CHAPTER V

RESULTS

Part I Characteristic of individual healthy donors and HIV infected patients

In this study, 12 healthy donors and 10 HIV infected patients were enrolled. Their characteristics were summarised in table 1. Control group was composed of 9 female and 3 male whose age ranging from 22 to 45 years with the mean of 27 years (Table 3). The patient group consisted of 8 female and 2 male at age between 20 to 38 years with the mean of 29 years, $CD4^+$ count ranging from 287 to 1,105 cells/mm^3 with the median of 465 cells/mm^3 and HIV RNA ranged from 1,262 to 92,044 copies/ml with the median of 9,756 copies/ml.

Part II Study of EBV serological responses

Both individual healthy donors and HIV infected patients were screened for the presence of EBV IgG antibody to viral capsid antigen (VCA) before enrolling into the study. According to the instruction of ELISA kit, anti-VCA IgG was considered positive when it reached 20 AU/ml. Therefore, the control group was divided into 2 subgroups based on their anti-VCA antibodies status, 10 persons with EBV positive anti-VCA IgG antibody and 2 persons without detectable anti-VCA IgG antibody. For HIV-infected patients, all of them were EBV seropositive. The antibody titre of EBV-seropositive healthy donors was ranged from 25 to 626 AU/ml with the mean of 285 AU/ml whilst 2 EBV seronegative donors had anti-VCA IgG with titre of less than 20 AU/ml (14 and 19 AU/ml, respectively with the mean of 16 AU/ml (Table 3). A anti-VCA antibody titre in HIV-patients was ranged from 219 to 1313 AU/ml with the mean of 643 AU/ml (Table 3 and Figure 9). The results showed significant greater anti-VCA IgG titre in HIV patients than that of EBV-seropositive healthy donor ($P<0.05$).

Part III Development of EBV-DNA estimation technique by real-time PCR

1. Standard EBV DNA

Three types of standard EBV-DNA were prepared. They were B95-8 EBV DNA, Namalwa EBV-DNA and plasmid containing EBNA-1 DNA. B95-8 EBV-DNA was extracted from B95-8 cells and amplified by real-time PCR (Figure 10). The melting temperature of the amplicon was approximately 68 °C (Figure 11) and the

product length was 297 bp as revealed by gel electrophoresis (Figure 12). The similar result was seen for Namalwa EBV-DNA amplification (data not shown).

For preparation of plasmid containing EBNA-1 DNA, the B95-8 EBV-DNA was amplified by real-time PCR. The amplicon was proved by melting temperature at 68 °C and the size of the amplificated product (297 bp). The PCR product was then purified resulting in a single clear band at 297-bp by electrophoresis (Figure 13). The DNA concentration was 177.50 µg/ml with 1.977 of the 260:280 OD ratio. Thereafter the DNA was cloned. The plasmid containing EBNA-1 DNA revealed the melting temperature at 68 °C. The recombinant plasmid was confirmed by sequencing (Figure 14 and 15), only 1 base (thymidine: T) at position 1520 was changed to cytosine (C) when compared to EBNA-1 gene sequences of B95-8 reference strains (genbank accession no.VO1555, NC001345 and AJ507799) (Appendix III).

Table 3 Characteristic and EBV serological responses of individual healthy donors and HIV infected patients

ID	Age	Sex	Anti-VCA (AU/ml)		CD4+ (cell/ml)	HIV RNA (copies/ml)
Healthy donors						
C1	23	F	315	+	ND	ND
C2	22	F	440	+	ND	ND
C3	28	M	25	+	ND	ND
C4	32	F	39	+	ND	ND
C5	23	F	127	+	ND	ND
C6	23	F	338	+	ND	ND
C7	23	F	401	+	ND	ND
C8	24	F	480	+	ND	ND
C9	25	M	62	+	ND	ND
C10	24	M	626	+	ND	ND
			Mean ± SD = 285*±210			
C11[a]	32	F	19	-	ND	ND
C12[a]	45	F	14	-	ND	ND
			Mean ± SD = 16±4			
	Mean ± SD = 27±6		Cut off for anti-VCA IgG seropositive was 20			
Patients						
P1	27	F	219	+	502	14,506
P2	20	F	287	+	294	3,864
P3	38	F	742	+	1,105	1,262
P4	29	F	375	+	453	7,072
P5	20	F	428	+	287	2,438
P6	25	F	1180	+	376	32,749
P7	33	M	455	+	1,030	9,758
P8	33	M	697	+	427	92,044
P9	33	F	1313	+	476	9,754
P10	27	F	734	+	531	12,009
	Mean ± SD = 29±6		Mean ± SD = 643*±368		Median = 465	Median = 9,756

NOTE. ND: not done, F: female, M: male, AU: arbitrary unit, +: EBV-seropositive donor, -: EBV-seronegative donor

* Significantly Difference at $P<0.05$

[a] These EBV-seronegative donors had an anti-VCA IgG titre less than positive cut off value (20 AU/ml).

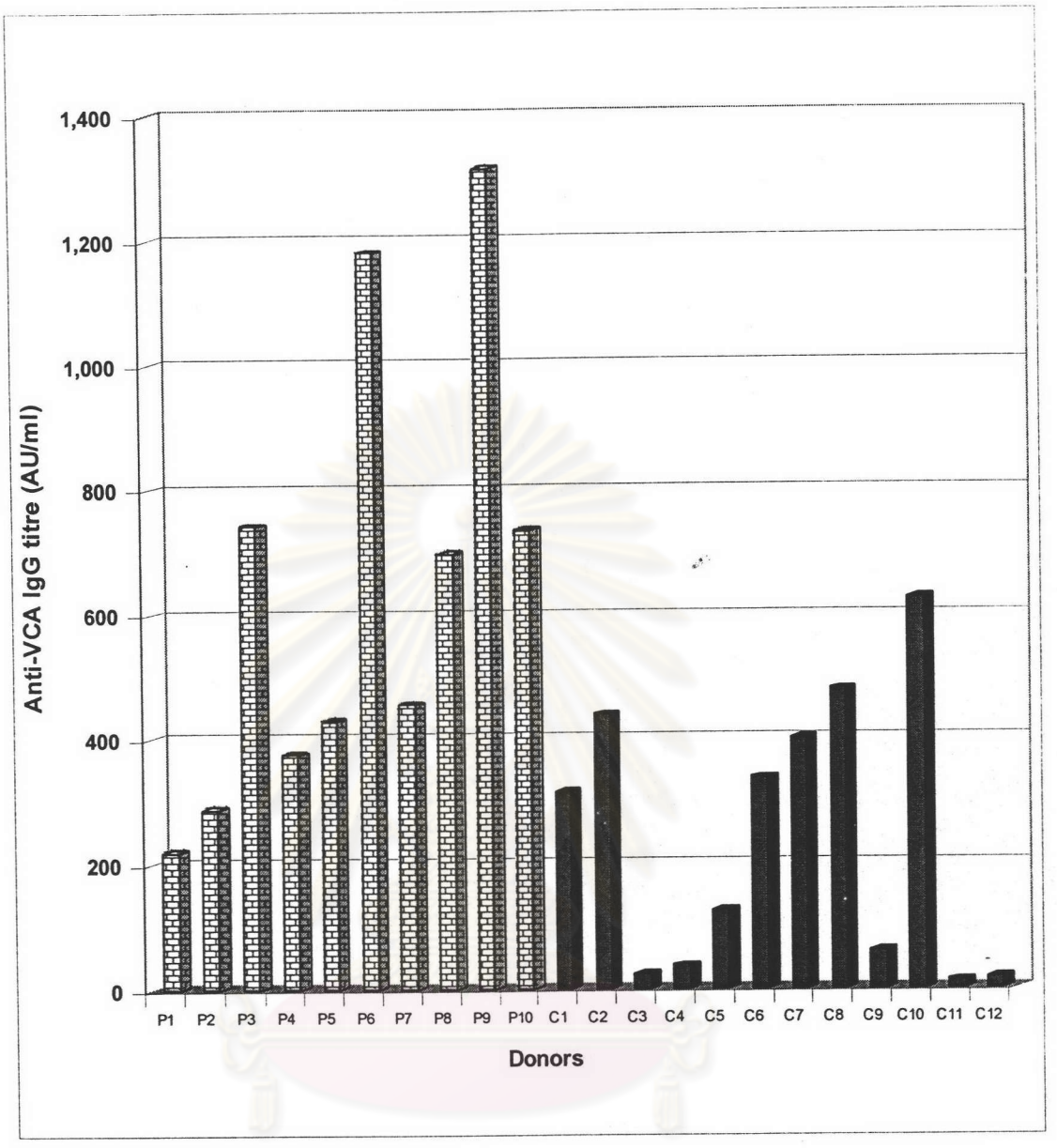


Figure 9. Anti-VCA IgG antibody responses of individual EBV-seropositive (C1-C10), EBV-seronegative (C11-C12) and HIV infected donors (P1-P10).

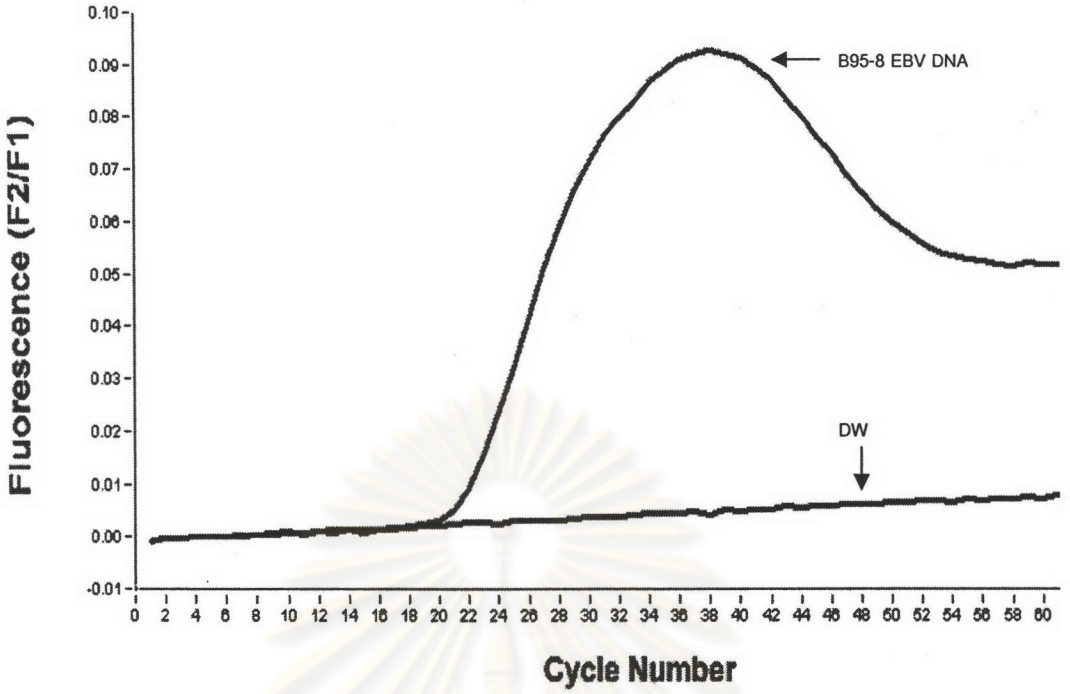
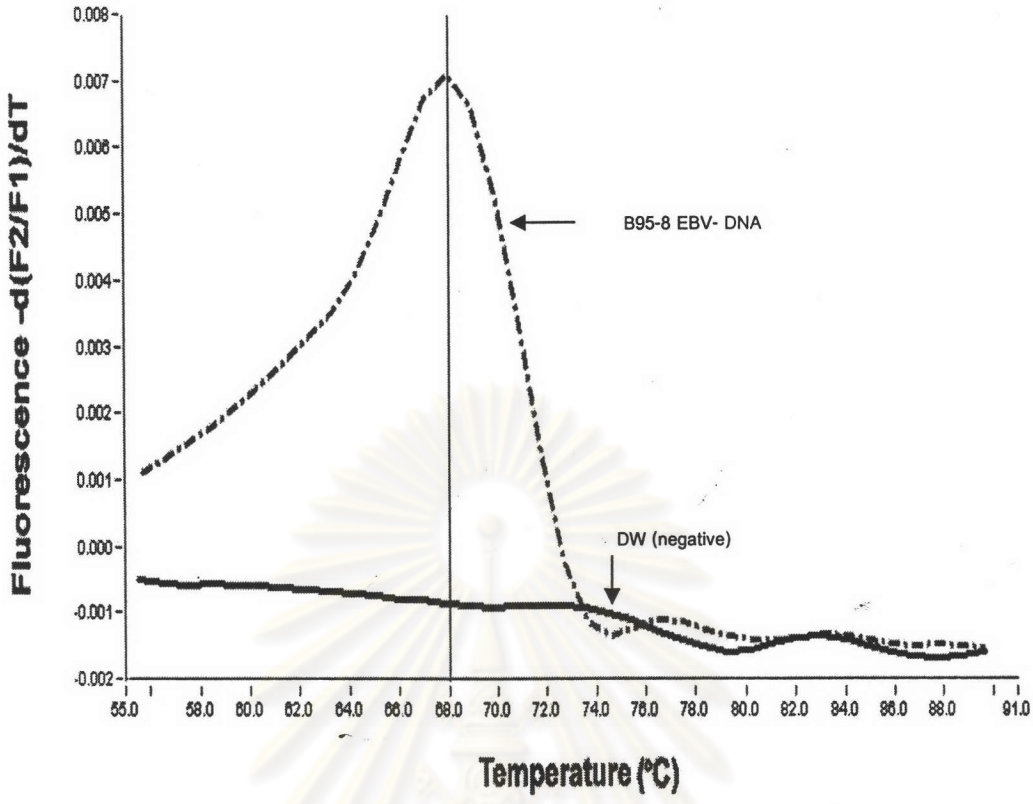


Figure 10. B95-8 EBV-DNA amplification curve, plotting against cycle number and fluorescence acquisition.



Figture11. Melting curve analysis of B95-8 EBV-DNA at 68°C.

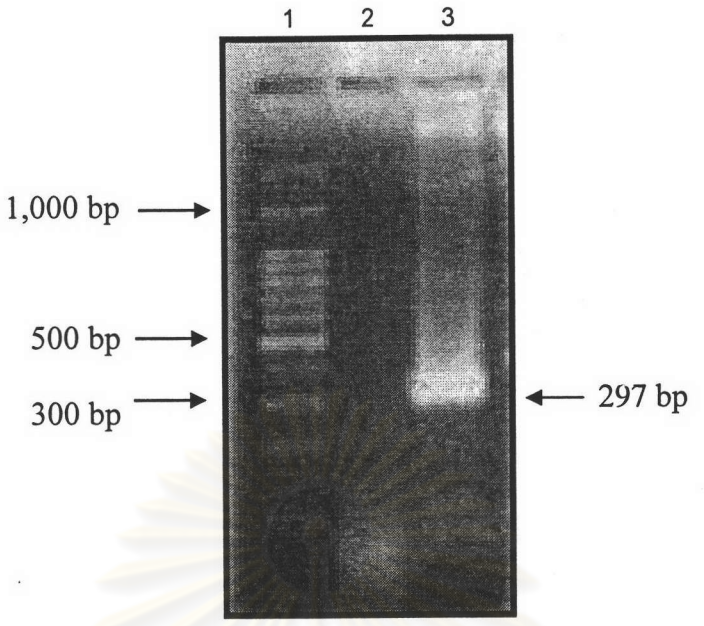


Figure 12. The amplified B95-8 EBV-DNA (EBNA-1) was confirmed by gel electrophoresis for 297 bp amplicon size after real-time PCR reaction. Lane1: 100 bp marker, lane2: DW and lane3: EBNA-1 PCR product from B95-8 EBV DNA.

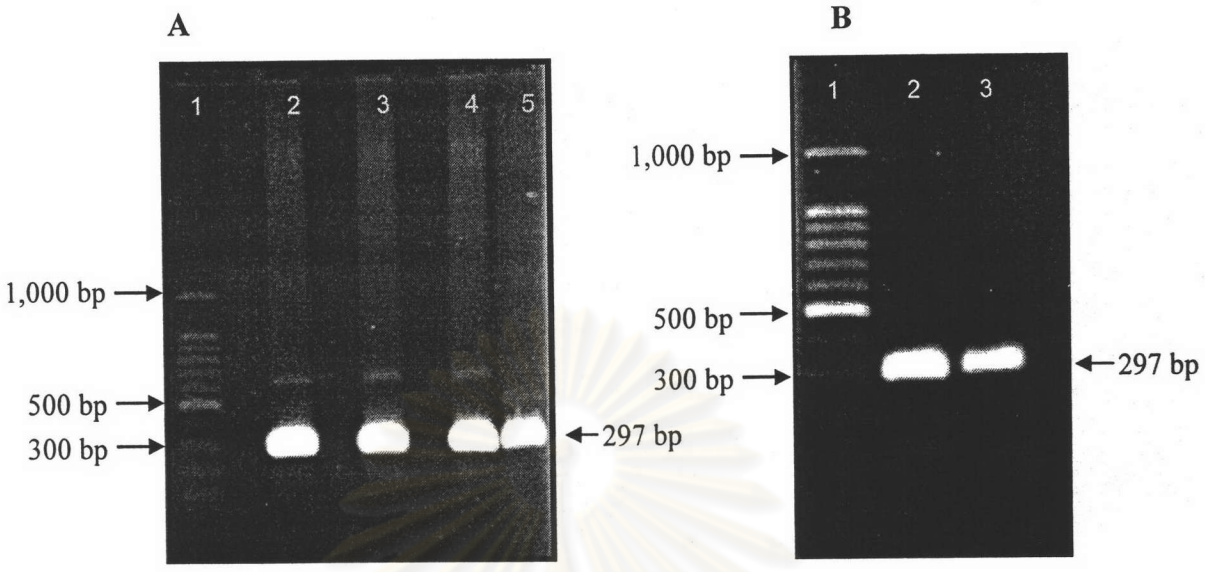


Figure 13. Gel electrophoresis of amplified EBNA-1 from B95-8 EBV-DNA used in preparation of plasmid containing EBNA-1 DNA before (A) and after purification (B). A; lane1: 100 bp marker, lane2-5: EBNA-1 PCR product of B95-8 EBV-DNA before purification. B; lane1: 100 bp marker, lane2 and 3: EBNA-1 PCR product of B95-8 EBV-DNA after purification.

```
                EB 30
1380    GAGGGTG GTTTGGAAAG CATCGTGGTC AAGGAGGTTC CAACCCGAAA TTTGAGAACA  1439

1440    TTGCAGAAGG TTTAAGAGCT CTCCTGGCTA GGAGTCACGT AGAAAGGACT ACCGACGAAG  1499

1500    GAACTTGGGT CGCCGGTGCG TTCGTATATG GAGGTAGTAA GACCTCCCTT TACAACCTAA  1559

                                                          3FL
1560    GGCGAGGAAC TGCCCTTGCT ATTCCACAAT GTCGTCTTAC ACCATTGAGT CGTCTCCCCT  1619

                          5LC                                  EB40
1620    TTGGAATGGC CCCTGGACCC GGCCCACAAC CTGGCCCGCT AAGGGAGTCC ATTGTCTGTT  1679
```

Figure 14. Nucleotide sequences of EBNA-1 PCR product that inserted to plasmid. The boxes at start and end of the sequence were primer EB30 and EB 40. The others (3FL and 5LC) were EBNA-1 specific probe.

```
NC001345              CGGGGTCAGGGTGATGGAGGCAGGCGCAAAAAAGGAGGGTGGTTTGGAAA 1400
AJ507799              CGGGGTCAGGGTGATGGAGGCAGGCGCAAAAAAGGAGGGTGGTTTGGAAA 1400
V01555                CGGGGTCAGGGTGATGGAGGCAGGCGCAAAAAAGGAGGGTGGTTTGGAAA 1400
297 bp-PCR-product    --------------------------------GAGGGTGGTTTGGAAA 16
                                                      ***************

NC001345              GCATCGTGGTCAAGGAGGTTCCAACCCGAAATTTGAGAACATTGCAGAAG 1450
AJ507799              GCATCGTGGTCAAGGAGGTTCCAACCCGAAATTTGAGAACATTGCAGAAG 1450
V01555                GCATCGTGGTCAAGGAGGTTCCAACCCGAAATTTGAGAACATTGCAGAAG 1450
297 bp-PCR-product    GCATCGTGGTCAAGGAGGTTCCAACCCGAAATTTGAGAACATTGCAGAAG 66
                      **************************************************

NC001345              GTTTAAGAGCTCTCCTGGCTAGGAGTCACGTAGAAAGGACTACCGACGAA 1500
AJ507799              GTTTAAGAGCTCTCCTGGCTAGGAGTCACGTAGAAAGGACTACCGACGAA 1500
V01555                GTTTAAGAGCTCTCCTGGCTAGGAGTCACGTAGAAAGGACTACCGACGAA 1500
297 bp-PCR-product    GTTTAAGAGCTCTCCTGGCTAGGAGTCACGTAGAAAGGACTACCGACGAA 116
                      **************************************************

NC001345              GGAACTTGGGTCGCCGGTGTGTTCGTATATGGAGGTAGTAAGACCTCCCT 1550
AJ507799              GGAACTTGGGTCGCCGGTGTGTTCGTATATGGAGGTAGTAAGACCTCCCT 1550
V01555                GGAACTTGGGTCGCCGGTGTGTTCGTATATGGAGGTAGTAAGACCTCCCT 1550
297 bp-PCR-product    GGAACTTGGGTCGCCGGTGCGTTCGTATATGGAGGTAGTAAGACCTCCCT 166
                      ******************.***********************************

NC001345              TTACAACCTAAGGCGAGGAACTGCCCTTGCTATTCCACAATGTCGTCTTA 1600
AJ507799              TTACAACCTAAGGCGAGGAACTGCCCTTGCTATTCCACAATGTCGTCTTA 1600
V01555                TTACAACCTAAGGCGAGGAACTGCCCTTGCTATTCCACAATGTCGTCTTA 1600
297 bp-PCR-product    TTACAACCTAAGGCGAGGAACTGCCCTTGCTATTCCACAATGTCGTCTTA 216
                      **************************************************

NC001345              CACCATTGAGTCGTCTCCCCTTTGGAATGGCCCCTGGACCCGGCCCACAA 1650
AJ507799              CACCATTGAGTCGTCTCCCCTTTGGAATGGCCCCTGGACCCGGCCCACAA 1650
V01555                CACCATTGAGTCGTCTCCCCTTTGGAATGGCCCCTGGACCCGGCCCACAA 1650
297 bp-PCR-product    CACCATTGAGTCGTCTCCCCTTTGGAATGGCCCCTGGACCCGGCCCACAA 266
                      **************************************************

NC001345              CCTGGCCCGCTAAGGGAGTCCATTGTCTGTTATTTCATGGTCTTTTTACA 1700
AJ507799              CCTGGCCCGCTAAGGGAGTCCATTGTCTGTTATTTCATGGTCTTTTTACA 1700
V01555                CCTGGCCCGCTAAGGGAGTCCATTGTCTGTTATTTCATGGTCTTTTTACA 1700
297 bp-PCR-product    CCTGGCCCGCTAAGGGAGTCCATTGTCTGTT------------------- 297
                      ******************************
```

Figure 15. Sequence alignment of 297 bp fragment of EBNA-1 gene amplified from recombinant plasmid (line 4) and the standard sequences of EBNA-1 from B95-8 strain (line 1 to 3), Genbank accession no. NC001345, AJ507799 and VO1555, respectively by ClustalW (http://www.ebi.ac.uk). The box indicated nucleotide mutation position (T 1520 C).

2. Real-time PCR

2.1. Specificity of the assay

To analyse specificity of the assay, other herpesviruses DNA such as CMV, HSV-1 and HSV-2 was amplified and quantitated in parallel with B95-8 EBV-DNA by real-time PCR. The DNA extracted from PBMC of EBV seronegative healthy donor was also performed in the same run as negative control. No cross-amplification was demonstrated either in DNA from other herpesviruses or DNA from the EBV-seronegative donor (Figure 16 and 17).

2.2. Standardisation of the assay

To generate standardisation curve, a serial 10-fold dilution of Namalwa EBV-DNA ranging from $1.4x10^4$ to 14 copies was carried out and quantified by real-time PCR. The results revealed that as few as 14 copies of Namalwa EBV-DNA could be detected by this real-time PCR-based assay (Figure 18). Amplicons were confirmed by melting curve analysis (Figure 19). The standardisation curve was then generated with linear regression (r^2) of -1.00 with an error value of 0.082 (Figure 20).

The number of EBV-DNA in plasmid containing 297 bp fragment of EBNA-1 was calculated against the standardisation curve. After that the plasmid containing EBV-DNA was used for establishing the standard curve for further analysis throughout the study. The concentration of recombinant plasmid was further proved by parallel run of Namalwa EBV-DNA at concentration of $2.8x10^4$ copies, as unknown sample, with the same run of serial 10-fold dilutions of EBV recombinant plasmid ranging from 10^8 to 10 copies. After automatically calculated by LightCycler software, the accuracy of the assay was revealed by the detection of Namalwa EBV-DNA at concentration of 2.77 $x10^4$ copies.

2.3. Sensitivity of the assay

To evaluate the sensitivity of the assay, serial 10-fold dilutions of EBV recombinant plasmid ranging from 10^8 to 10 copies were performed (Figure 21). The reaction was confirmed by melting temperature of 68°C as similar to other standard EBV DNA. As few as 10 copies of EBV-DNA could be detected by the assay. Due to amplification curve in Figure 21, the crossing point of each cycle at which fluorescence line entered the log-

linear phase and distinguished from background were plotted against log concentration to construct standard curve used for quantitation of the sample. The standard curve was established under -1.00 and 0.0347 of linear regression value and error, respectively (Figure 22).

2.4. Reproducibility and precision of the assay

The estimation of intra- and inter-assay variabilities were carried out in 2 independent experiments by amplifying *EBNA-1* recombinant plasmid at concentration 10^6, 10^4 and 10^2 copies in duplicate (Figure 23). The results are summarised in Table 4. All intra- and inter-assay variabilities were shown to be within a 0.5 $\log_{10}$ difference.

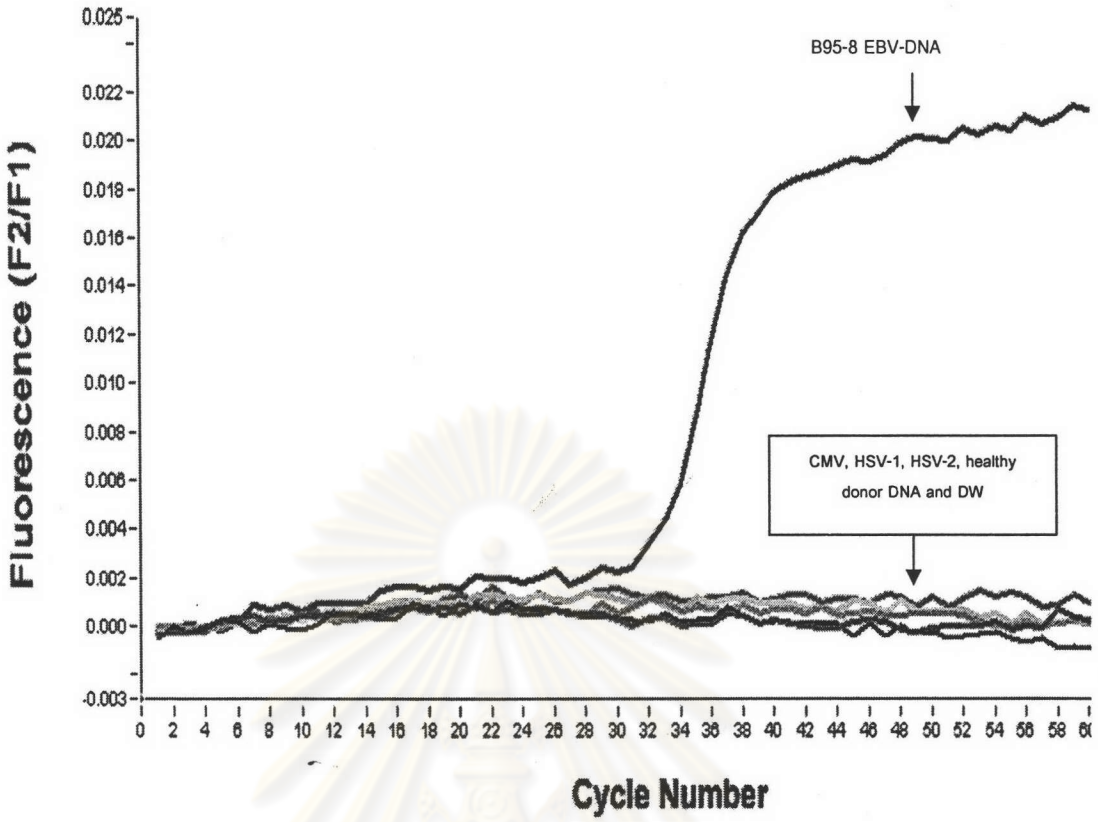


Figure 16. Amplification curve of DNA of EBV (B95-8), CMV, HSV-1, HSV-2 and EBV seronegative healthy donor. DW was included as negative control.

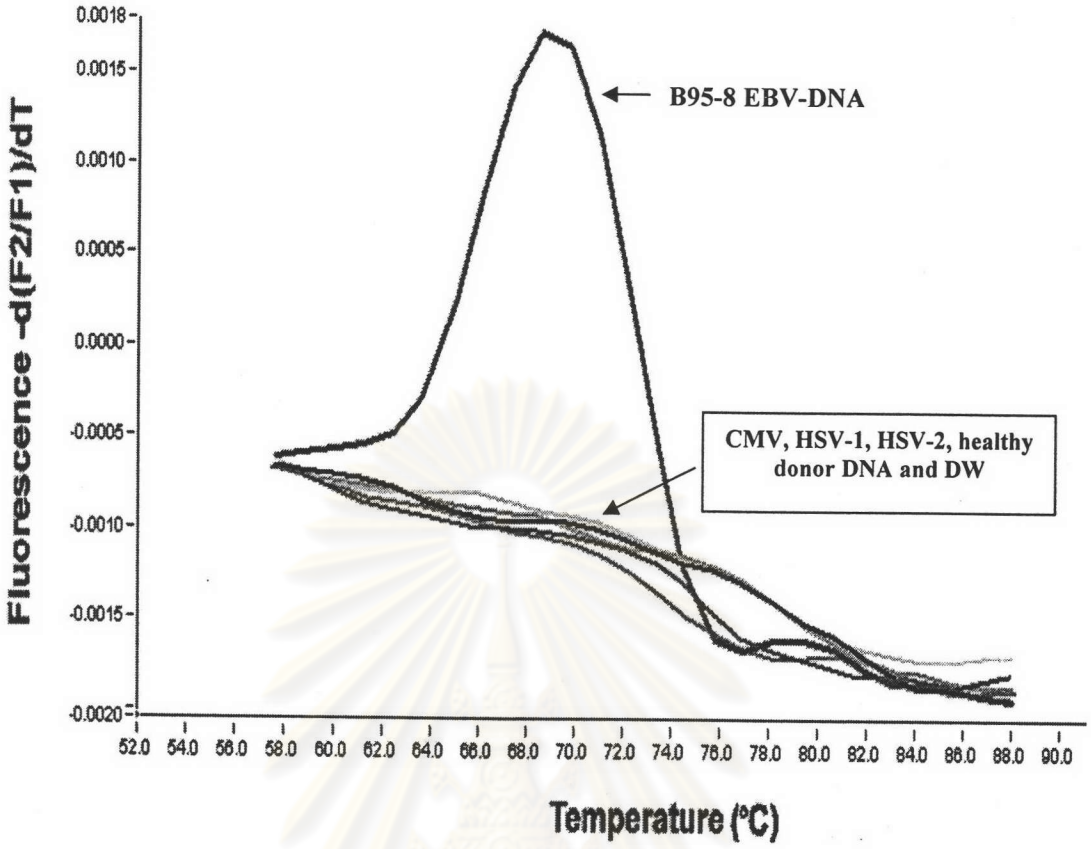


Figure 17. Melting curve of DNA of EBV (B95-8), CMV, HSV-1, HSV-2 and EBV from seronegative healthy donor. DW was induced as negative control and DW. No cross reaction was found.

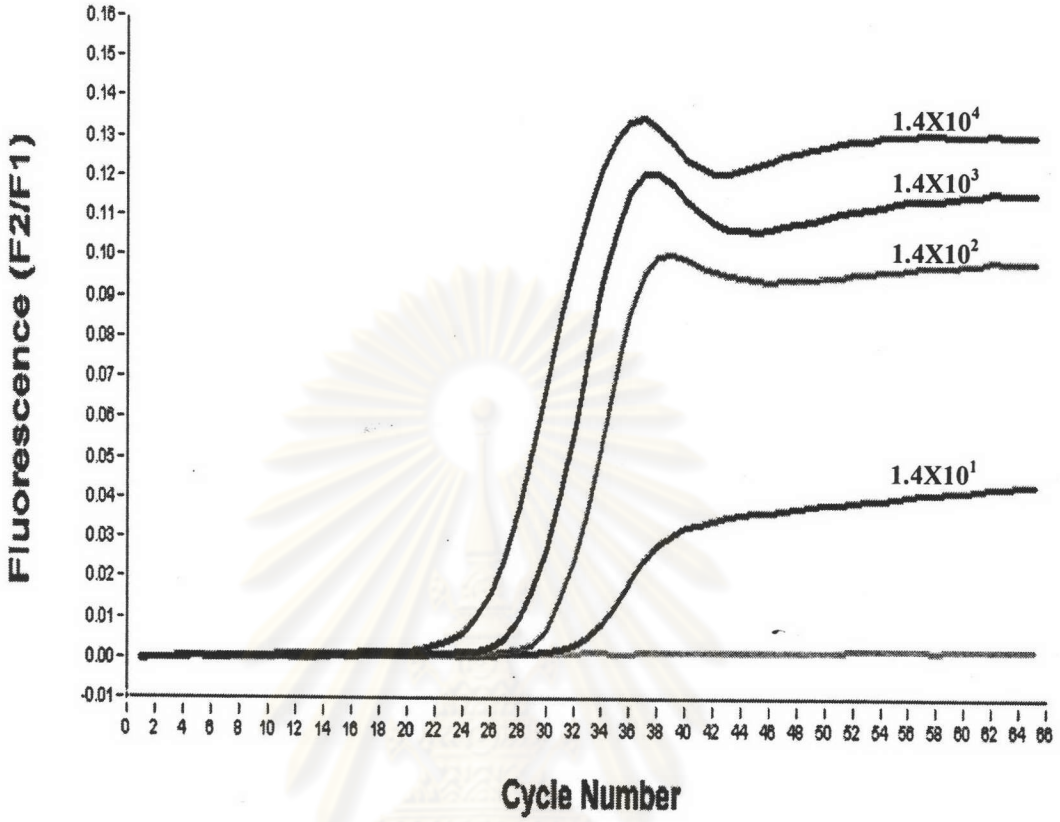


Figure 18. Amplification curve of Namalwa EBV-DNA at concentration of $1.4x10^4$, $1.4x10^3$, $1.4x10^2$ and 14 copies.

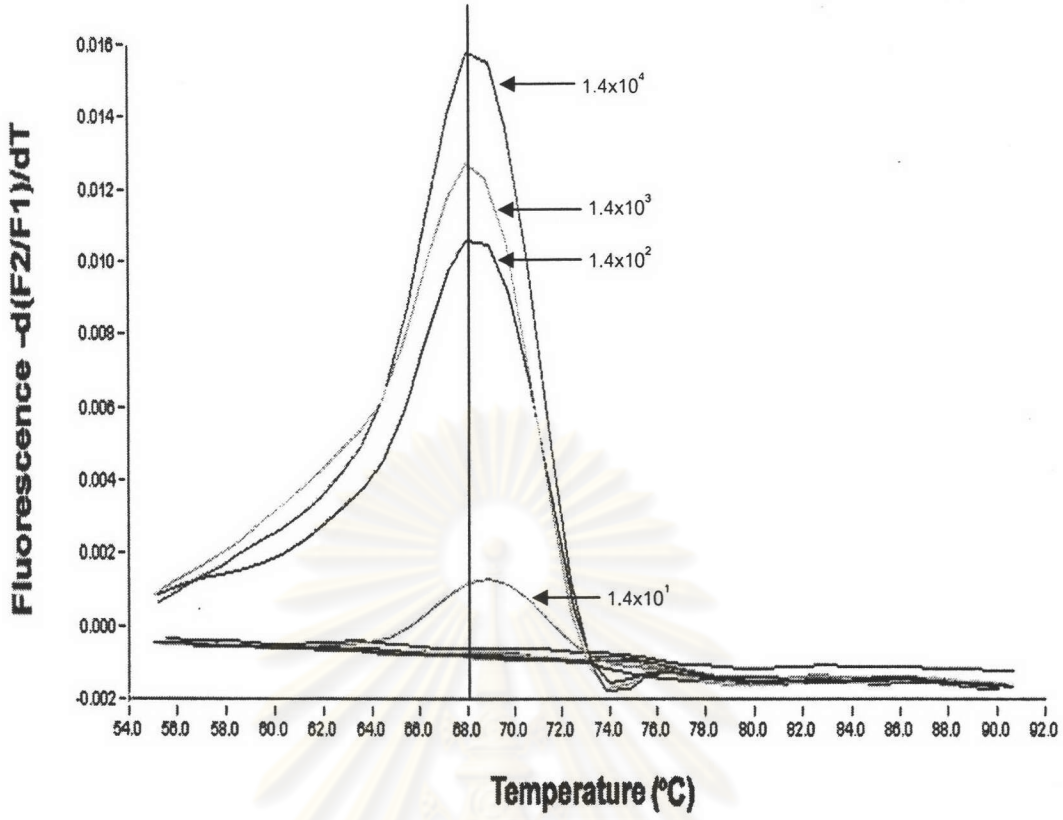


Figure 19. Melting curve analysis of Namalwa EBV-DNA at concentration of $1.4x10^4$, $1.4x10^3$, $1.4x10^2$ and 14 copies, respectively. They were all revealed melting temperature approximately at 68°C.

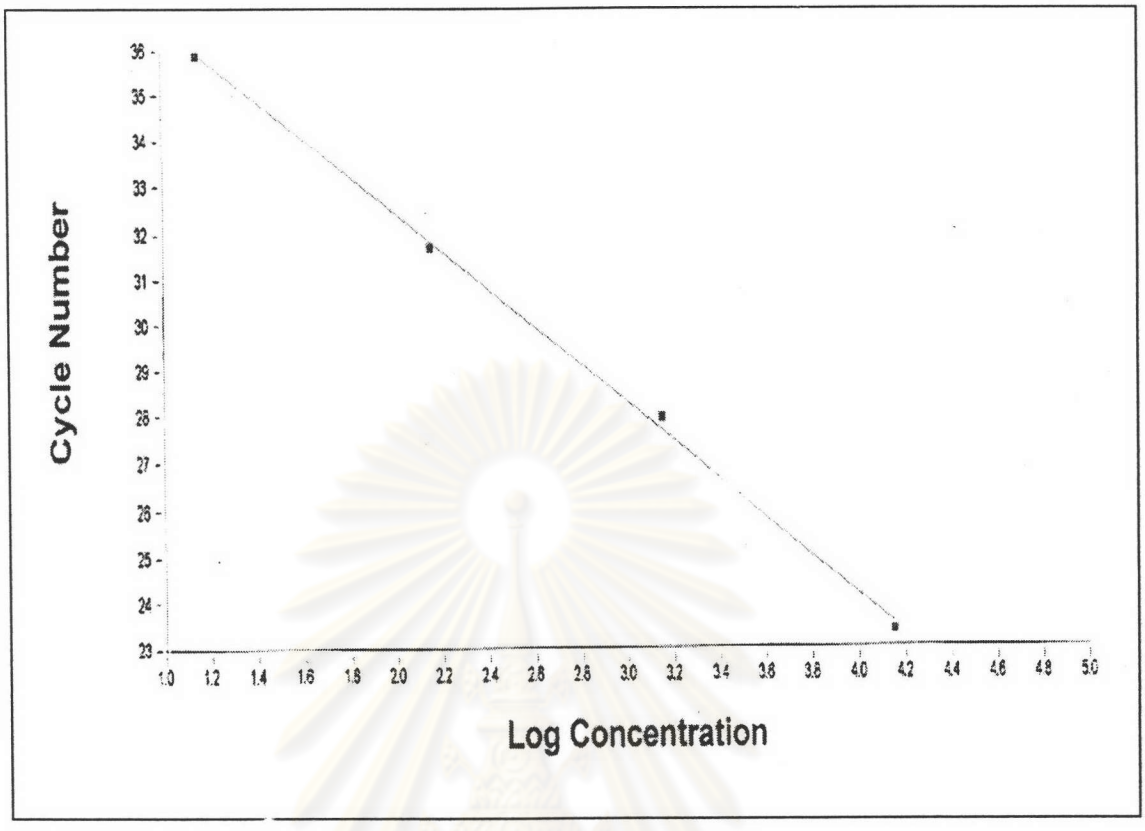


Figure 20. The standardisation curve was obtained by serial 10-fold dilutions of Namalwa EBV-DNA. Each point indicates a log dilution from $1.4x10^4$ to 14 copies per reaction plotted against cycle number. These gave an error value 0.082 with a linear regression (r^2) of -1.00.

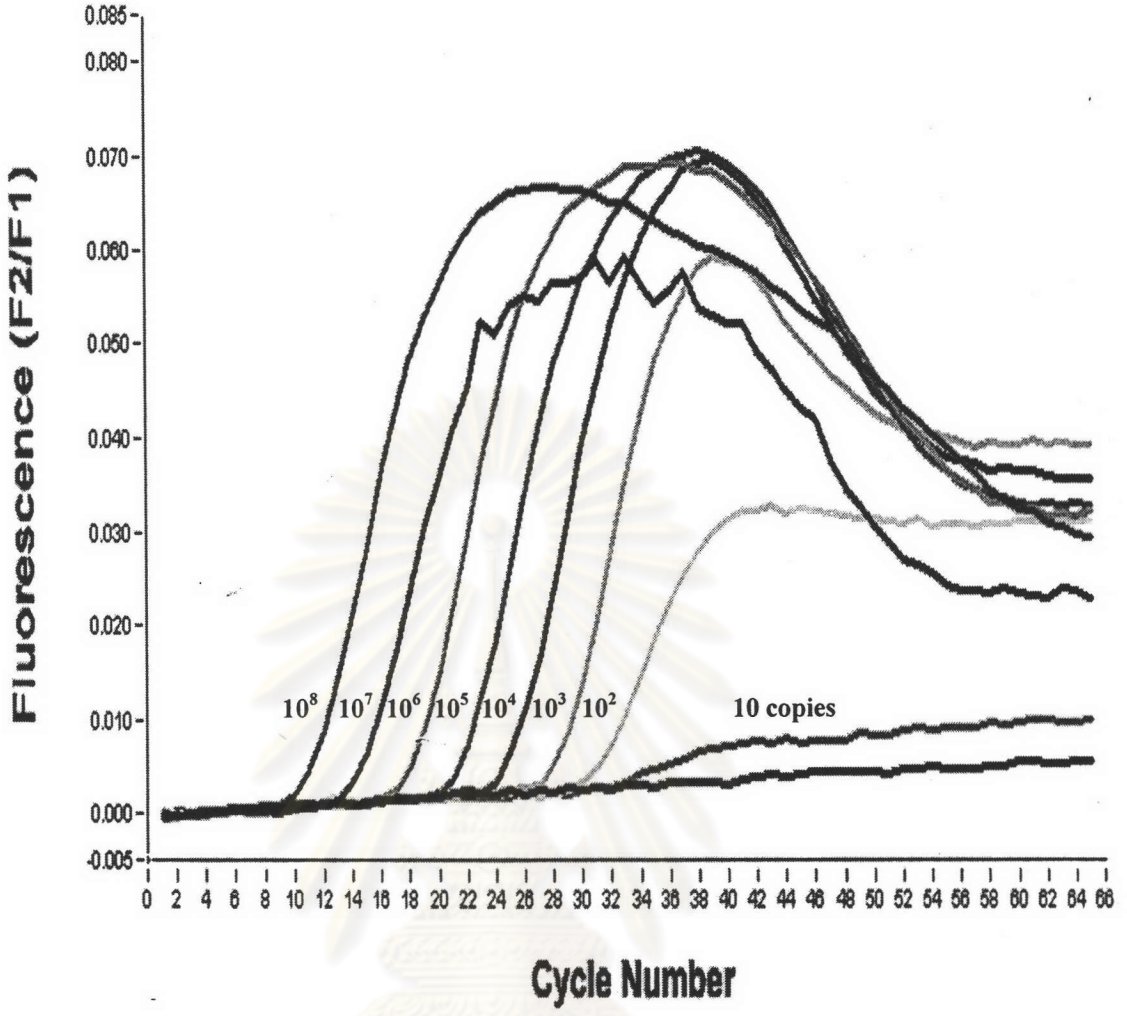


Figure 21. Amplification curve of *EBNA-1* recombinant plasmid obtained from a serial 10-fold dilution of recombinant plasmid ranging from 10^8 to 10 copies per reaction.

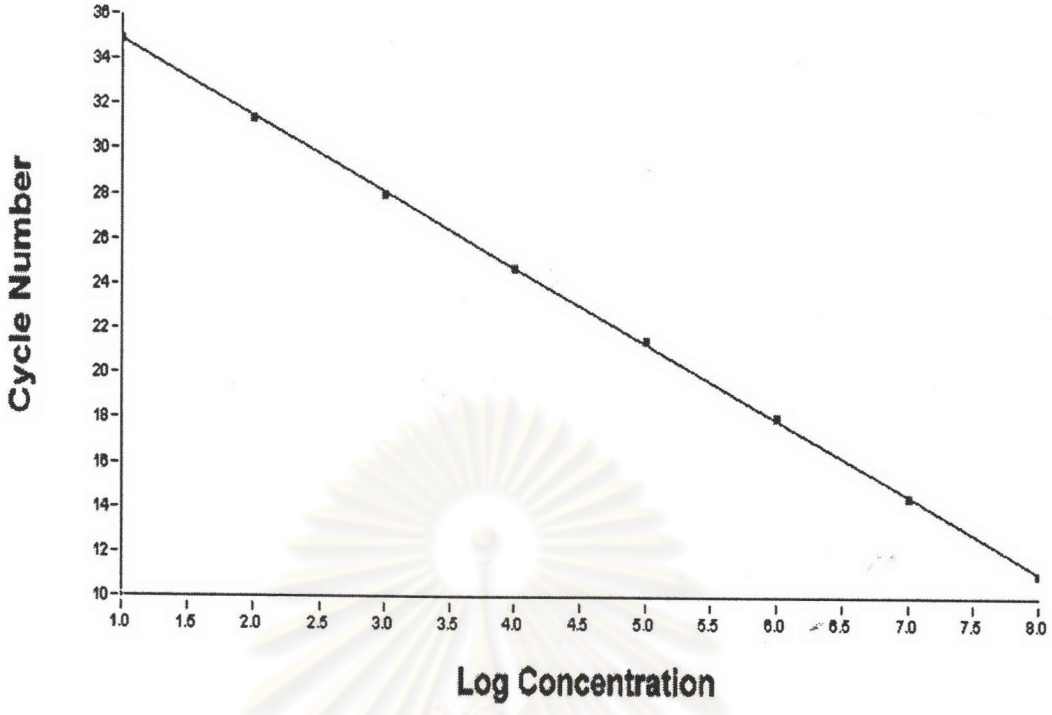


Figure 22. Standard curve obtained by serial 10-fold dilutions of EBNA-1 recombinant plasmid. The curve plots cycle number versus $\log_{10}$ concentration of standardised plasmid with an error value of 0.0347 and a linear regression value (r^2) of –1.00.

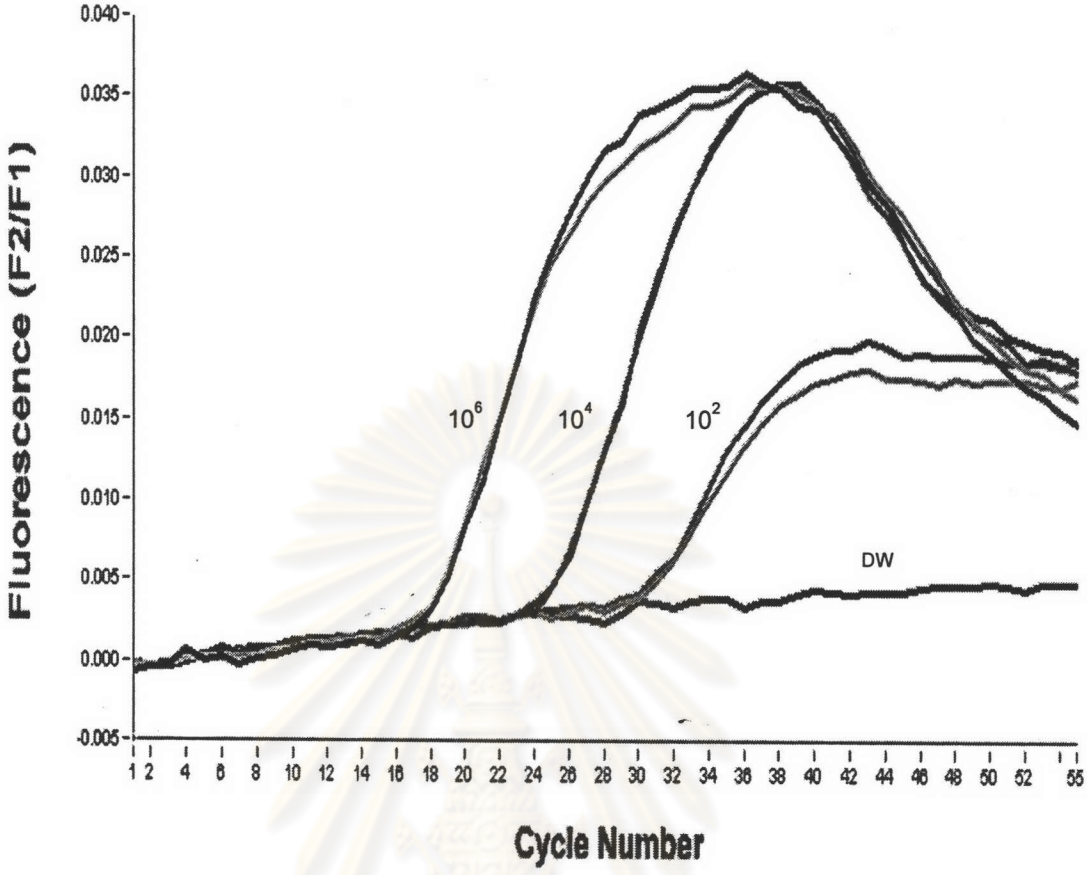


Figure 23. Reproducibility of the assay. The curve plots cycle number versus fluorescence acquisition of standardised plasmid at duplicative concentration of 10^6, 10^4 and 10^2 copies. DW was induced as negative control (DW).

Table4. Intra- and Inter-assay variability

	Intra-Assay		**Inter-Assay**	
Dilution (copy number)	**Range**	**Log_{10} difference**	**Range**	**Log_{10} difference**
1,000,000	1,000,000 - 1,229,000	0.09	929,000 1,229,000	0.12
	1,000,000 - 1,003,000	0.00	1,000,000 1,003,000	
	mean	0.05	1,229,000	
10,000	10,310 -11,600	0.05	9,830 10,310	0.11
	10,000 -12,790	0.11	11,600 10,000	
	mean	0.08	12,790	
100	51 - 57	0.05	51 57	0.45
	102 - 144	0.15	108 144	
	mean	0.10	101	

Part IV Quantitation of EBV-DNA in clinical specimens

To quantify EBV DNA in PBMC of 10 healthy EBV-infected, 2 healthy EBV uninfected and 10 immunocompromised donors. The real-time PCR assay was performed. EBV load of each sample was calculated based on the crossing point of the standard and sample curves. The median of EBV load in EBV seropositive healthy adults was <10 copies per μg DNA (ranged from <10 to 54 copies/μg DNA) whereas the median of EBV DNA in immunocompromised patients was 38 copies per μg DNA (ranged from <10 to 3,785 copies/μg DNA). Both seronegative healthy donors had EBV load less than 10 copies/μg DNA. The EBV DNA in HIV-infected patients was significantly higher than EBV-DNA in EBV-seropositive donors ($p<0.05$). Results were summarised in Table 5.

Part V Study of EBV-CTL responses

1. Propagation and titration of recombinant vaccinia containing EBV genes

   A total of eight recombinant vaccinia containing EBV genes and one wild type vaccinia were propagated and titrated in TK^-143 cell line. The vaccinia virus could infect TK^-143 cell line and cause cytopathic effect (CPE) visible under microscope (Figure 24). Plaque titration assay was then performed to quantitate the recombinant vaccinia (Figure 25). The concentration of wild type and recombinant vaccinia used in this study was ranged from $3x10^7$ to $2.1x10^8$ pfu/ml (Table 6).

Table5. Epstein-Barr virus copy number of peripheral blood mononuclear cell DNA in healthy donors and immunocompromised patients

ID	EBV DNA (copies /µg DNA)	ID	EBV DNA (copies /µg DNA)
EBV seropositive healthy donors		HIV infected patients	
C1	54	P1	52
C2	<10	P2	11
C3	16	P3	<10
C4	<10	P4	17
C5	57	P5	<10
C6	<10	P6	853
C7	36	P7	909
C8	<10	P8	3,785
C9	<10	P9	24
C10	<10	P10	77
EBV seronegative healthy donors			
C11	<10*		
C12	<10*		
Median	<10	Median	38

* EBV seronegative healthy donors were not induced in the statistic calculation.

A

B

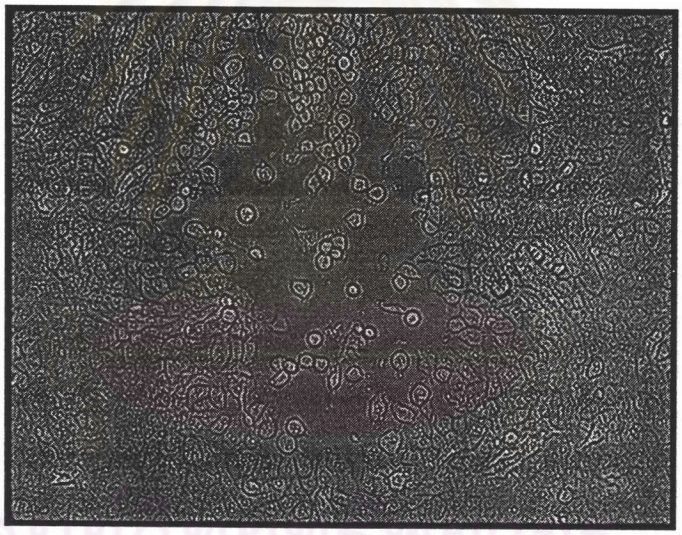
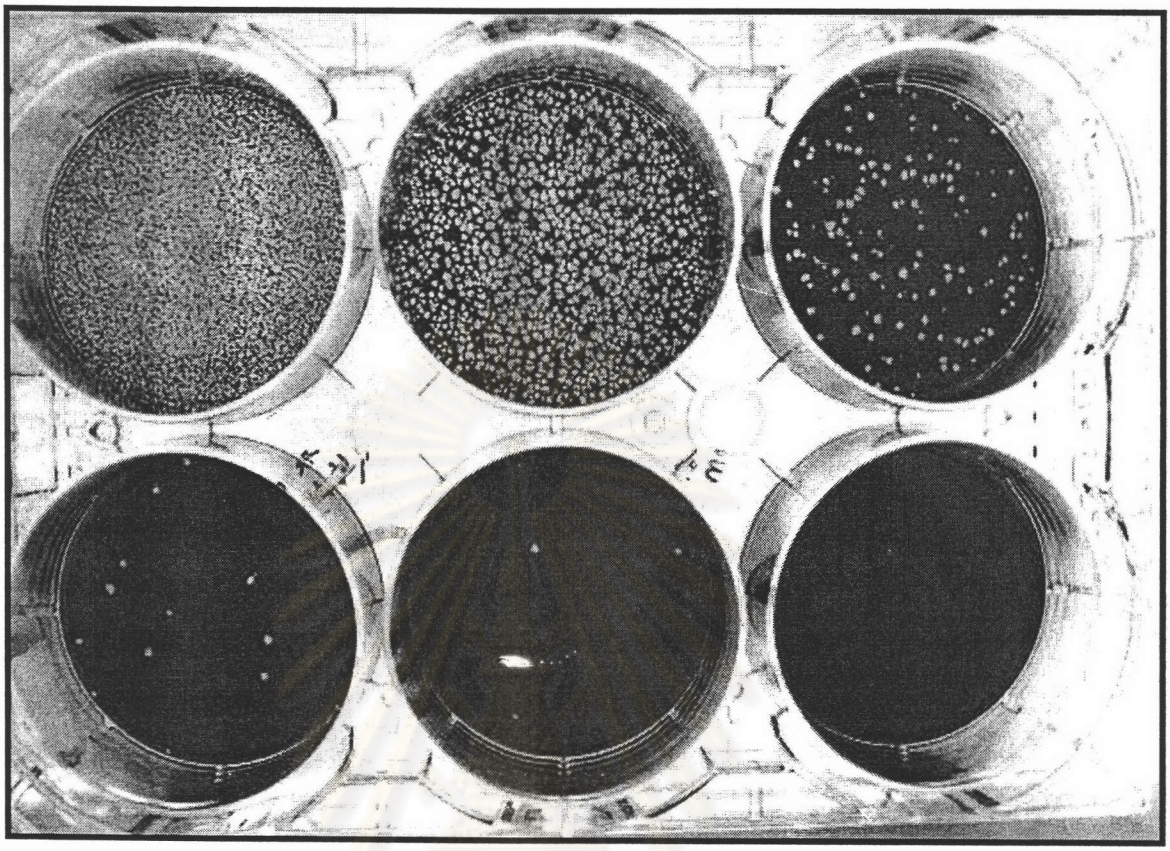


Figure 24. TK$^-$143 cell line (A) and cytopathic effect caused by vaccinia virus (B) Magnification x100

Dilution: 10^{-4} 10^{-5} 10^{-6}



Dilution: 10^{-7} 10^{-8} 10^{-9}

Figure 25. Plaque titration assay, the assay obtained by serial 10 fold dilution of recombinant vaccinia ranging from 10^{-4} to 10^{-9}. The titre of recombinant vaccinia was calculated from the dilution which obtained plaque between of 10 to 300.

Table 6. The concentration of wild type and eight recombinant vaccinia viruses expressing EBV genes used in the study

Vaccinia expressing EBV gene	Titre (pfu/ml)
1. LMP-1	2.0×10^8
2. LMP-2A	0.4×10^8
3. EBNA-3A (E3a)	1.1×10^8
4. EBNA-3B (E3b)	1.1×10^8
5. EBNA-3C (E3c)	0.8×10^8
6. EBNA-1 (E1)	0.5×10^8
7. EBNA-LP (LP)	0.3×10^8
8. BHRF-1	2.1×10^8
9. Wild type (WT)	2.0×10^8

2. Analysis of EBV-specific T cell responses by IFN-γ producing ELISpot assay

We decided to use recombinant vaccinia expressing EBV antigens-based ELISpot to enumerate EBV-specific T cells. However, the protocol for vaccinia-based ELISpot assay was not been established to quantify EBV-specific T cells. We therefore needed to optimise the condition for the assay.

2.1. Optimisation of ELISpot assay

In order to achieve optimum condition for the ELISpot assay, optimisation of PBMC concentration, Interleukin supplementation and infection dose of recombinant vaccinia has required. Details of optimisation were discussed.

2.1.1. Optimal PBMC concentration

PBMC of an EBV seropositive healthy donor who mediated strong T cell responses against EBNA-3 in screening was selected for optimisation of PBMC concentration. ELISpot was performed by co-cultured recombinant vaccinia expressing EBNA-3 (E3-vac) and wild-type vaccinia (wt-vac) at MOI of 2 with PBMC at concentration $2.5x10^5$ and $5.0x10^5$ cells per well. The results revealed the higher spots number in the well containing $5.0x10^5$ cells however, the more background was observed. Therefore, the ELISpot assay in this study was performed using the PBMC concentration at $2.5x10^5$ cells per well.

2.1.2. Interleukin supplementation

To see whether cytokines were needed to obtain optimal condition of the ELISpot assay, the assay was performed by culturing $2.5x10^5$ PBMC with wt-, E3a-vac at MOI of 2, 5 and 10. The assay was supplemented by interleukins in 3 conditions i) IL-2 at concentration of 50 U/ml (IL2-ELISpot) ii) IL-7 at concentration of 25 ng/ml (IL7-ELISpot) iii) Both IL-2 and Il-7 ELISpot at the previously-indicated concentration (IL2/IL7-ELISpot). The control contained only PBMC, recombinant vaccinia and R10(10% fetal bovine serum in RPMI). The magnitude of EBV-specific T cell responses in the control ranged from 330 to 466 with the mean of 419 SFU/10^6 PBMC, whereas the responses IL2-ELISpot ranged from 554 to 616 with the mean of 584 SFU/10^6 PBMC. For IL7-ELISpot, the responses ranged from 482 to 564 with the mean of 524 SFU/10^6 PBMC. Surprisingly, the magnitude

of responses in IL2/IL7-ELISpot ranged from 166 to 208 with the mean of 189 SFU/10^6 PBMC. Since IL2-ELISpot gave higher number of spot-forming unit when compared with conventional assay (control) ($P<0.05$), IL7-ELISpot and IL2/IL7-ELISpot (Table 7), IL2-ELISpot was selected to supplement in ELISpot assay to run throughout this study.

2.1.3. Infection dose of recombinant vaccinia

In order to establish the optimal dose of recombinant vaccinia, ELISpot was performed by co-culturing $2x10^5$ PBMC with wt-, E3a-, E3b- and E3c-vac at MOI of 2, 4 and 6 in R10 supplemented with IL-2 at final concentration 50 U/ml. The magnitude of EBV-specific T cell responses in assay containing vaccinia at MOI of 2 ranged from 23 to 69 with the mean of 41 SFU/10^6 PBMC, where as the responses in assay containing vaccinia at MOI of 4 ranged from 54 to 82 with the mean of 66 SFU/10^6 PBMC . For assay containing vaccinia at MOI of 6, the responses ranged from 39 to 79 with the mean of 53 SFU/10^6 PBMC. The results revealed the vaccinia at MOI of 4 gave the highest responses, albeit not statistically significant (Table 8 and Figure 27).

Taken together, the condition for quantitation of EBV-specific T cells was optimised whereby $2x10^5$ PBMC was stimulated with recombinant vaccinia at the MOI of 4 in the presence of 50 U/ml of IL-2.

Table 7. ELISpot assay optimisation

Type of vaccinia and infection dose	EBV-specific $CD8^+$T cell responses (SFU/10^6 PBMC) in reaction containing			
	R10	IL-2[a]	IL-7[b]	IL-2[a] & IL-7[b]
WT-vac at MOI of 2	76	72	110	62
E3a-vac at MOI of 2*	330	554	528	192
WT-vac at MOI of 5	62	80	64	56
E3a-vac at MOI of 5*	462	582	564	166
WT-vac at MOI of 10	106	84	78	38
E3a-vac at MOI of 10*	466	616	482	208
Mean**.	419***	584***	524	189

NOTE. MOI: multiplicity of infection, WT-vac: wild type vaccinia, E3a-vac: recombinant vaccinia expressing EBNA-3a

* The number of spot in each condition was presented after subtraction with wild type vaccinia control.

** Number of spot mediated by WT-vac was not included in statistic calculation.

*** Significant at $P<0.05$

[a] R10 supplemented with IL-2 at final concentration of 50 U/ml

[b] R10 supplemented with IL-7 at final concentration of 25 ng/ml

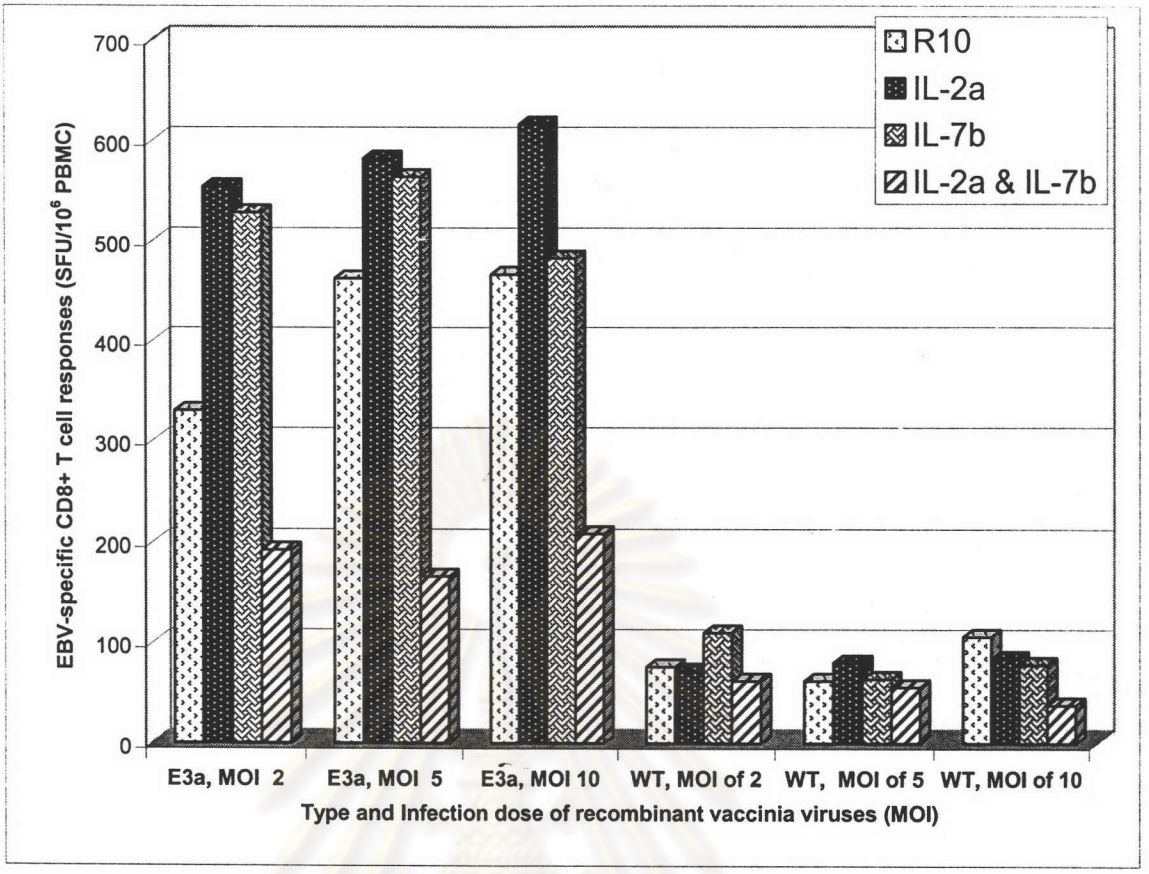


Figure 26. EBV-specific $CD8^+$ T cell responses were enumerated when IL-2, IL-7 or both cytokines supplemented in the ELIspot assay. The number of spot in each condition was presented after subtraction with wild type vaccinia control

Table 8. ELISpot assay of infectious dose of vaccinia optimisation

	EBV-specific $CD8^+$ T cell responses ($SFU/10^6$ PBMC)*				
Infection dose of vaccinia (MOI)	WT-vac**	E3a-vac	E3b-vac	E3c-vac	Mean±SD
2	55	69	23	32	41±21
4	62	82	62	54	66±12
6	79	79	40	39	53±22

NOTE. MOI: multiplicity of infection, WT-vac: wild type vaccinia, E3a-vac: recombinant vaccinia expressing EBNA-3a, E3b-vac: recombinant vaccinia expressing EBNA-3b, E3c-vac: recombinant vaccinia expressing EBNA-3c gene

* The number of spot in each condition was presented after subtraction with wild type vaccinia control.

** Number of spot mediated by WT-vac was not included in statistic calculation.

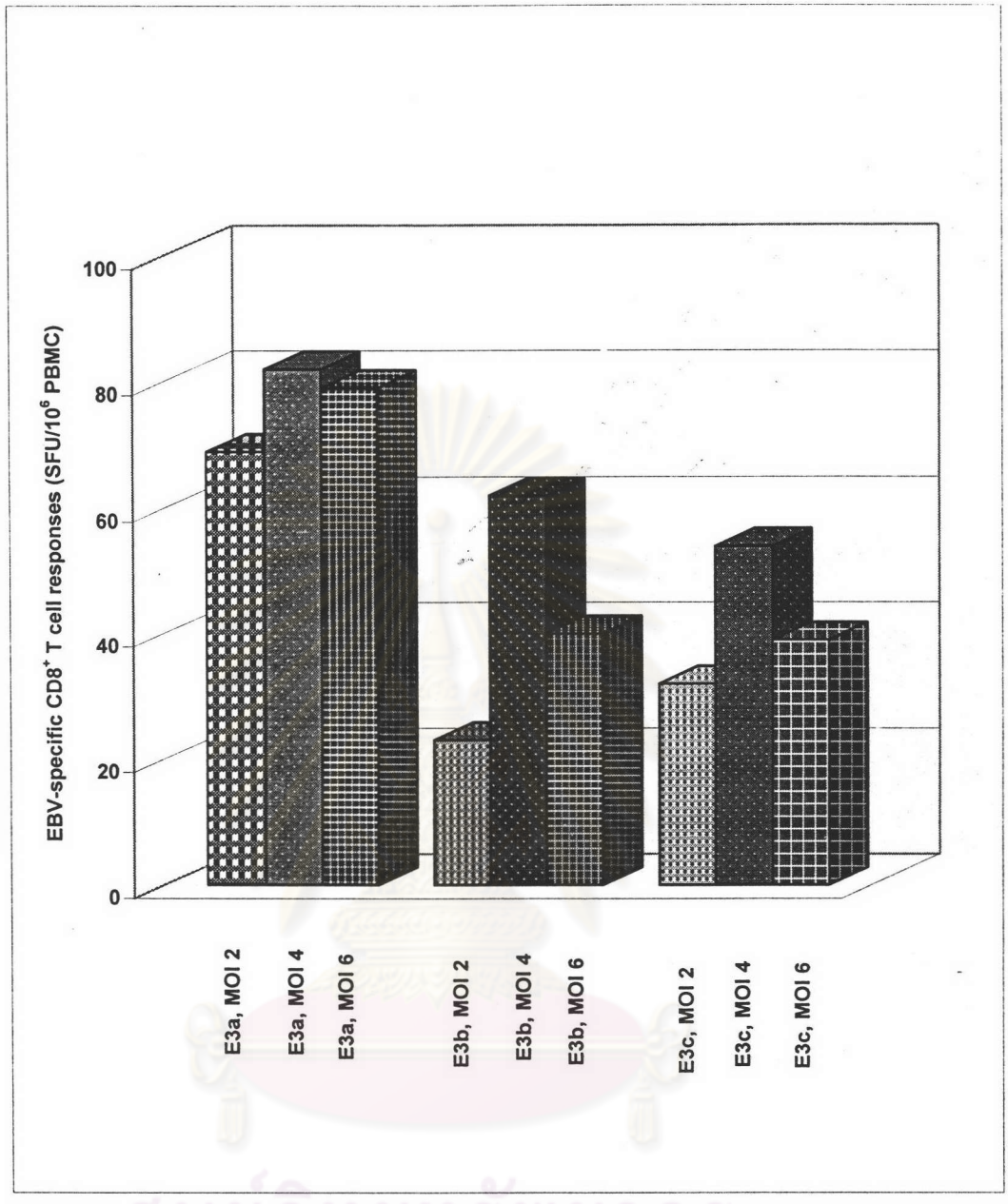


Figure 27. EBV-specific $CD8^+$ T cell responses were enumerated in ELISpot containing difference recombinant vaccinia doses (MOI of 2, 4 and 6 respectively). The number of spot in each condition was presented after subtraction with wild type vaccinia control.

2.2. Enumeration of EBV-specific $CD8^+$T cell responses

To study EBV-specific $CD8^+$ T cell responses, we enrolled 2 EBV-seronegative, 10 EBV seropositive and 10 HIV-infected donors into the experiment. The T cell responses were analysed by vaccinia-based ELISpot assay performing as previously described. The EBV-specific $CD8^+$ T cell responses were defined as positive if the number of spot was higher than 20 spot/well and 2.5 times more than negative control. The responses of EBV-specific $CD8^+$ T cell in this study was divided into two typed, individual responses and cumulative responses. The individual response was a level of response from each donor to a single protein. The cumulative responses were calculated by summation of the individual positive responses.

2.2.1. Overall EBV-specific $CD8^+$T cell responses

Neither of EBV-seronegative donors (C11 and C12) had EBV-specific $CD8^+$ T cell responses (Table 9 and Figure 28). On the other hand, 15 of 20 (75%) donors, including 10 control and 10 HIV-infected donors had the EBV-specific $CD8^+$ T cell responses to at least one protein (Table 10 and 11). Indeed only 5 of 8 proteins (63%) were recognised by the EBV-seropositive and HIV-infected donors (20 donors). The most frequently recognised EBV protein was EBNA 3 protein which 13 out of 20 donors has response to. In fact, EBNA-3b (E3b) seemed to be the most immunodominant protein in this study. As many as 50% donors (10 of 20) recognised this protein with magnitude of responses of 76 to 1,092 with the mean of 123 ± 249 SFU/10^6 PBMC. When the responses to E3b by these 20 donors were summed up (the cumulative response to E3b), we found the cumulative of E3b from all recognised donors was 2,463 SFU/10^6 PBMC with the mean of 246± 311 SFU/10^6 PBMC (Table 10 and 11).

2.2.2. EBV-specific $CD8^+$T cell responses in EBV-soropositive healthy donors

All of EBV-seropositive donors (C1–C10) had detectable EBV-specific T cell responses which varied amongst individuals (Table 10 and Figure 28). The cumulative EBV-specific $CD8^+$ T cell responses individual ranged from 96 to 426 with the mean of 228 ± 115 SFU/10^6 PBMC. The EBV-seropositive donors had a EBV-specific $CD8^+$ T cell responses to 5 proteins including E3a, E3b, E3c, LP and BHRF-1. Indeed most of them (60%) had the response against only one EBV protein (Table 10). The broadest T cell response was demonstrated in donor C6 who recognised three EBV proteins comprising of E3a, E3b and E3c with the magnitude of responses of 94, 117 and 114 SFU/10^6 PBMC, respectively. Eventhough donor C6 gave the broadest responses but the highest magnitude of responses belonged to C1 who had cumulative responses of 462 SFU/10^6 PBMC.

Table 9. EBV-specific $CD8^+$ T cell responses in EBV-seronegative donors

r-VV/donors	*LMP-2A*	*LMP-1*	*E3a*	*E3b*	*E3c*	*E-1*	*LP*	*BHRF-1*	**Total donors**
C11	4	0	8	0	0	0	0	20	-
C12	2	2	0	0	10	0	10	0	-
Total r-VV	-	-	-	-	-	-	-	-	-

NOTE: The number of spot in each condition was presented after subtraction with wild type vaccinia control.

: Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive, therefore no positive responses was observed in both EBV-seronegative donors.

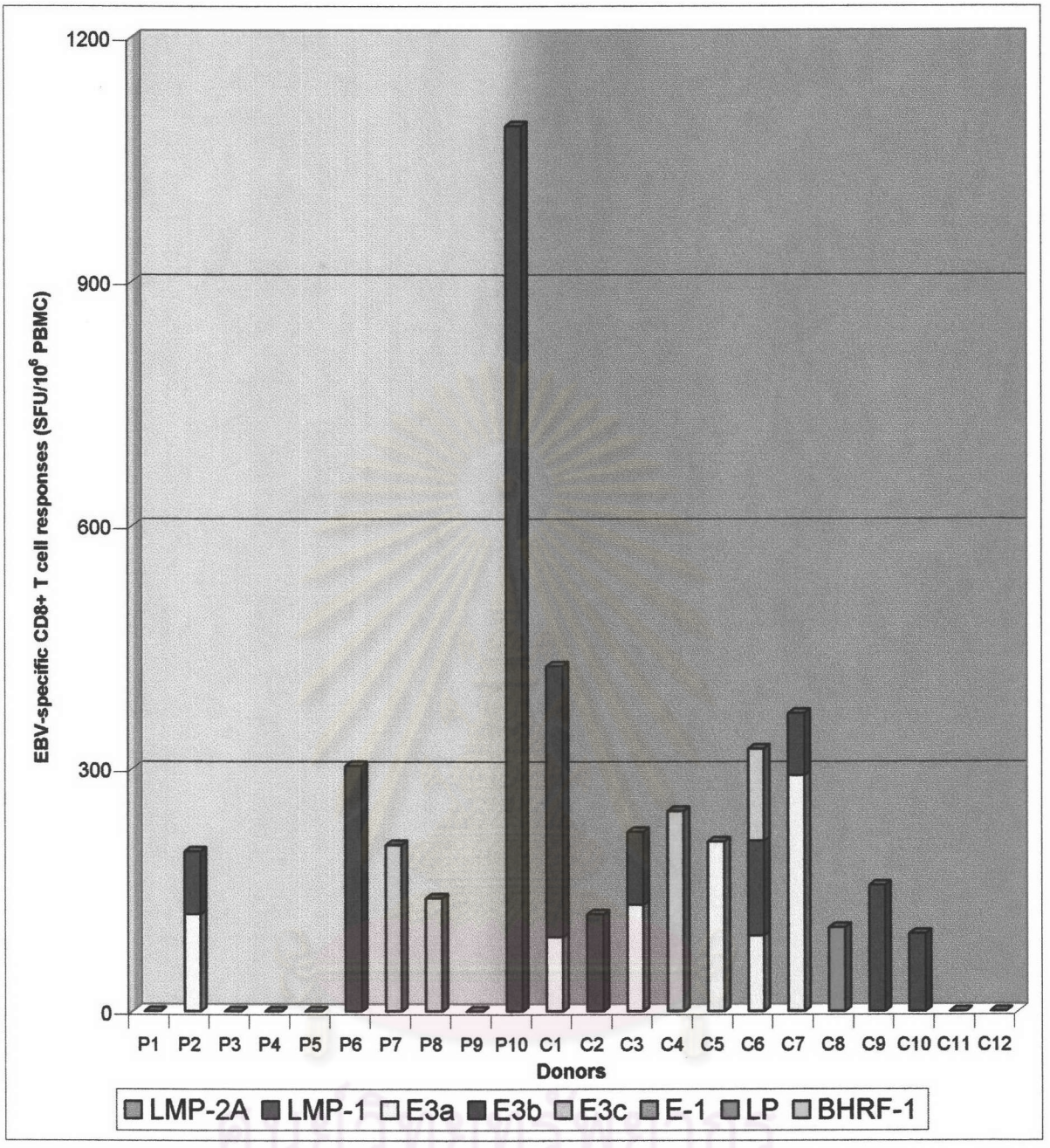


Figure 28. EBV-specific CD8+ T cell responses in HIV infected (P1-P10), EBV seropositive (C1-C10) and EBV seronegative donors (C11 and C12) individual. The number of spot in each condition was presented after subtraction with wild type vaccinia control.

Table 10. EBV-specific $CD8^+$ T cell responses in EBV-seropositive donors

Proteins/Donors	*LMP-2A*	*LMP-1*	*E3a*	*E3b*	*E3c*	*E-1*	*LP*	*BHRF-1*	**Cumulative responses to each donor**	Mean	Min	Max
C1	0	0	**92**	**334**	0	0	0	0	426	53±118	0	334
C2	0	0	0	**120**	0	0	0	0	120	15±42	0	120
C3	0	0	**132**	**90**	0	0	0	0	222	28±53	0	132
C4	0	0	0	0	0	0	0	**248**	248	31±88	0	248
C5	0	0	**210**	0	0	0	0	0	210	26±74	0	210
C6	0	0	**94**	**117**	**114**	0	0	0	325	41±56	0	117
C7	0	0	**292**	**76**	0	0	0	0	368	46±103	0	292
C8	0	0	0	0	0	0	**104**	0	104	13±37	0	104
C9	0	0	0	**156**	0	0	0	0	156	20±55	0	156
C10	0	0	0	**96**	0	0	0	0	96	12±34	0	96
Cumulative responses to each protein	NA	NA	820	989	114	NA	104	248	2,275			
Mean±SD	NA	NA	82±104	99±100	11±36	NA	10± 33	25± 78				
Min	NA	NA	0	0	NA	NA	0	0				
Max	NA	NA	292	334	NA	NA	104	104				

NOTE.

: NA, Not applicable

: Results were presented as SFU of EBV-specific CD8+ T cell responses after subtracting with wild type vaccinia.

: Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive (printed in bold)

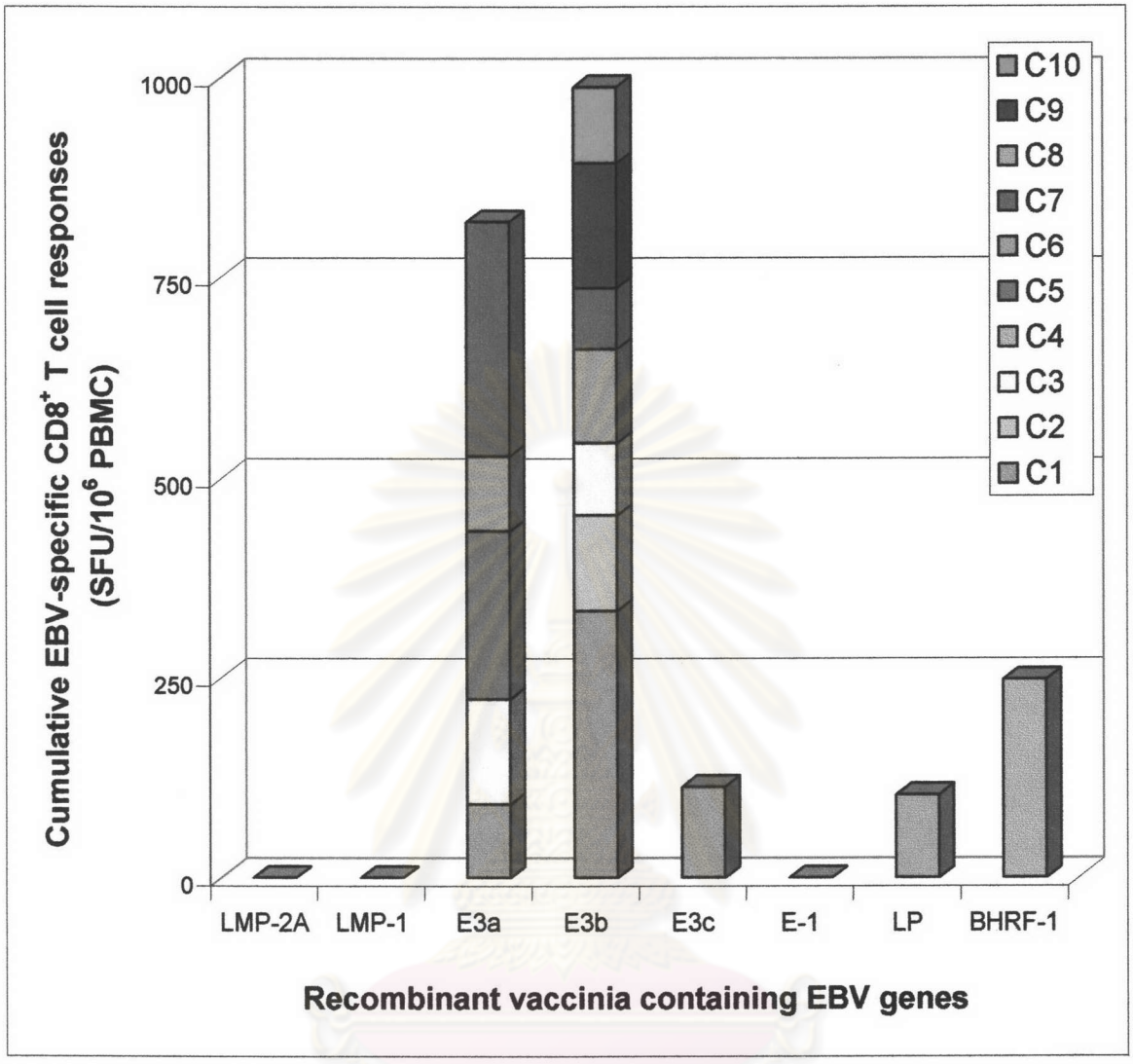


Figure 29. The cumulative EBV-specific CD8+ T cell responses to each EBV protein by healthy donors. The number of spot in each condition was presented after subtraction with wild type vaccinia control

2.2.3. EBV-specific $CD8^+$T cell responses in HIV-infected donors

2.2.3. EBV-specific $CD8^+$T cell responses in HIV-infected donors

On the other hand only 5 of 10 (50%) HIV-infected patients (P2, P6, P7, P8 and P10) had detectable EBV-specifric T cell responses (Table 11 and Figure 28). Their cumulative T cell responses ranged from 198 to 1,092 with the mean of 194 ± 335 S FU/10^6 PBMC. The HIV-infected patients had EBV-specific $CD8^+$ T cell responses to only 3 proteins including E3a, E3b and E3c. However, the highest T cell response in this study (1,092 SFU/106 PBMC) was observed in one patient (P10). The number of EBV proteins recognised per donor was ranged from one and two, most responsive persons (80%) recognised only one EBV protein. These included 4 donors who recognised E3b (P6 and P10) and E3c (P7 and P8). The broadest EBV-specific response in this study was from P2 who recognised E3a and E3b with a magnitude of responses of 120 and 78 SFU/10^6 PBMC, respectively. On the other hand, the highest EBV-specific responses was from the patient P10 whose the magnitude responses to E3b was 1,092 SFU/10^6 PBMC. There were 5 patients (P1, P3, P4, P5 and P9) who did not recognise any EBV proteins.

2.2.4. Immunodominance of EBV protein in EBV-seropositive healthy donors

The EBV-specific $CD8^+$ T cell response to EBV protein was analysed. We found that five of eight EBV proteins (63%) were recognised by EBV-seropositive healthy donors (Table 10 and Figure 29). The two most frequently recognised were E3b (80%) and E3a (50%). Whilst the magnitude responses of E3b ranged from 76 to 334 with the mean of 99±100 SFU/10^6 PBMC, the responses of E3a ranged from 92 to 292 with the mean of 88±104 SFU/10^6 PBMC. In fact, the mean of responses of E3b and E3a were 414±89 and 164±86 SFU/10^6 PBMC, respectively if the mean of responses from responsive donors were taken into account. In addition, there were 3 proteins which were less frequently targeted. These proteins were E3c, LP and BHRF-1 which had the magnitude of responses of 114, 104 and 248 SFU/10^6 PBMC, respectively. On the other hand, there were three EBV proteins the responses of which could not be detected in this study. These proteins were LMP-2a, LMP-1 and E-1.

2.2.5. Immunodominance of EBV protein in HIV-infected donors

In HIV-infected patients, only three (38%) of eight EBV protein were recognised by HIV-infected donors (Figure 30). The frequency of the HIV-infected donor who recognised EBV protein were 30% for E3b (3 donors), 20% for E3c (2 donors) and 10% for E3a (1 donor). The magnitude responses of E3b and E3c ranged from 78 to 1,092 and 104 to 206 with the mean of 147±345 and 35±75 SFU/10^6 PBMC, respectively. If considering only positive responses, the mean of magnitude responses of E3b and E3c was 491±532 and 173±47 SFU/10^6 PBMC. The cumulative responses of E3b from all HIV-infected donors were 1,474 SFU/10^6 PBMC whereas the responses of E3a and E3c were 346 and 120 SFU/10^6 PBMC, respectively. The EBV proteins responses of which could not be detected in the HIV-infected donor were LMP-2a, LMP-1, E-1and BHRF-1.

Table 11. EBV-specific $CD8^+$ T cell responses in HIV-infected patients

Proteins/donors	*LMP-2A*	*LMP-1*	*E3a*	*E3b*	*E3c*	*E-1*	*LP*	*BHRF-1*	**Cumulative responses to each donor**	Mean	Min	Max
P1	0	0	0	0	0	0	0	0	NA	NA	NA	NA
P2	0	0	**120**	**78**	0	0	0	0	198	25±47	0	120
P3	0	0	0	0	0	0	0	0	NA	NA	NA	NA
P4	0	0	0	0	0	0	0	0	NA	NA	NA	NA
P5	0	0	0	0	0	0	0	0	NA	NA	NA	NA
P6	0	0	0	**304**	0	0	0	0	304	38±107	0	304
P7	0	0	0	0	**206**	0	0	0	206	26±72	0	206
P8	0	0	0	0	**140**	0	0	0	140	18±49	0	140
P9	0	0	0	0	0	0	0	0	NA	NA	NA	NA
P10	0	0	0	**1,092**	0	0	0	0	1,092	137±386	0	1,092
Cumulative responses to each protein	NA	NA	120	1,474	346	NA	NA	NA	1,940			
Mean	NA	NA	12±38	147±345	35±75	NA	NA	NA				
Min	NA	NA	0	0	0	NA	NA	NA				
Max	NA	NA	120	1,092	206	NA	NA	NA				

NOTE: The number of spot in each condition was presented after subtraction with wild type vaccinia.

:Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive (printed in bold)

: NA, Not applicable

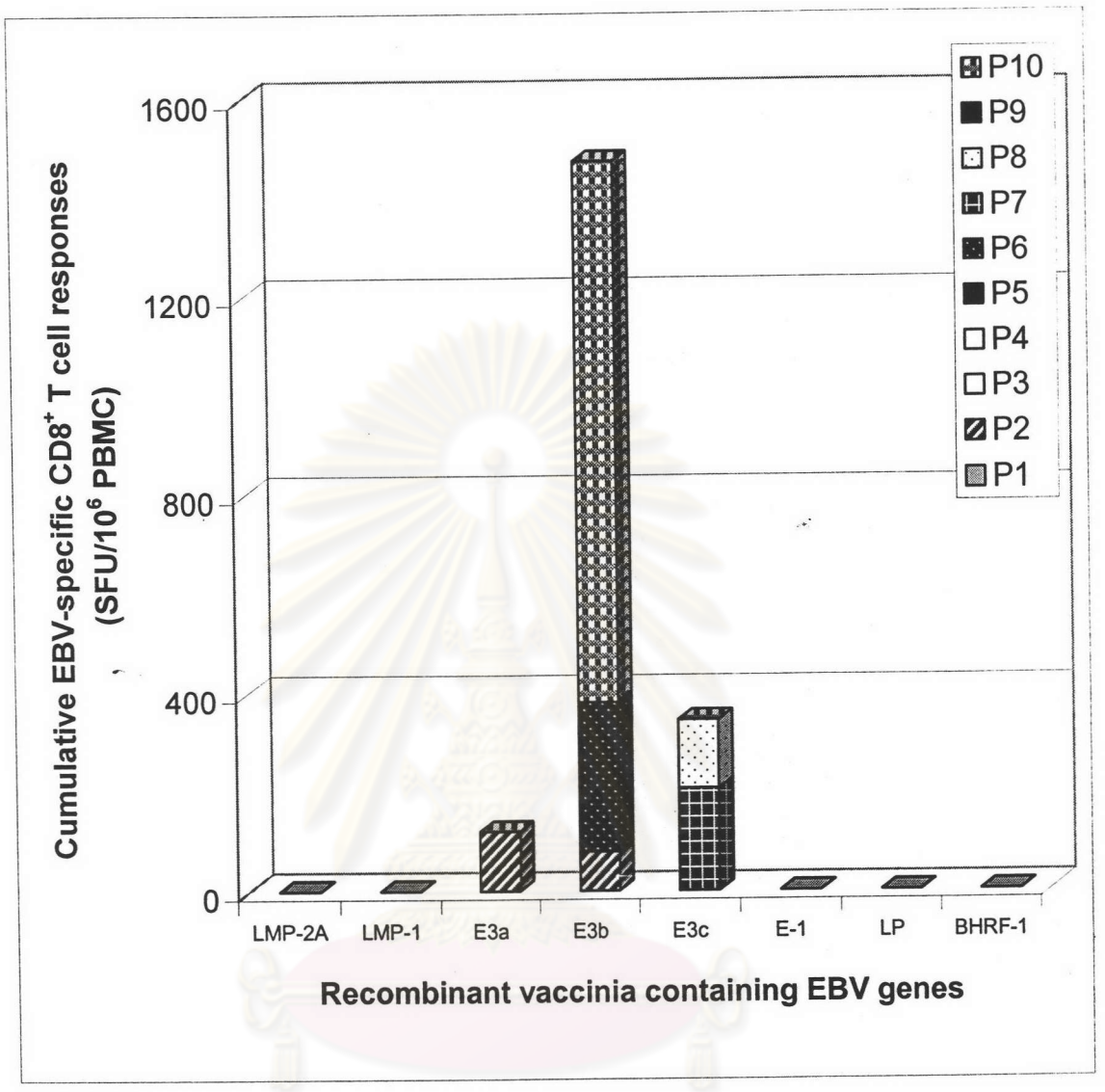


Figure 30. The cumulative EBV-specific CD8+ T cell responses in HIV infected donors. The number of spot in each condition was presented after subtraction with wild type vaccinia control

2.3. Correlation between EBV-specific CD8+ T cell responses and EBV DNA load.

In this study we need to know whether EBV-specific $CD8^+$ T cell responses might play a role in the control of EBV infection. We tried to establish the correlation between EBV-specific $CD8^+$ T cell responses obtained from ELISpot and EBV DNA load in PBMC. The correlation was tried to generated between either cumulative T cell responses or the individual responses from EBV immnunodominant proteins (E3a, E3b and E3c) and EBV-DNA load in both EBV-seropositive and HIV-infected group.

Whilst there was positive trend of correlation between EBV-DNA and EBV-specific $CD8^+$ T cell responses in the healthy control donors (Figure 31 and 32), the negative trend was observed in HIV-infected patients (Figure 31 and 33)

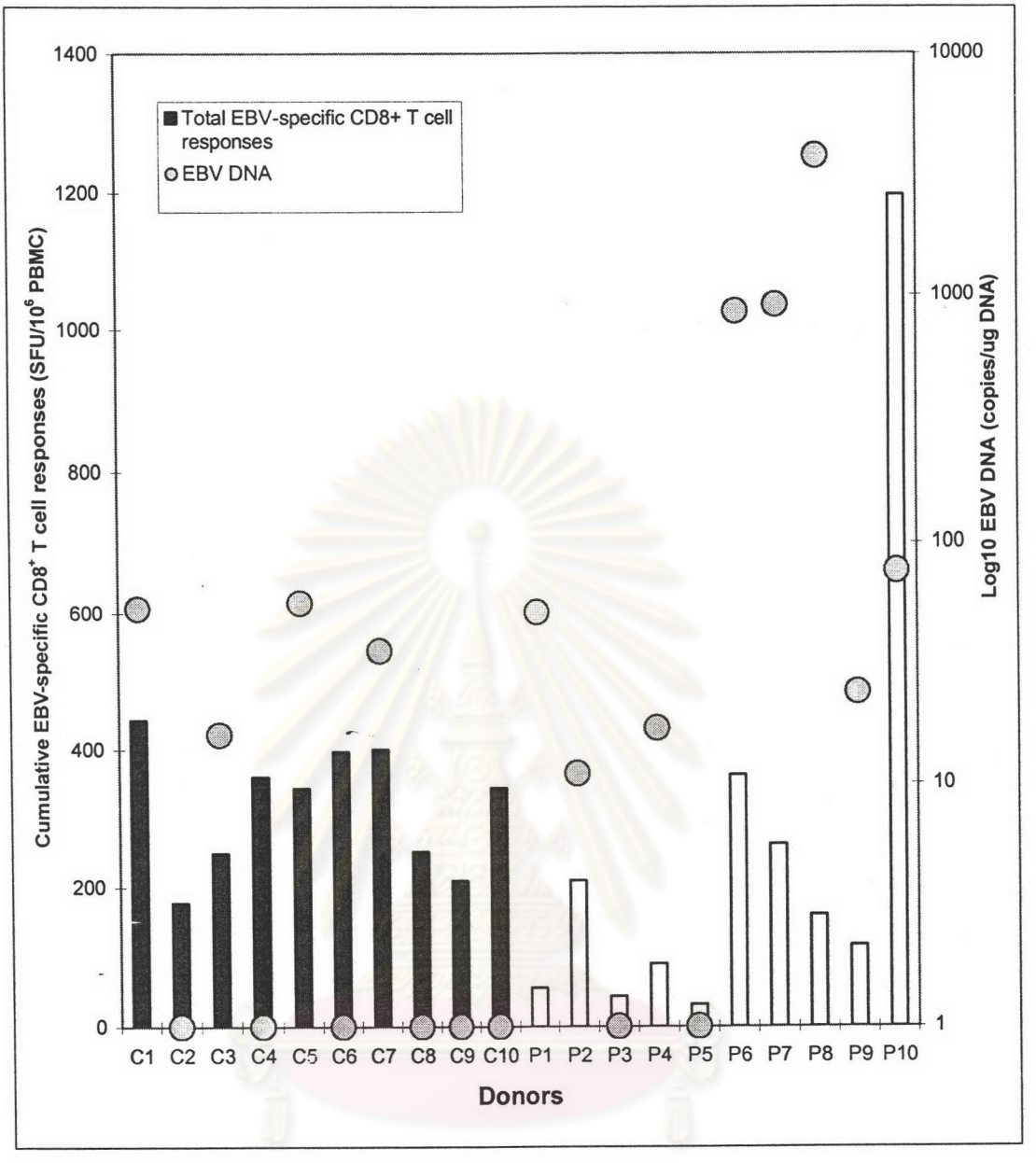


Figure 31. Magnitude of cumulative EBV-specific T cell responses by ELISpot and EBV load of individual EBV-seropositive healthy donors (C1-C10) and HIV-infected patients (P1-P10).

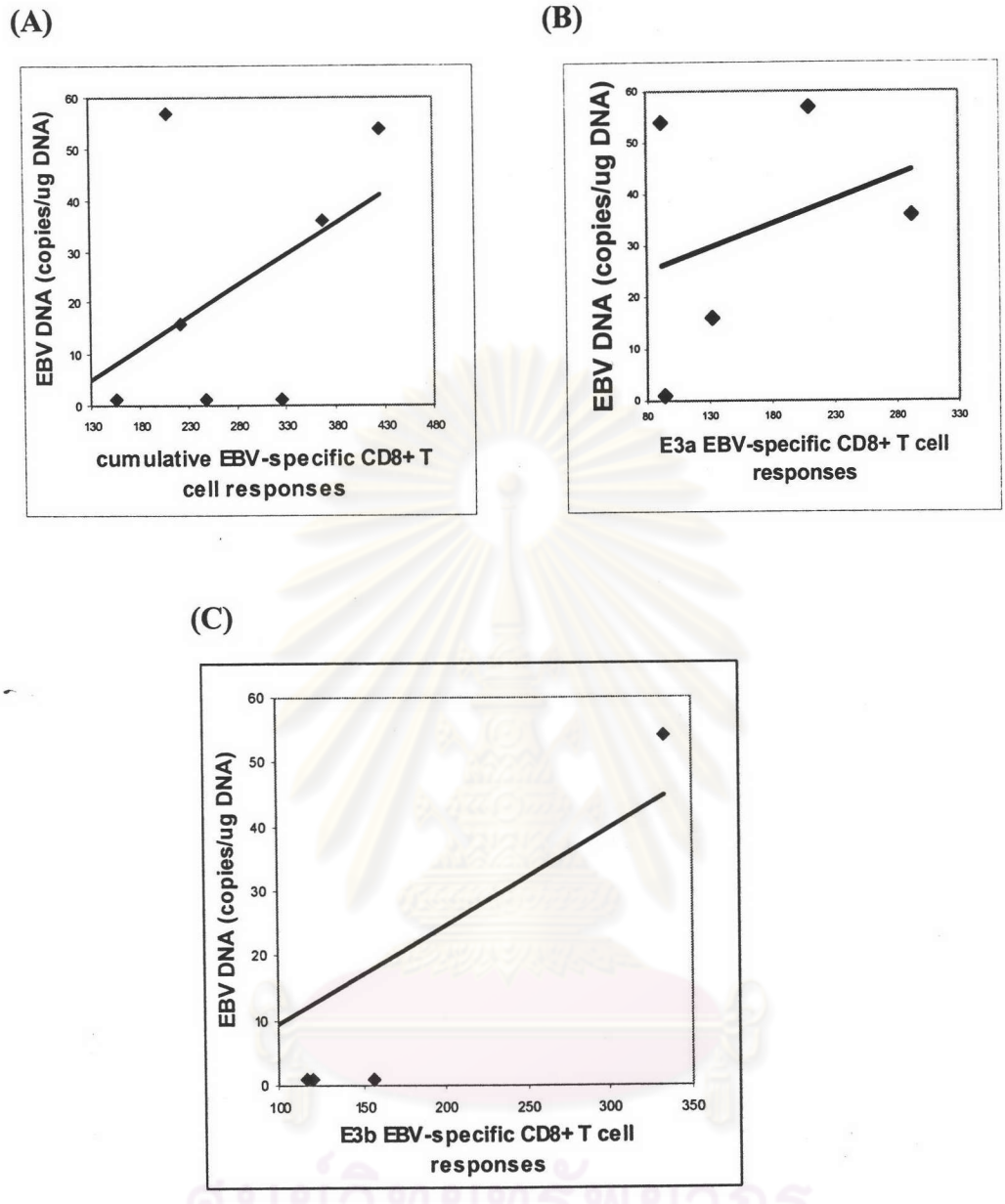


Figure. 32 The positive trend of correlation between EBV-DNA load in PBMC and cumulative EBV-specific CD8+ T cell responses (A), E3a EBV-specific CD8+ T cell responses (B) and E3b EBV-specific CD8+ T cell responses (C)

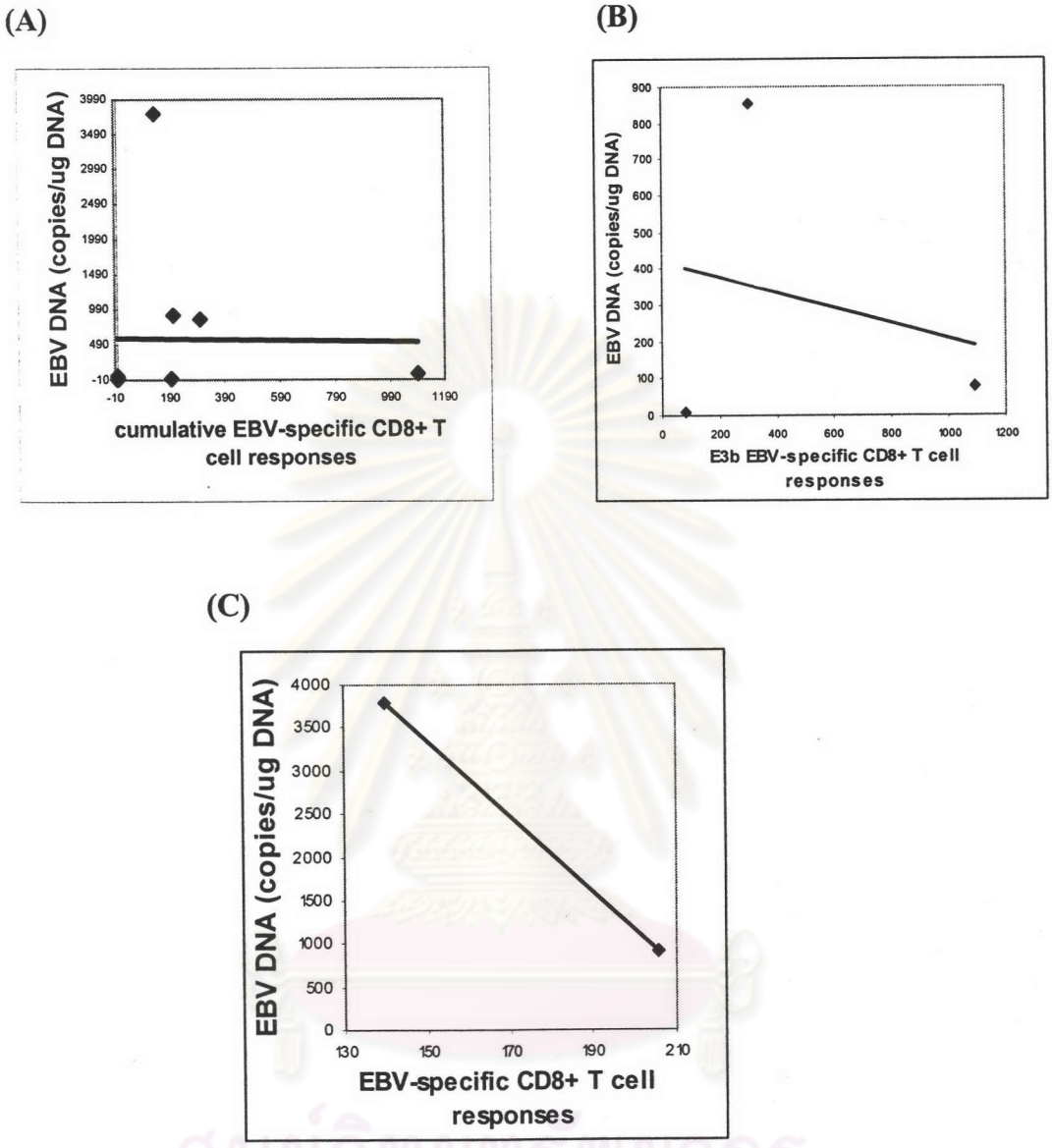


Figure. 33 The negative trend of correlation EBV-DNA load in PBMC of HIV-infected patients and total EBV-specific CD8+ T cell responses (A), E3b EBV-specific CD8+ T cell responses (B) and E3c EBV-specific CD8+ T cell responses (C)

2.4. CD8+ cells depletion experiment

Since IFN-γ could be released from $CD4^+$ or $CD8^+$ lymphocytes or NK cells, we need to prove whether the responses detected in this experiment were mediated through $CD8^+$ T cell by performing $CD8^+$ depletion experiments using anti-$CD8^+$ immunomagnetic bead. We selected 2 EBV-seropositive (C5 and C9) and 3 HIV-infected donors (P6, P8 and P10) who had T cell responses against recombinant vaccinia expressing EBV gene by previous ELISpot assay. After the $CD8^+$depletion, we found the levels of responses were significantly reduced. The results revealed more than 93% of spot were abrogated by CD8+ depletion, suggesting that the responses in the ELISpot assay were mediated by CD8+ T cells. (Table 12).

Table 12. The EBV-specific T cell responses after depletion of $CD8^+$ T cells by immunomagnetic beads of EBV-seropositive donors (C5 and C9) and HIV-infected donors (P6, P8 and P10).

Magnitude of EBV-specific T cell responses (SFU/10^6 PBMC)[a]															
Vaccinia expressing EBV genes /donors	**C5**			**C9**			**P6**			**P8**			**P10**		
	Pre	Post	%R	Pre	Post	%R	Pre	Post	%R	Pre	Post	%R	Pre	Post	%R
E3a	**210**	**0**	**100**	-	-	-	-	-	-	-	-	-	-	-	-
E3b	-	-	-	**156**	**8**	**94**	**304**	**0**	**100**	-	-	-	**1092**	**2**	**100**
E3c	-	-	-	-	-	-	-	-	-	**140**	**10**	**93**	-	-	-

NOTE. Pre: Before $CD8^+$ cell depletion, Post: After $CD8^+$ cell depletion, %R: Percent reduction

[a] The number of spot in each condition was presented after subtraction with wild type vaccinia control. Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive (printed in bold)

3. Establishment of EBV-transformed B- Lymphoblastoid cell line (BLCL)

We were able to establish 20 BLCL lines from all EBV-seropositive donors enrolled into this study (Figure 34). The timing required for establishing BLCL of EBV-seropositive healthy donors was between 4 to 6 weeks while establishing time of HIV-infected BLCL (HIV-BLCL) was approximately 3 to 4 weeks.

4. Establishment of EBV specific cytotoxic T lymphocyte lines

PBMC from all donors, including of 10 EBV-seropositive healthy donors and 10 immunocompromised patients were collected and used in establishment of EBV-CTL (Figure 35).

We were able to establish EBV-CTL from all HIV-infected donors. When phenotype of cells the were analysed by flow cytometry, the results revealed that most of CTL lines from 9 patients were mainly composed of $CD8^+$ T cells ranging from 70 to 88% with the mean of 80%. The lines also consisted of minority population which were $CD4^+$ T cells (ranging from 0 to 11 with the mean of 3%) and NK^+ cells (less than or equal to 1%). Interestingly, one CTL line from the patients P2 contained 48% of $CD8^+$ T cells, 4% of $CD4^+$ T cells and 48% of undefined $CD3^+$ cells (Table 13).

On the other hand, we were able to establish EBV-CTL from only 7 out of 10 EBV-seropositive healthy donors. The culture from 6 donors was composed of $CD8^+$ T cells, as a major cell phenotype, ranging from 13 to 88% with the mean of 61%, $CD4^+$ T cells ranging from 7 to 49 with the mean of 15%, NK^+ cells ranging from 0 to 6 with the mean of 2 and $CD19^+$ cells ranging from 0 to 5 with the mean of 1%. Surprisingly, one CTL line from donors C6 had $CD4^+$ T cell population up to 99.5 % without NK^+ and $CD8^+$ T cells (Table 13).

5. Analysis of EBV-specific cytotoxic T cell responses by chromium release assay

The EBV-specific killing functions of all CTL lines were analysed by chromium release assay. Each CTL line was co-cultured with radioactive chromium-labelled autologous BLCL at E:T ratio of 50:1, 25:1, 12.5:1 and 6.25:1 (Table 14).

All the cytotoxic lymphocyte lines showed cytotoxic activity with a specific lysis ranging from 11 to 100%. Spontaneous lysis was less than 25% in all experiments. The highest CTL responses in this study was from patient P6 who revealed the specific lysis of 100% at E:T ratio of 50:1 (Figure 36). On the average,

the EBV-CTL lines had specific lysis of 53% lysis at an E:T ratio of 50:1, 49% lysis at 25:1, 43% lysis at 12.5:1 and 37% at 6.25:1, respectively.

Indeed, the cytotoxic lymphocyte lines of HIV-infected patient showed very high cytotoxic activity with specific lysis ranging from 36 to 100% at E:T ratio of 50:1 (Table 14) with a mean of EBV-specific killing of 62, 59, 52 and 49% at E:T ratio of 50:1, 25:1, 12.5:1 and 6.25:1, respectively (Figure 36 and 37). The majority of these CTL lines (80%) were able to recognised EBV-infected cells with high EBV-specific cytotoxic activity (50% of specific lysis) at E:T ratio of 50:1.

On the other hand, the CTL line from healthy donor revealed the specific lysis ranging from 11 to 70% with a mean of EBV-specific killing of 40, 34, 28 and 23% at E:T ratio of 50:1, 25:1, 12.5:1 and 6.25:1, respectively (Figure 36 and 37). Unlike what was observed in HIV-infected patients, there were only 2 CTL lines (23%) from EBV-seropositive had a specific killing more than 50% (70 and 68%). Interestingly, the highest cytotoxic activity (70%) was observed in CTL of donor C6 who obtain greater 99% of $CD4^+$ cells.

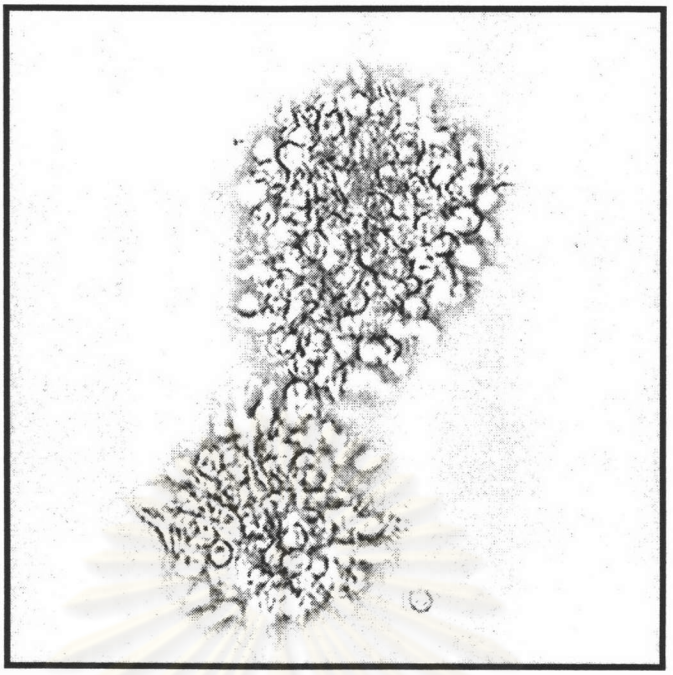


Figure 34. EBV-transformed B- Lymphoblastoid cell line, Magnification x100

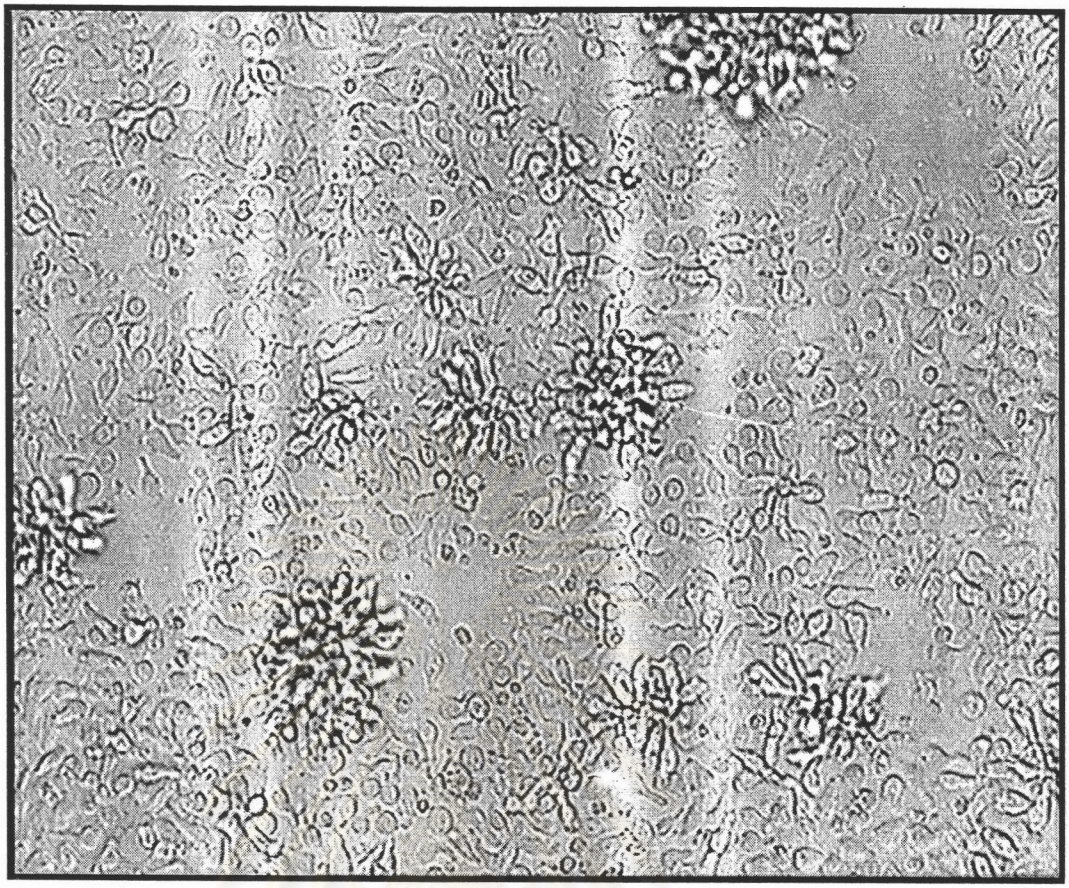


Figure 35. EBV specific cytotoxic T lymphocyte lines ready for performing Cr release assay. Magnification x100

Table 13. Summary results in establishment of BLCL, EBV-CTL and Cr-release assay of each donor.

Donors	Establishment of			Flow cytometry			
	BLCL	EBV-CTL	Cr-release	$CD4^+$	$CD8^+$	NK^+	$CD19^+$
C1	DONE	DONE	DONE	9	13	1	0
C2	DONE	UE	ND	ND	ND	ND	ND
C3	DONE	DONE	DONE	5	85	0	0
C4	DONE	UE	ND	ND	ND	ND	ND
C5	DONE	UE	ND	ND	ND	ND	ND
C6	DONE	DONE	DONE	99	1	0	0
C7	DONE	DONE	DONE	10	88	0	0
C8	DONE	DONE	DONE	11	48	6	5
C9	DONE	DONE	DONE	7	82	1	0
C10	DONE	DONE	DONE	49	51	1	1
P1	DONE	DONE	DONE	1	78	0	0
P2	DONE	DONE	DONE	3	48	0	0
P3	DONE	DONE	DONE	ND	ND	ND	ND
P4	DONE	DONE	DONE	1	82	0	0
P5	DONE	DONE	DONE	1	77	0	0
P6	DONE	DONE	DONE	1	79	0	0
P7	DONE	DONE	DONE	11	88	0	0
P8	DONE	DONE	DONE	5	70	1	0
P9	DONE	DONE	DONE	0	80	0	0
P10	DONE	DONE	DONE	2	86	0	0

C_n: EBV seropositive healthy donors, P_n: immunocompromised patients, UE: unable to establish, at least 3 times trying, ND: not done

Table 14. Percentage of specific killing at E:T ratio of 50:1, 25:1, 12.5:1 and 6.25:1

Donor	% specific lysis at E:T ratio of			
	50:1	25:1	12.5:1	6.25:1
C1	31	24	18	15
C3	16	9	8	7
C6	70	57	45	39
C7	42	38	31	28
C8	11	8	6	5
C9	68	68	60	46
Mean ± SD	40 ± 25	34 ± 25	28 ± 22	23 ± 17
P1	67	66	61	53
P2	48	43	38	36
P3	36	35	32	30
P4	73	72	60	56
P5	52	49	45	38
P6	100	99	86	70
P7	58	55	53	45
P8	51	46	39	31
P9	57	53	50	43
P10	73	69	58	52
Mean ± SD	62 ± 19	59 ± 19	52 ± 16	46 ± 13

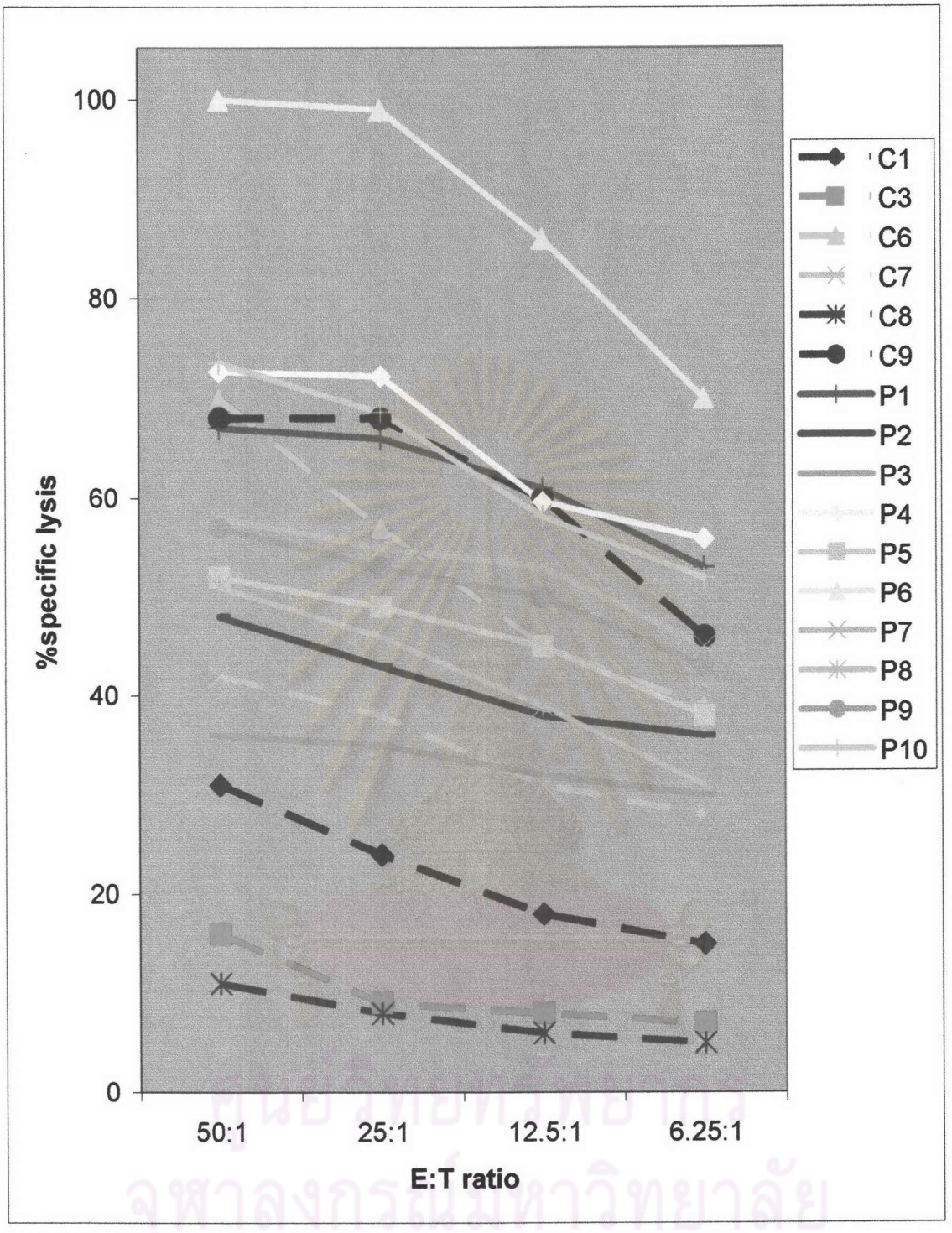
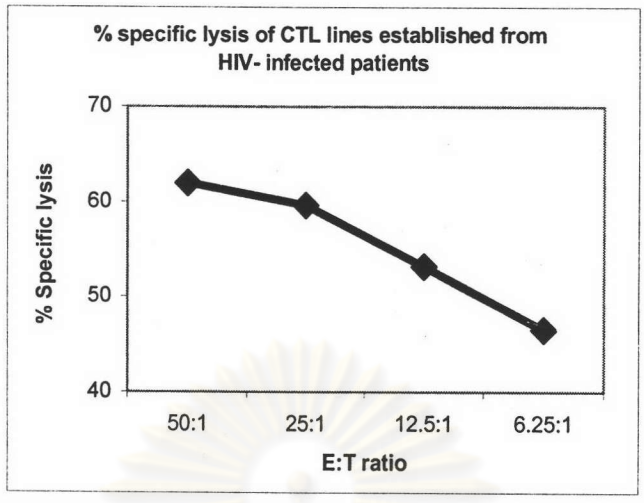


Figure 36. % specific killing at E:T ratio of 50:1, 25:1, 12.5:1 and 6.25:1 of both EBV-seropositive donors and immunocompromised patients

(A)



(B)

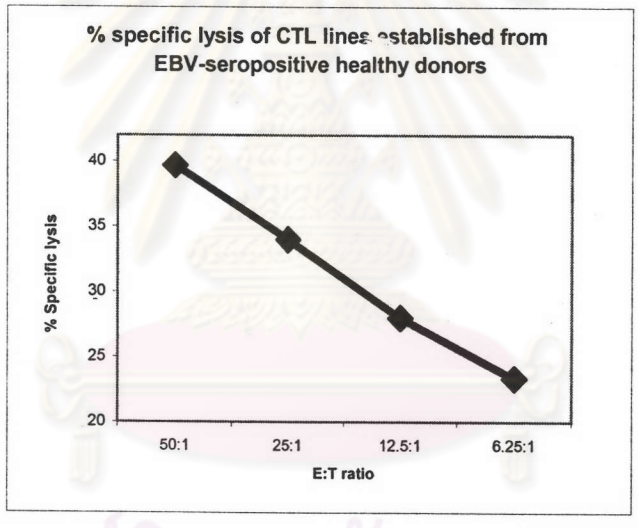


Figure 37. The CTL assay shown were performed by co-culturing the CTL with chromium-absorbed-BLCL at an effector: target (E:T) ratio of 50:1, 25:1, 12.5:1, and 6.25:1. (A) The mean CTL responses of HIV-infected patients. (B) The mean CTL responses of EBV-seronegative donors.