CHAPTER IV

MATERIALS AND METHODS

Materials

Part I Population and subjects

Study groups and sample size determination

There were 2 study groups in this study. The first group consisted of 40 HIV-1-seropositive Thais who were enrolled through the Anonymous Clinic of Thai Red Cross AIDS Research Centre. All subjects were clinically asymptomatic with $CD4^+$ T cell counts more than 300 cells/mm^3, and antiretroviral naïve. The subjects who had active infection and/or opportunistic infections were excluded from the study.

The second group consisted of 10 HIV-1-seronegative Thais who had multiple HIV exposure (i.e., couples in a stable relationship with HIV sero-discordant status). They were also enrolled through the Anonymous Clinic of Thai Red Cross AIDS Research Centre. All HIV-1-seronegative Thais were healthy.

Part II Specimen collections and processing

1. Specimen collection

30 ml of heparinised blood was taken from each donors after IRB-approved consent was obtained.

2. Specimen processing

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll hypaque density gradient centrifugation method. Heparinised blood (30 ml) was 1:1 diluted with RPMI (GIBCO, U.S.A.) and layered on ficoll hypaque reagent and centrifuged at 1,500 round per minute (rpm) for 30 minutes at room temperature. PBMC was isolated, washed twice with 10 ml of RPMI for 10 min at 2,000 rpm and 1,800 rpm, respectively. The pellets were resuspended in RPMI with 10% fetal-bovine serum (R10)

supplemented with L-glutamine, penicillin 100 U/ml, streptomycin 100 µg/ml. Then PBMC were counted and adjusted to desired cell concentration.

3. HLA typing

All HLA typing was performed by polymerase chain reaction using sequence-specific primers (PCR-SSP) at Professor Sarah Rowland-Jones' Laboratory, MRC Human Immunology Unit, University of Oxford, UK.

Methods

Part I Peptide preparation

1. Peptide selection

A panel of 21 overlapping Nef peptides (20 amino acid long overlapping by 10-amino acids) designed from HIV-1 CRF01_AE strain CM240 and 49 overlapping Gag peptides (20 amino acid long overlapping by 10-amino acids) designed from HIV-1 subtype A strain 92UG037 were kindly provided by Associate Professor Kiat Ruxrungtham, Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

2. Design of peptide matrices

The use of peptide pools for initial screening offers the minimisation of specimen usage. In this study, we used matrix system for peptides preparation. Matrices consisted of pools of peptides in which each peptide was represented in two different peptide pools, allowing for the identification of the respective peptide by responses in the two corresponding pools. The number of pools per matrix and the number of peptides per pool depended on the total number of peptides spanning each protein. The example of Pol matrix was shown in table 1 where Pol peptide 9 (Pol 9) belonging to pool D and pool G was identified as the responding peptide.

In our study, overlapping peptides were included in two different peptide matrix systems, with Nef and Gag as individual peptide matrices. The 49 overlapping Gag peptides were pooled into fourteen two-dimension matrices such that each peptide was presented in two different pools. The 21 overlapping Nef peptides were pooled into nine two-dimension matrices such that each peptide was presented in two different pools. All peptides matrix setup are summarised in Table 2-5. All $CD8^+$ T lymphocyte responses to peptides identified by the matrix approach were subsequently reconfirmed individually in an ELISpot assay.

Table 2 Example of peptide matrix setup for Pol[a].

	Pool A	Pool B	Pool C	Pool D	Pool E
Pool F	Pol 1	Pol 2	Pol 3	Pol 4	Pol 5
Pool G	Pol 6	Pol 7	Pol 8	**Pol 9**	Pol 10
Pool H	Pol 11	Pol 12	Pol 13	Pol 14	Pol 15
Pool I	Pol 16	Pol 17	Pol 18	Pol 19	Pol 20

[a] Example, shown in bold : a positive response to pools D and G would reflect a positive responses in peptide Pol 9.

Table 3 Peptides matrix setup.

A Peptides matrix setup for Nef.

	MPN1	MPN2	MPN3	MPN4	MPN5	
MPN6	N1	N2	N3	N4	N5	
MPN7	N6	N7	N8	N9	N10	
MPN8	N11	N12	N13	N14	N15	
MPN9	N16	N17	N18	N19	N20	N21
	N21					

MPN, matrix pool Nef

B Peptides matrix setup for Gag.

	MPG1	MPG2	MPG3	MPG4	MPG5	MPG6	MPG7
MPG8	G1	G2	G3	G4	G5	G6	G7
MPG9	G8	G9	G10	G11	G12	G13	G14
MPG10	G15	G16	G17	G18	G19	G20	G21
MPG11	G22	G23	G24	G25	G26	G27	G28
MPG12	G29	G30	G31	G32	G33	G34	G35
MPG13	G36	G37	G38	G39	G40	G41	G42
MPG14	G43	G44	G45	G46	G47	G48	G49

MPG, matrix pool Gag

Table 4 Nef peptide sequences

Matrix	NO.	Amino acid sequences	Position	Matrix	NO.	Amino acid sequences	Position
MPN1	N1	MGGKWSKSSIVGWPQVRERI	1-20	MPN2	N2	VGWPQVRERIKQTPPATEGV	10-30
	N6	NNADCVWLRAQEEEEVGFPV	49-70		N7	AQEEEEVGFPVRPQVPLRPM	60-79
	N11	LDGLIYSKRRQEILDLWVY	97-115		N12	RQEILDLWVYNTQGFFPDW	106-124
	N16	KLVPVDPREVEEDNK	144-158		N17	DPREVEEDNKGENNCLLHPM	149-168
	N21	LARKHIARELHPEYYKDCA	189-207	MPN4	N4	GAVSQDLDKHGAVTSSNM	31-48
MPN3	N3	KQTPPATEGVGAVSQDLDKH	21-40		N9	TYKGAFDLSFFLKEKGGL	80-97
	N8	VRPQVPLRPMTYKGAFDLSF	70-89		N14	WQNYTPGPGIRYPLCFGWCF	124-143
	N13	YNTQGFFPDWQNYTPGPGIR	115-134		N19	SQHGIEDEEREVLMWKFDSA	169-188
	N18	GENNCLLHPMSQHGIEDEER	159-178	MPN6	N1	MGGKWSKSSIVGWPQVRERI	1-20
MPN5	N5	KHGAVTSSNMNNADCVWLRA	39-60		N2	VGWPQVRERIKQTPPATEGV	10-30
	N10	SFFLKEKGGLDGLIYSKRR	88-106		N3	KQTPPATEGVGAVSQDLDKH	21-40
	N15	RYPLCFGWCFKLVPVDPREV	134-153		N4	GAVSQDLDKHGAVTSSNM	31-48
	N20	EVLMWKFDSALARKHIAREL	179-198		N5	KHGAVTSSNMNNADCVWLRA	39-60
MPN7	N6	NNADCVWLRAQEEEEVGFPV	49-70	MPN8	N11	LDGLIYSKRRQEILDLWVY	97-115
	N7	AQEEEEVGFPVRPQVPLRPM	60-79		N12	RQEILDLWVYNTQGFFPDW	106-124
	N8	VRPQVPLRPMTYKGAFDLSF	70-89		N13	YNTQGFFPDWQNYTPGPGIR	115-134
	N9	TYKGAFDLSFFLKEKGGL	80-97		N14	WQNYTPGPGIRYPLCFGWCF	124-143
	N10	SFFLKEKGGLDGLIYSKRR	88-106		N15	RYPLCFGWCFKLVPVDPREV	134-153
MPN9	N16	KLVPVDPREVEEDNK	144-158				
	N17	DPREVEEDNKGENNCLLHPM	149-168				
	N18	GENNCLLHPMSQHGIEDEER	159-178				
	N19	SQHGIEDEEREVLMWKFDSA	169-188				
	N20	EVLMWKFDSALARKHIAREL	179-198				
	N21	LARKHIARELHPEYYKDCA	189-207				

MPN, matrix pool Nef; N, Nef peptide; No., peptide number

Table 5 Gag peptide sequences

Matrix	NO.	Amino acid sequences	Position	Matrix	NO.	Amino acid sequences	Position
MPG1	G1	MGARASVLSGGKLDAWEKIR	1-20	MPG2	G2	GKLDAWEKIRLRPGGKKKYR	11-30
	G8	GTEELRSLYNTVATLYCVHQ	71-90		G9	TVATLYCVHQRIEVKDTKEA	81-100
	G15	GQMIHQSLSPRTLNAWVKVIE	140-160		G16	TLNAWVKVIEEKALSPEVIP	151-170
	G22	EWDRLHPVHAGPVAPGQMRE	211-230		G23	GPVAPGQMREPRGSDIAGTT	221-240
	G29	SILDIKQGPKEPFRDYVDRF	281-300		G30	EPFRDYVDRFFKTLRAEQAT	291-310
	G36	CQGVGGPGHKARVLAEAMSQV	350-370		G37	RVLAEAMSQVQHTNIMMQR	361-379
	G43	HQMKDCTERQANFLGKIWPS	419-438		G44	ANFLGKIWPSSKGRPGNFPQ	429-448
MPG3	G3	LRPGGKKKYRLKHLVWASRE	21-40	MPG4	G4	LKHLVWASRELERFALNPSL	31-50
	G10	RIEVKDTKEALDKIEEIQKK	91-110		G11	LDKIEEIQKKSKQKTQQAAA	101-120
	G17	EKALSPEVIPMFSALSEGAT	161-180		G18	MFSALSEGATPQDLNMMLNI	171-190
	G24	PRGSDIAGTTSTPQEQIAWM	231-250		G25	STPQEQIAWMTGNPPIPVGD	241-260
	G31	FKTLRAEQATQEVKGWMTET	301-320		G32	QEVKGWMTETLLIQNANPDC	311-330
	G38	VQHTNIMMQRGNFKGQKRI	370-388		G39	GNFKGQKRIKCFNCGKEGHL	380-390
	G45	SKGRPGNFPQSRPEPTAP	439-456		G46	SRPEPTAPPAAEIFGMREE	449-467
MPG5	G5	LERFALNPSLLETTEGCQQI	41-60	MPG6	G6	LETTEGCQQIMEQLQSALRT	51-70
	G12	SKQKTQQAAADTGSSSKVSQ	111-130		G13	DTGSSSKVSQNYPIVQNAQG	121-140
	G19	PQDLNMMLNIVGGHQAAMQM	181-200		G20	VGGHQAAMQMLKDTINEEAA	191-210
	G26	TGNPPIPVGDIYKRWMILGL	251-270		G27	IYKRWMILGLNKIVRMYSPV	261-280
	G33	LLIQNANPDCKSILRALGAG	321-340		G34	CKSILRALGAGATLEEMMTA	331-349
	G40	KCFNCGKEGHLAKNCRAPRKK	389-408		G41	LAKNCRAPRKKGCWKCGREG	399-418
	G47	PAAEIFGMREEIVSPPKQEQN	457-477		G48	IVSPPKQEQNDRDQNPPSVSL	468-488
MPG7	G7	MEQLQSALRTGTEELRSLYN	61-80	MPG8	G1	MGARASVLSGGKLDAWEKIR	1-20
	G14	NYPIVQNAQGQMIHQSLSPR	131-150		G2	GKLDAWEKIRLRPGGKKKYR	11-30
	G21	LKDTINEEAAEWDRLHPVHA	201-220		G3	LRPGGKKKYRLKHLVWASRE	21-40
	G28	NKIVRMYSPVSILDIKQGPK	271-290		G4	LKHLVWASRELERFALNPSL	31-50
	G35	ATLEEMMTACQGVGGPGHKA	341-360		G5	LERFALNPSLLETTEGCQQI	41-60
	G42	KGCWKCGREGHQMKDCTERQ	409-428		G6	LETTEGCQQIMEQLQSALRT	51-70
	G49	DRDQNPPSVSLKSLFGNDLLSQ	478-499		G7	MEQLQSALRTGTEELRSLYN	61-80

MPG, matrix pool Gag; G, Gag peptide; No., peptide number

Table 5 Gag peptide sequences (continued).

Matrix	NO.	Amino acid sequences	Position	Matrix	NO.	Amino acid sequences	Position
MPG9	G8	GTEELRSLYNTVATLYCVHQ	71-90	MPG10	G15	GQMIHQSLSPRTLNAWVKVIE	140-160
	G9	TVATLYCVHQRIEVKDTKEA	81-100		G16	TLNAWVKVIEEKALSPEVIP	151-170
	G10	RIEVKDTKEALDKIEEIQKK	91-110		G17	EKALSPEVIPMFSALSEGAT	161-180
	G11	LDKIEEIQKKSKQKTQQAAA	101-120		G18	MFSALSEGATPQDLNMMLNI	171-190
	G12	SKQKTQQAAADTGSSSKVSQ	111-130		G19	PQDLNMMLNIVGGHQAAMQM	181-200
	G13	DTGSSSKVSQNYPIVQNAQG	121-140		G20	VGGHQAAMQMLKDTINEEAA	191-210
	G14	NYPIVQNAQGQMIHQSLSPR	131-150		G21	LKDTINEEAAEWDRLHPVHA	201-220
MPG11	G22	EWDRLHPVHAGPVAPGQMRE	211-230	MPG12	G29	SILDIKQGPKEPFRDYVDRF	281-300
	G23	GPVAPGQMREPRGSDIAGTT	221-240		G30	EPFRDYVDRFFKTLRAEQAT	291-310
	G24	PRGSDIAGTTSTPQEQIAWM	231-250		G31	FKTLRAEQATQEVKGWMTET	301-320
	G25	STPQEQIAWMTGNPPIPVGD	241-260		G32	QEVKGWMTETLLIQNANPDC	311-330
	G26	TGNPPIPVGDIYKRWMILGL	251-270		G33	LLIQNANPDCKSILRALGAG	321-340
	G27	IYKRWMILGLNKIVRMYSPV	261-280		G34	CKSILRALGAGATLEEMMTA	331-349
	G28	NKIVRMYSPVSILDIKQGPK	271-290		G35	ATLEEMMTACQGVGGPGHKA	341-360
MPG13	G36	CQGVGGPGHKARVLAEAMSQV	350-370	MPG14	G43	HQMKDCTERQANFLGKIWPS	419-438
	G37	RVLAEAMSQVQHTNIMMQR	361-379		G44	ANFLGKIWPSSKGRPGNFPQ	429-448
	G38	VQHTNIMMQRGNFKGQKRI	370-388		G45	SKGRPGNFPQSRPEPTAP	439-456
	G39	GNFKGQKRIKCFNCGKEGHL	380-390		G46	SRPEPTAPPAAEIFGMREE	449-467
	G40	KCFNCGKEGHLAKNCRAPRKK	389-408		G47	PAAEIFGMREEIVSPPKQEQN	457-477
	G41	LAKNCRAPRKKGCWKCGREG	399-418		G48	IVSPPKQEQNDRDQNPPSVSL	468-488
	G42	KGCWKCGREGHQMKDCTERQ	409-428		G49	DRDQNPPSVSLKSLFGNDLLSQ	478-499

MPG, matrix pool Gag; G, Gag peptide; No., peptide number

Part II Study of $CD8^+$ T lymphocyte responses

1. Peptide-based IFN-γ ELISpot assay

A 96-well nitrocellulose bottom plates (Millititer, Millipore, Bedford, MA) were precoated with 50 µl/well of 10 µg/ml anti-IFN-γ mAb, 1-DIK (Mabtech, Stockholm, Sweden) for 3 hours at 37 °C, 5% CO_2.

The antibody-coated plates were washed six times with phosphate-buffered saline (PBS, Sigma) and blocked with 200 µl/well of RPMI medium (Gibco, Life Technologies, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, Md.) (R10) over 1 hour at room temperature.

The plates were washed six times with PBS , and PBMC were then transferred along with 10 µg/ml HIV peptides (10 µl each) into the precoated ELISpot plates at a final concentration of $2.5x10^5$ cells per well. Wells containing 25,000 PBMC per well with 2 µl of 1 µg/ml phytohemagglutinin (PHA) served as positive controls while wells containing PBMC at a final concentration of $2.5x10^5$ cells per well with medium alone served as negative controls. All tests were run in duplicate.

After 15 hours incubation at 37 °C, 5% CO_2, cells were removed from the plates by washing six times with PBS containing 0.05% Tween 20 (Sigma, Deisenhofen, Germany) (PBS/0.05% Tween) and the last time with PBS. After washing, the second, biotinylated anti-IFN-γ mAb, 7-B6-1 biotin (Mabtech, Stockholm, Sweden) was added at 1 µg/ml in 50 µl/well for 3 hours at room temperature.

The plates were washed six times with PBS/0.05% Tween and the last time with PBS, followed by 1 hour of incubation with streptavidin-conjugated alkaline phosphatase (Mabtech, Stockholm, Sweden) at 1 µg/ml in 50 µl/well at room temperature.

After washing step, alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA) was added at 1 µg/ml in 50 µl/well for 10-15 minutes at room temperature. The wells were washed with tap water to terminate the colorimetric reaction and plates were then air-dried.

After drying at room temperature the colour spots were counted with a dissecting microscope. HIV-1-specific IFN-γ responses were reported as number of spot-forming unit (SFU) per 10^6 PBMC, after substraction of background IFN-γ secretion (negative control values). Responses were regarded as positive if the spots were more than 20 spots

per well (80 spots per million PBMC) and had at least 2.5 times the number of SFU in the negative control wells.

2. $CD8^+$ T lymphocyte depletion assay

In order to confirm that the responses detected in study were $CD8^+$ T cell mediated, the $CD8^+$ T lymphocyte depletion experiment were performed. Briefly, 10 million PBMC in 1 ml were incubated with 72 µl of anti-$CD8^+$ Ab-coated-immunomagnetic beads (Dynabead HLA cell prep I; Dynal Inc., Lack Success, New York, USA) at 4 °C for 20 minutes, mixed during incubation every 5 minutes. Cells were washed twice with 5 ml of RPMI at 1,500 rpm for 5 min and then resuspened with R10 before testing by ELISpot assay. Significant reduction of an ELISpot response was defined as 50% or greater reduction in HIV-1-specific IFN-γ release after $CD8^+$ T lymphocyte depletion.

3. Cultured ELISpot assay

In some experiments, we stimulated HIV-specific $CD8^+$ T cell responses by co-cuturing cryopreserved $10x10^6$ PBMC with 200 µl of HIV-Nef or Gag pooled overlapping peptides (2 µg/peptide) (Mimotope, Australia) and 12 µl of IL-7 (330 U/ml) (R&B, UK.) and plated out into 24-wells plate (IWAKI, Japan) with R10 at 37 °C in 5% CO_2 for 3 days. On day 3 and day 7, IL-2 was added at the final concentration of 100 U/ml (R&B, UK.)). On day 9, the CTL line was washed three times and left them to rest overnight at 37 °C in 5% CO_2 in R10. On day 10, the CTL line was washed and resuspended in 2 ml of R10. The CTL line was counted and transfered along with 10 µg/ml overlapping peptides (10 µl each) into ELISpot plates at a final concentration of 25,000 cells per well for peptide-based IFN-γ ELISpot assay.

Part III HIV DNA preparation

1. PHA blast

PBMC were stimulated with 10 µl of 1 µg/ml PHA at 37 °C, 5% CO_2 overnight in duplicate wells of a 24-wells Coastar plate at a final concentration of $2.5x10^6$ cells per well.

2. Dried packed cells preparation

PHA-stimulated cells were harvested into cryotube and then centrifuged at 1300 rpm for 1 minute. Supernatant was discarded and then dried pack cells were stored at -70 °C.

3. HIV DNA extraction

HIV DNA was extracted from the PHA-stimulated cells by QIAamp®DNA Mini Kit. The procedure of DNA extraction was followed the recommendation of the company. Dried packed cells was added with 40 µl proteinase K, 400 µl buffer AL and mixed by pulse-vortexing for 15 seconds. After lysis for 10 minutes at 56 °C to reach DNA yield, DNA was precipitated by 400 µl absolute ethanol and mixed again by pulse-vortexing for 15 seconds. The tube was spun at 8,000 rpm for 1 minute to remove drops from the inside of the lid. The mixture was transferred to QIAamp spin column and centrifuged at 8,000 rpm for 1 minute. QIAamp spin column was then washed with 500 µl buffer AW1 and 500 µl buffer AW2 at 8,000 rpm for 1 minute and 3 minutes, respectively. Finally, eluate with 20 µl TE buffer as performed at 8,000 rpm for 1 minute once and another elution was done after incubating at room temperature for 5 minutes. The eluate containing HIV DNA was stored at -20 °C until used.

4. Quantitation the amount of extracted DNA

The amount of extracted HIV DNA was determined using an ultraviolet spectrophotometer (SmartSpec™ 3000, Bio-Rad, U.S.A.). Concentration of DNA in the eluate was measured by absorbance at 260 nm. An OD of 1 corresponds to approximately 50 µg/ml for dsDNA. The ratio of absorbance at 260 nm to absorbance at 280 nm (A_{260}/A_{280}) between 1.7 and 1.9 indicates purity of the nucleic acid. The lower ratio, on the other hand, reflects contamination.

Part IV Producing PCR product

1. Nef gene amplification

A 703-bp-*nef* was amplified from extracted HIV DNA using the primers previously described by S. Reungdehsuwan, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.: Outer primer 5'GGTGGAACTTCTGGGACACAGC and 5'GGAGTTGGGGAGTAGCTAACCC and Inner primer 5'CCTAGAAGAATCAGACAGGGCTTAG and 5'GCTGGGGACTTTCCAGGGGA. Each DNA template was amplified for 30 cycles in separate reactions. The first round of amplification were performed in a total volume of 25 µl using 200 ng DNA extract and the second rounds of amplification were performed in a total volume of 50 µl using 2 µl of first PCR product. PCR reaction mixture composed of 10X Taq Buffer with $(NH_4)_2SO_4$ (750 mM Tris-HCL pH8.8 at 25 °C, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 1.5 mM $MgCl_2$, 200 µM deoxynucleosidetriphosphate (dNTP), 1.5 unit of Taq polymerase and 10 µM of each primers. The first and the second PCR cycling conditions were as follows: an initial denaturation step at 92 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 1.30 minutes, and a final extension of 5 minutes at 72 °C. The amplified product was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for 30 minutes. The positive PCR sample was used as positive control and distilled water was used as the negative control.

2. Gag gene amplification

Similarly, extracted HIV DNA was used as the template for nested PCR to amplify a 1,702-bp-*gag* using the primers previously described by Associate Professor Dr. Arunee Thitithanyanont, Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand: Outer primer 5'TCTCGACGCAGGACTCGGCTTGCT and 5'CCTCCAATTCCCCCTATCATTTTTGG and Inner primer 5'GCTGAGGTACACACAGCAATA GGC and 5' CCTCCAATTCCCCCTATCATTTTTGG. Each DNA template was amplified 30 cycles in separate reactions. The first round of amplification were performed in a total volume of 25 µl using 200 ng DNA extract and the second rounds of amplification were performed in a total volume of 50 µl using 2 µl of first PCR product. PCR reaction mixture composed of 10X Taq Buffer with $(NH_4)_2SO_4$ (750 mM Tris-HCL pH8.8 at 25

°C, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 3 mM $MgCl_2$, 200 μM deoxynucleosidetriphosphate (dNTP), 1.5 unit of Taq polymerase and 10 μM of each primers. The first PCR cycling conditions were as follows: an initial denaturation step at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72°C for 1.30 minutes, and a final extension of 7 minutes at 72 °C. The second PCR cycling conditions were as follows: an initial denaturation step at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 1 minute, and a final extension of 7 minutes at 72 °C. The amplified product was electrophoresed on a 0.9% agarose gel and stained with ethidium bromide for 30 minutes. The positive PCR sample was used as positive control and distilled water was used as the negative control.

Part V HIV DNA cloning

1. PCR Purification

PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Germany), according to the manufacturer's instructions. PCR products were added with 200 µl of buffer PB. The mixtures were gently mixed and applied to the QIAquick column and then centrifuged at 13,000 rpm for 1 minute. DNA was washed twice with 350 µl buffer PE and centrifuged at 13,000 rpm for 1 minute. The QIAquick column was placed to 1.5 microcentrifuge tube. Finally, DNA was eluted by incubating with 20 µl buffer EB for 3 minutes and centrifuged at 13,000 for 1 minute. The eluted DNA was stored at -20 °C until used.

2. Competent cells Preparation

Escherichia coli strain DH5α was prepared as competent cells. *E. coli* was kindly provided by Assistant Professor Dr. Chintana Chirathaworn, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. A single colony from a plate of freshly grown culture was transferred into 5 ml of LB broth and incubated at 37 °C overnight. To expand the cell culture, 500 µl of the cell suspension was added into 50 ml of LB broth. After shaking at 225 rpm at 37 °C until the cell turbidity was at an O.D. of 0.4-0.5 at the wavelength 600 nm., the cells were harvested by centrifugation at 5,000 rpm, 4 °C for 8 minutes. The cells were washed twice with 10 ml of 15% glycerol in 50 mM $CaCl_2$ (Ca/Glycerol solution) by placed on ice for 2 hours at the first time. The cells were harvested by centrifugation at 5,000 rpm, 4 °C for 8 minutes. The supernatant was discarded and cold Ca/Glycerol solution was added. The suspension was aliquoted in 100 µl in microcentrifuge tubes and stored at -70 °C until used.

3. Cloning Nef gene into pCR®2.1

Fresh purified PCR products were cloned into vector using the TA system (Invitrogen, USA), according to the manufacturer's instructions. The kit was composed of pCR®2.1 vector and T4 DNA ligase enzyme. pCR®2.1 is linearized vector containing ampicillin resistance gene and 3'-thymidine (T) overhang within the LacZα fragment. As the knowledge, Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of their PCR products. This allows

A-tail of target PCR products complementary bound to T-tail of vector. The ligation reactions composed of 2-3 µl of PCR product (concentration of PCR products varies by length of DNA and gave the proper ratio of 1:1 (vertor:insert)), 10X ligation buffer, 50 ng of pCR®2.1 vector, 4.0 Weiss units of T4 DNA ligase, and sterile distilled water to top up to the final volume of 10 µl. The mixtures were incubated at 14 °C for 16 hours and then transformed into the competent cells or stored at -20 °C until used.

4. Transforming Nef gene into competent cells

Vials containing the ligation products were briefly centrifuged and placed on ice. The competent cells were thawed on ice and 4 µl of ligation product was added. The transformation mixture was mixed gently by stirring with the pipette tip and incubated on ice for 30 minutes. After that, the cells were heat shocked for 30 seconds at 42 °C and immediately placed the vials to ice. The vials were horizontally shaked at 37 °C for 1 hour at 225 rpm after adding 250 µl of room temperature S.O.C. medium. Finally, each transformation vial was spread on LB agar plates containing 100 µg/ml ampicillin which were coated with 100 µl of 100 mM IPTG (isopropyl-b-D-thiogalactopyranoside) and 20 µl of 100 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Fisher Scientific, USA). As the knowledge, LacZ was encoded for β-galactosidase that metabolized colourless substrate, X-gal, into galactose and a blue insoluble product. This reaction was activated by IPTG which was an inducer of lac operon. Then five transformed bacteria, had vector containing Nef gene indicating by white colony, were selected and transferred into LB broth and shaked for 16-24 hours at 37 °C. The plasmid extraction was performed for DNA sequencing. The remaining transformed bacteria were frozen down in aliquot with 20% glycerol at -70 °C until used.

5. Cloning Gag gene into pDrive Cloning Vector

Fresh purified PCR products were cloned into vector using the TA system (QIAgen, Germany), according to the manufacturer's instructions. The kit was composed of pDrive Cloning Vector and ligation master mix (2X) which contains all reagents and cofactors required for ligation in a convenient pre-mixed format. pDrive Cloning Vector is linearized vector containing ampicillin resistance gene and U overhangs. As the knowledge, Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of their PCR products. This allows

A-tail of target PCR products hybridize to the U overhang of pDrive Cloning Vector. The ligation reactions composed of 2-3 µl of PCR product (concentration of PCR products varies by length of DNA and gave the proper ratio of 1:1 (vertor:insert)), 2X Ligation Master, 50 ng of pDrive Clonong Vector, and sterile distilled water to top up to the final volume of 10 µl. The mixtures were incubated at 4-16 °C for 1-2 hours and then transformed into the competent cells or stored at -20 °C until used.

6. **Transforming Gag gene into competent cells**

Vials containing the ligation products were briefly centrifuged and placed on ice. The competent cells were thawed on ice and 5 µl of ligation product was added. The transformation mixture was mixed gently by flicking a few times and incubated on ice for 10 minutes. After that, the cells were heat shocked for 30 seconds at 42 °C and immediately placed the vials to ice. The vials were horizontally shaked at 37 °C for 1 hour at 225 rpm after adding 250 µl of room temperature S.O.C. medium. Finally, each transformation vial was spread on LB agar plates containing 100 µg/ml ampicillin which were coated with 100 µl of 100 mM IPTG (isopropyl-b-D-thiogalactopyranoside) and 20 µl of 100 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Fisher Scientific, USA). As the knowledge, LacZ was encoded for β-galactosidase that metabolized colourless substrate, X-gal, into galactose and a blue insoluble product. This reaction was activated by IPTG which was an inducer of lac operon. Then five transformed bacteria, had vector containing Gag gene indicating by white colony, were selected and transferred into LB broth and shaked for 16-24 hours at 37 °C. The plasmid extraction was performed for DNA sequencing. The remaining transformed bacteria were frozen down in aliquot with 20% glycerol at -70 °C until used.

7. Plasmid extraction

Plasmids of transformed bacteria were extracted using QIAprep® Miniprep Kit (QIAgen, Germany), according to the manufacturer's instructions. The suspended cells were centrifuged at 4,000 rpm for 10 minutes. The pellet was resuspended with digestion buffer (250 µl of buffer P1, 250 µl of buffer P2 and 350 µl of buffer N3), mixed gently by inverting the tube, and centrifuged at 13,000 for 1 minute. The supernatant was transferred into the QIAprep spin column and centrifuged at 13,000 for 1 minute. The trace nuclease activity was removed by washing with 500 µl buffer PB and then washed

twice with 350 buffer PE. Finally, DNA was eluted by incubating with 25 µl buffer EB for 5 minutes and centrifuged at 13,000 for 1 minute. The eluted DNA was stored at -20 °C until used for sequencing.

Part VI HIV DNA sequencing

The eluted DNA was sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), according to the manufacturer's instructions. Briefly, the reactions carried out with a total volume of 10 µl containing DNA template (concentration of eluted DNA varies by length of DNA : 150-300 ng Nef DNA or 200-400 ng Gag DNA), 10 µM Nef inner primer or 10 µM Gag inner primer, 1 µl of buffer and 3 µl of BigDye. The sequencing cycling conditions were performed for 25 cycles of an initial denaturation step at 96 °C for 30 seconds, followed by annealing at 55 °C for 10 minutes and final extension at 60 °C for 4 minutes. The extension product was precipitated with 1 µl of 0.25 M EDTA and 30 µl of absolute ethanol. Then, centrifugation at 14,000 rpm for 30 minutes after incubating at 4 °C for 30 minutes was done. The supernatant was removed from the microcentrifuge tube and the DNA was washed by 60 µl of 70% ethanol for 10 minutes at 14,000 rpm. Finally, the supernatant was discarded and dried at 95 °C for 2 minutes. The amplified product was sequenced using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, U.S.A.). DNA sequence was analysed by using the software Chromas 1.45, ClustalX, Genedoc and Genetyx.