKP based on Anchor residues (continued).