CHAPTER 3

Materials and Methods

3.1 Study population

This study was to determine the molecular epidemiology of HIV-1 subtypes and the genetic characterization of inter-subtype recombination of HIV-1 infected patients in Northern Thailand that composed of Thai citizens and non-Thai citizens. While Thai population used was divided into HIV-1 treatment-experienced patients and HIV-1 infected naïve–treatment or newly diagnosed HIV-1 patients, non-Thai citizens were HIV-1 treatment-experienced patients. All samples were taken from routine and could not be traced back to patients, including three groups of HIV-1 infected patients.

In the first group sampled, the total population was 2,400 treatment-experienced HIV-1 infected patients who participated in the National AIDS Program (NAP) by National Health Security Office of Thailand (NHSO). This program established a treatment program for all Thai citizens. All patients were from 90 hospitals in 16 provinces including Chiang Rai, Nan, Payao, Chiang Mai, MaeHongSon, Phrae, Lampang, Lamphun, Uttaradit, Phitsanulok, Sukhothai, Phetchabun, Phichit, NakhonSawan, UthaiThani and Tak. The regions of Thailand were based on the Bureau of policy and strategy, Ministry of Public Health that are shown in Figure 3.1.

Figure 3.1 The regions of Thailand were based on the Bureau of policy and strategy, Ministry of Public Health [153].

In 2009 to 2012, all samples with a viral load ranging from 2,000 to 750,000 copies/mL were routinely tested for HIV drug resistance at the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Numbers of HIV-1 infected patients are shown in Table 3.1. In routinely HIV drug resistances testing, plasma samples were *gag-pol* genotyped by ViroSeq® HIV-1 Genotyping Assay version 2.0 (Abbott Molecular, USA) following the manufacturer's instructions. The ViroSeq kit contained all the necessary reagents for RNA extraction, reverse transcription, PCR amplification, PCR product purification, and sequencing reactions. RNA was extracted and reverse transcribed to cDNA that was a template to amplify the whole HIV-1 protease coding region (codons 1-99) and codons 1-335 of the reverse transcriptase, which about two thirds of that gene

was in the *pol* open reading frame. Then, 1.8 kb of the final PCR product was made into a template of sequencing that generated approximately 1.2 kb of sequence data by using seven sequencing primers (A, B, C, D, F, G, and H). The amplification and sequencing plot of the ViroSeq HIV-1 genotyping assay version 2.0 are shown in Figure 3.2.

Table 3.1 Number of the first treatment-experienced HIV-1 infected patients who participated in the NAP by NHSO that register with hospitals throughout Northern Thailand. The data was clarified by province and the year of sample collection.

Number	Province	Number of HIV-1 infected patients				Total
		2009	2010	2011	2012	
1	Chiang Rai	82	111	151	120	464
2	Nan	7	1	-	-	8
3	Payao	6	10	15	9	40
4	Chiang Mai	133	219	262	298	912
5	MaeHongSon	7	14	33	40	94
6	Phrae	13	21	19	21	74
7	Lampang	4	2	-	-	6
8	Lamphun	50	29	31	35	145
9	Uttaradit	-	39	36	33	108
10	Phitsanulok	36	73	107	70	286
11	Sukhothai	-	8	4	2	14
12	Phetchabun	38	18	20	24	100
13	Phichit	7	6	37	23	73
14	NakhonSawan	-	-	6	-	6
15	UthaiThani	-	-	2	-	2
16	Tak	-	9	9	11	29
17	NA	21	17	1		39
	Total	404	**577**	**733**	**686**	**2,400**

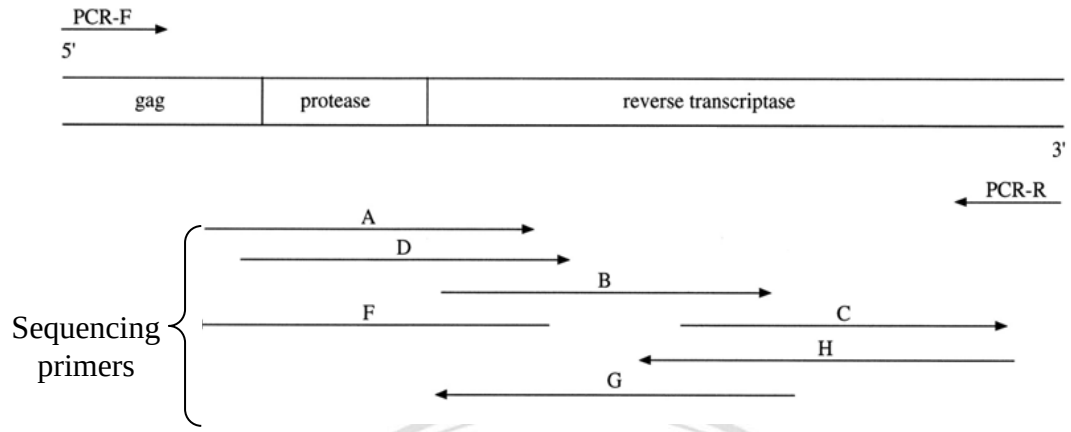

Figure 3.2 Diagram represents the amplification and sequencing strategies of the ViroSeq HIV-1 genotyping assay version 2.0. The positions and directions of the seven sequencing primers (A, B, C, D, F, G, and H) are shown within the protease and the reverse transcriptase regions.

In the second group, the study population was treatment-experienced HIV-1 infected patients who participated in the National Access to Antiretroviral Program for People living with HIV/AIDS or NAPHA EXTENSION program by Global Fund AIDS Care. This program established a treatment program for noncitizen or people without a Thai ID which included migrants, minorities, migrant workers and homeless people. All 50 patients were from 26 hospitals in 5 provinces including Chiang Rai, Tak, MaeHongSon, Lamphun, and Chiang Mai that throughout Northern Thailand. All patients were routinely HIV drug resistance tested at Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University from 2010 to 2013. All samples were kindly provided by Asst. Prof. Sakchai Dettrairat. Numbers of HIV-1 infected patients are shown in Table 3.2. In routine HIV drug resistance testing, plasma samples with a viral load of ≥1,000 copies/ml of RNA were extracted by QIAamp viral extraction kit (Qiagen Inc., USA), and were *gag-pol* genotyped by TRUGENE® HIV-1 Genotyping Assay (Siemens Healthcare, Germany) following the manufacturer's instructions. Afterwards, viral RNA was extracted from the plasma, reverse transcribed to cDNA and amplified in a single-tube RT-PCR reaction. The amplified product was sequenced with a modified Sanger technique using fluorescent dye-labeled primers that generated four coupled fragments. Primers used in DNA sequencing reactions for the protease and reverse

transcriptase gene regions are shown in Figure 3.3. The Trugene assay sequences the protease gene from codons 4 to 99 and the RT gene from codons 38 to 248, which is about 1.3 kb of sequence data.

Table 3.2 Number of the second population, treatment-experienced HIV-1 infected patients who participated in the NAPHA EXTENSION program

Number	Province	Number of HIV-1 infected patients				Total
		2010	2011	2012	2013	
1	Chiang Rai	1	11	9	1	22
2	Chiang Mai		9	9		18
3	MaeHongSon		1	4		5
4	Lamphun			1		1
5	Tak			4		4
	Total	1	21	27	1	50

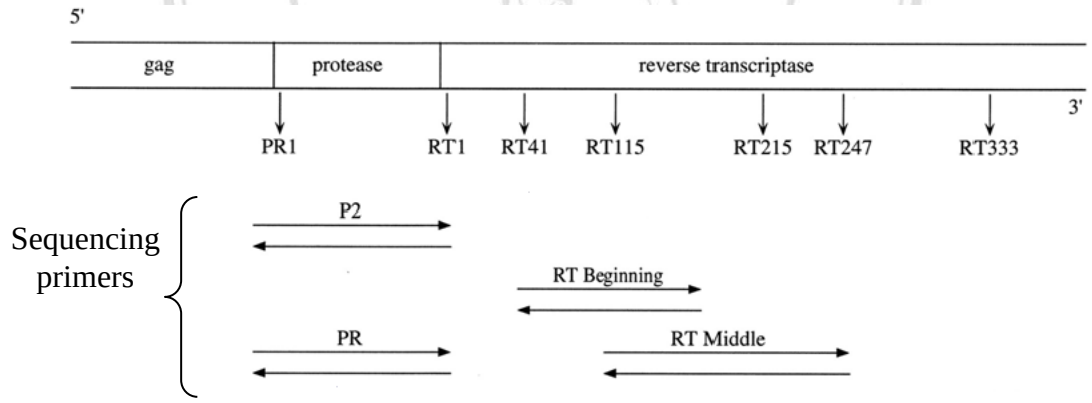

Figure 3.3 Diagram displays the amplification and sequencing strategies of the TRUGENE® HIV-1 Genotyping Assay, and the diagram of primers used in DNA sequencing reactions for the protease and the reverse transcriptase regions.

In the third group, the study population was naïve-treatment HIV-1 infected patients or newly diagnosed HIV patients. All patients were blood donors that donated their blood at the 10th Regional Blood Center, Thai Red Cross Society, Chiang Mai and 85 branch hospitals in 6 provinces throughout Northern Thailand including 92 patients from Chiang Rai, Chiang Mai, Lamphun, Lampang, Phrae and MaeHongSon from 2012

to 2013. All patients had seropositive as diagnosed for HIV antigen and antibodies by ARCHITECT HIV Ag/Ab Combo assay (Abbott Laboratories, USA) and Determine™ HIV-1/2 (Abbott Laboratories, USA) that followed by guideline of the National Blood Center, Thai Red Cross Society. All samples were approved by the director of the National Blood Center, Thai Red Cross Society.

3.1.1 Sample collection and storage

All samples remained anonymous and could not be traced back to patients. This study was approved to be a project exempt from the provisions number 005EXP/56 by the ethics committee from the Faculty of Associated Medical Sciences, Chiang Mai University. All samples required a minimum volume of 500 mL, stored at -70 ◦C and newly labeled by researcher.

3.1.2 Research design

The methods to determine HIV-1 subtype and genetic characterization of inter-subtype recombination that were selected for this study were V3 serotyping by subtype-specific enzyme immunoassay (SSEIA) and HIV-1 genotyping by REGA HIV-1 Subtyping Tool version 2.0 http://dbpartners.stanford.edu/RegaSubtyping/, Recombinant Identification Program: RIP http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html and Phylogenetic trees by Neighbour-joining method (MEGA version 5.0 programs) [154] .

All samples were examined for HIV-1 serotype by SSEIA that detect antibodies binding to the V3 gp120 region of HIV-1 subtypes B and E (CRF01_AE). The *gag-pol* sequence data that was collected from routinely HIV drug resistance testing examined HIV-1 genotyping. The results of HIV-1 V3 serotypes and *gag-pol* genotypes were compared to each other. In cordant results or similar results between HIV-1 V3 serotypes and *gag-pol* genotypes, HIV-1 subtypes could be concluded. In discordant results or non-similar results, RNA would be reverse transcribed and amplified *env* gp120 C2-V3-C3 region about 430 base pairs. The amplified products were sequenced and analyzed to determine HIV-1 genotype. All steps of this study are shown in Figure 3.4.

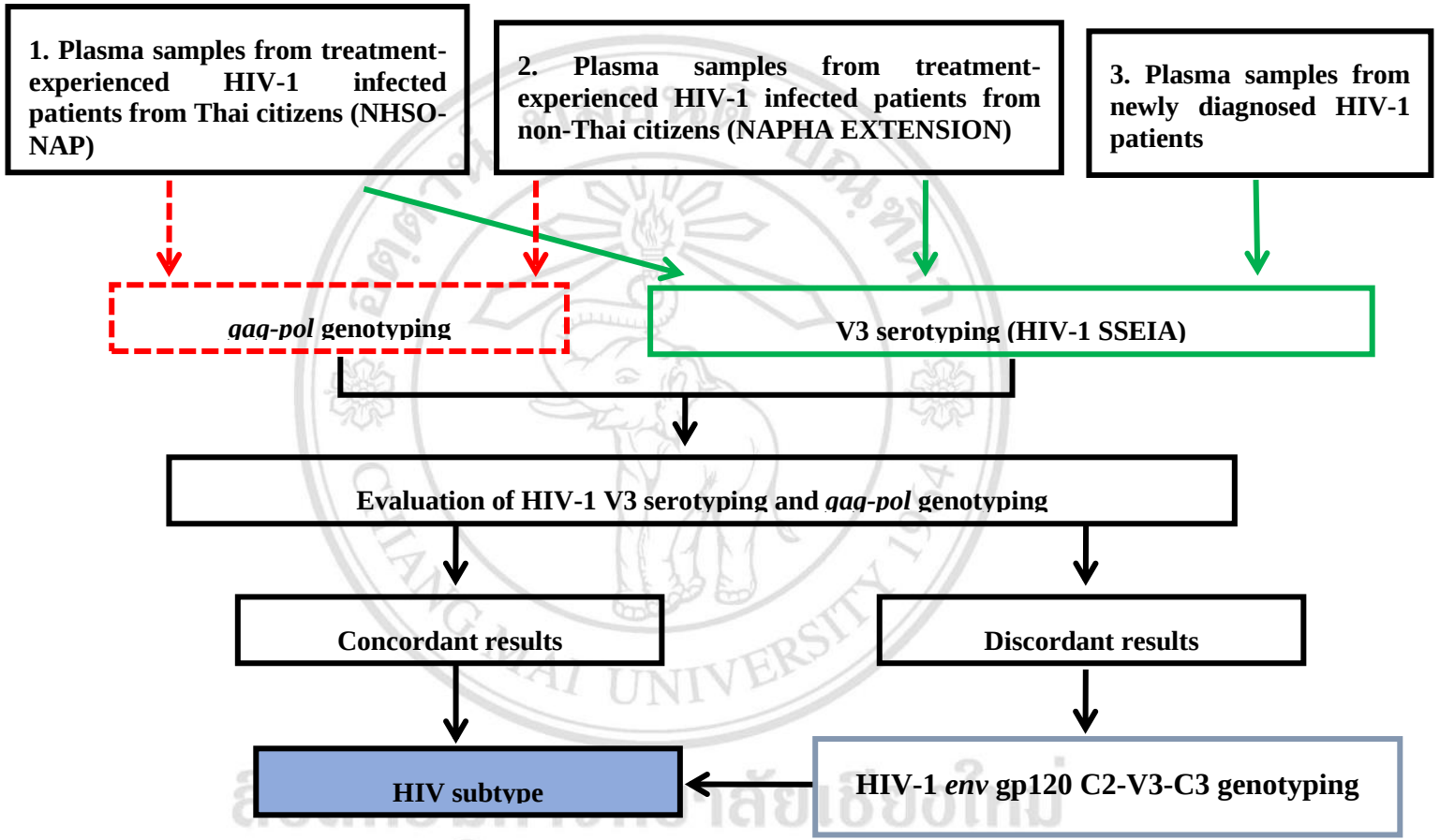

Figure 3.4 The diagram presents the research design of this study

3.2 Materials and methods

3.2.1 V3 serotyping by subtype-specific enzyme immunoassay (SSEIA) [150]

SSEIA is a competitive indirect ELISA that detect specific antibodies based on V3 gp120 region that use the short synthetic peptide corresponding to consensus sequences in the V3 region of HIV-1/2 subtypes. The short synthetic peptides that were used as an antigen source in SSEIA include peptides 30 and 28 amino acids. This study used synthetic peptide including subtype B and E (CRF01_AE). Sequences of the synthetic peptides are shown in Table 3.3.

Table 3.3 Sequences of the synthetic peptides that using in HIV-1 SSEIA composes of peptides 28 and 30 amino acids of the subtype B and CRF01_AE.

Length of peptides	Subtype	Sequences
30 amino acids	B	N N T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C
	E	N N T R T S I T I G P G Q V F Y R T G D I I G D I R Q A H C
28 amino acids	B	T R K S I H I G P G R A F Y T T G E I I G D I R Q A H C
	E	T R T S I T I G P G Q V F Y R T G D I I G D I R Q A H C

3.2.1.1 Microplate coating

The coating solution (mixture of peptide B+E 30 amino acid, 2 µg/mL) was added to the microplate at 100 µL/well. After incubation at 37˚C overnight, the microplate was washed 3 times with washing buffer (PBS 1X. 0.5% tween 20) that added 200 µL/well. Then 200 µL surcoating buffers (PBS 1X. 0.5% tween 20. 2%FCS) were added in each well. After incubation at 37˚C for 1 hour, the microplate was washed 3 times. Finally, the microplate dried at room temperature for 30 minutes and wrapped with aluminum foil, and stored at -20˚C up to 1 year**.**

3.2.1.2 Subtype-specific enzyme immunoassay

The coating microplate was allowed to come to room temperature for 30 minutes, and labeled sample ID. The samples, negative control (NC), positive control

subtype B (PC-B) and positive control subtype E (PC-E) were diluted with dilution buffer for titer 1:100, using 10 µL of sample and 990 µL of dilution buffer (PBS 1X FCS with tween 20). A 10 µL of blocking solution B (peptide B 28 amino acid, 100 µg/mL) was added in each well of lanes A and E. A 10 µL of blocking solution E (peptide E 28 amino acid, 100 µg/mL) was added in each well of lanes B and F. A 10 µL of blocking solution B+E (peptide B+E 28 amino acid, 100 µg/mL) was added in each well of lanes C and G. A 10 µL of dilution buffer was added in each well of lanes D and H. Then 100 µL of diluted negative control (NC) was added in each well A1-D1. 100 µL of diluted positive control subtype B (PC-B) was added in each well A2-D2. 100 µL of diluted positive control subtype E (PC-E) was added in each well A3-D3. 100 µL of diluted samples were added to 4 wells (well A4-D4 for the first sample, well A5-D5 for the second sample…). The example of locations in the microplate that including negative control, positive control subtype B, positive control subtype E and sample are shown in Table 3.4. After incubation 30 minutes at room temperature (20-22˚C), the microplate was washed 5 times. Then 100 µL of Peroxidase-conjugated goat F (ab') 2 anti-human Ig solution (5 µL of conjugate and 25 mL of buffer solution) was added in each well and incubated 30 minutes at room temperature. About 5 minutes before the end of incubation, substrate (Hydrogen peroxide-o-phenylenediamine, H_2O_2-OPD) was prepared by dissolving 1 tablet OPD and 1 tablet buffer urea H_2O_2 in 20 mL of water (kept in darkness until adding). After the microplate was washed 5 times, 100 µL of substrate was added into each well and incubated for 15 minutes at room temperature in darkness. Finally, 50 µL of 2N H_2SO_4 was added in each well to stop the reaction and the microplate read the OD value at 492 nm.

Table 3.4 The example of locations in the microplate that including negative control, positive control subtype B, positive control subtype E and sample

	1	2	3	4
A	blocking solution B + diluted NC	blocking solution B + diluted PC-B	blocking solution B + diluted PC-E	blocking solution B + diluted sample
B	blocking solution E + diluted NC	blocking solution E + diluted PC-B	blocking solution E + diluted PC-E	blocking solution E + diluted sample
C	blocking solution B+E + diluted NC	blocking solution B+E + diluted PC-B	blocking solution B+E + diluted PC-E	blocking solution B+E + diluted sample
D	dilution buffer + diluted NC	dilution buffer + diluted PC-B	dilution buffer + diluted PC-E	dilution buffer + diluted sample

3.2.1.3 Evaluation of SSEIA

The results of each sample could only be evaluated if they followed the validation. This included the OD of well that without blocking (well D) had to be more than 0.2, the difference between the OD of well D and the OD of well is the presence of 2 peptides (well C) that had to be more than 0.15, and four wells of negative control (well A1 to D1) should be clear and colorless.

The inhibition percentage induced by each peptide (B or E) for every sample is calculated by the formula:

$$\% \text{ Inhibition of peptide B} = \frac{\text{OD without blocking - OD in presence of peptide B x100}}{\text{OD without blocking-OD in presence of 2 peptides}}$$

The subtype was indicated by the highest inhibition percentage between peptide B and peptide E, which had to be higher than 60%, and the difference between the percentage of inhibition of peptide B and peptide E, which had to be more than 20%, but if less than 20% then classified into non-typable group. In this group and the other

invalid result, the sample was repeated by dilution titer 1:50 or 1:20. If this sample did not interpret the subtype in the new dilution titer, that meant indeterminate serotypes by SSEIA, *env* gp120 C2-V3-C3 was amplified and analyzed for determination of HIV-1 genotype.

3.2.2 Determination of HIV-1 *gag-pol* genotyping

The nucleotide sequences of the HIV-1 *gag-pol* gene that resulted from routinely drug resistance testing were saved as Fasta file and were analyzed by REGA HIV-1 Subtyping Tool version 2.0 http://dbpartners.stanford.edu/RegaSubtyping/ and Recombinant Identification Program: RIP http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html

3.2.3 Evaluation of HIV-1 V3 serotype and gag-pol genotyping

The results from HIV-1 V3 serotype and *gag-pol* genotype were compared to conclude the subtype. This example is shown in Table 3.5. Since case 1 and 2 had cordant results, the HIV-1 subtype could be concluded. In the other, case 3 and 4 had discordant results, so the RNA was reverse transcribed and amplified *env* gp120 C2-V3-C3 region for HIV-1 genotyping.

Table 3.5 The example of comparing results of HIV-1 V3 serotype and *gag-pol* genotype

Case	SSEIA V3 serotype	*gag-pol* genotype	Subtype
1	E	CRF01_AE	CRF01_AE
2	B	B	B
3	E	B	CRF01_AE /B (Discordant result)
4	B	CRF01_AE	B/CRF01_AE (Discordant result)

3.2.4 HIV-1 env gp120 C2-V3-C3 genotyping

In case indeterminate serotypes by SSEIA and discordant result between HIV-1 V3 serotype and *gag-pol* genotype, RNA would be genotyped in *env* gp120 C2-V3-C3 region. Additionally, the case that *gag-pol* genotype showed inter-subtype

recombination or the different subtype from CRF01_AE and subtype B, RNA would be genotyped in *env* gp120 C2-V3-C3 region for confirmation. Normally RNA was exacted in routine at Division of Clinical Immunology and Division of Clinical Microbiology and stored at -70 ◦C. RNA was reverse transcribed to cDNA and amplified with gp120 V5 specific primers [155] (C20F1 and C30R1) by SuperScript® III First-Strand Synthesis System (Invitrogen, USA). Then, the first PCR product was amplified *env* gp120 C2-V3-C3 by Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, USA) with gp120 C2 specific primers (C20F1 and C2IF2) and gp120 C3 specific primers (C3OR1 and C3IR2).

3.2.4.1 cDNA synthesis and first PCR amplification by SuperScript® III First-Strand Synthesis System

The PCR reaction mixture was performed in a total volume of 50 µL containing autoclaved distilled water 16 µL, 2X Reaction Mix 25 µL, C2OF1 primer (10 µM) 1 µL, C3OR1 primer (10 µM) 1 µL, SuperScript R III RT/ Platinum R Taq Mix 2 µL and Template RNA (0.01 pg–1 µg) 5 µL

The reaction was cDNA synthesis at 55°C for 30 minutes, denaturation at 94°C for 2 minutes and PCR amplification for 40 cycles in the thermal cycler. Each cycle consisted of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and extended at 68°C for 45 seconds. After the last cycle, the extension step was extended for another at 68°C for 5 minutes.

3.2.4.2 Second PCR amplification by Platinum® Taq DNA Polymerase High Fidelity

The PCR reaction mixture was performed in a total volume of 50 µL containing autoclaved distilled water 23.8 µL, 10X PCR buffer (minus Mg) 5 µL, 25 Mm $MgCl_2$ 4 µL, 1.25 Mm dNTP mixture 8 µL, C2IF2 primer (10 µM) 2 µL, C3IR2 primer (10 µM) 2 µL, 5U/µL *taq* DNA polymerase 0.2 µL and first PCR product 5 µL

The reaction denaturation was at 95°C for 1 minute, and PCR amplification was for 35 cycles in the thermal cycler. Each cycle consisted of

denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extended at 72°C for 45 seconds. After the last cycle, the extension step was extended for another at 72°C for 10 minutes.

The amplified products were ran and check compared with 100 bp markers in 2% Agarose gel electrophoresis in TAE buffer at 100 volts for 45 minutes. Then the gel was stained with 2 µL/ml of Ethidium bromide, destained, and photographed under UV light.

3.2.4.3 Purification of DNA

The DNA was purified by a PCR clean-up (Macherey-Nagel, USA). Step one, 100 µL PCR reactions was mixed with 200 µL buffer NTI. Step two, a PCR clean-up column was placed into a collection tube (2 mL) and loaded up a 700 µL sample. The collection tube was centrifuged for 30 seconds at 11,000 x g., then discarded the flow-through and placed the column back into the collection tube. Step three, 700 µL buffer NT3 was added to the PCR clean-up column. The collection tube was centrifuged for 30 seconds at 11,000 x g., then discarded the flow-through and placed the column back into the collection tube. The fourth step, the collection tube was centrifuged for 1 minute at 11,000 x g. to remove buffer NT3 completely. The final step, the PCR clean-up column was placed into a new 1.5 mL microcentrifuge tube. About 15–30 µL buffer NE was added. The new microcentrifuge tube was incubated at room temperature (18–25°C) for 1 minute and centrifuged for 1 minute at 11,000 x g.

3.2.4.4 DNA sequencing of *env* gp120 C2-V3-C3

DNA was sequenced by Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with C2IF2 primer.

The sequencing reaction mixture was performed in a total volume of 10 µL containing autoclaved distilled water 3 µL, Reaction Mix 1 µL, C2IF2 primer (2 µM) 3.2 µL, and purified DNA 2.8 µL

The reaction was denaturation at 96°C for 1 minutes and cycle sequencing for 30 cycles. Each cycle consisted of denaturation at 96°C for 15 seconds, annealing at 50°C for 15 seconds and extended at 60°C for 4 minutes.

Protocol of precipitation started with 70% and Isopropanol 40 µL was added in each well and kept at 4°C overnight. Then, the plate was centrifuged at 2,000 x g for 45 minutes and inverted to get rid of the Isopropanol. After 70% of Isopropanol 150 µL was added in each well, it was centrifuged at 2,000 x g. for 10 minutes. Next, the plate was inverted and centrifuged with papers at 700 x g for 1 minute. Finally, 10 µL Formamide was added in each well. Lastly, the microplate was analyzed and sequenced in ABI PRISM® 3100 Genetic Analyzer.

3.2.5 Determination of HIV-1 *env* gp120 C2-V3-C3 genotyping

The nucleotide sequences of HIV-1 *env* gp120 C2-V3-C3 were edited and saved as Fasta file. Then these sequences were analyzed for HIV subtype by REGA HIV-1 Subtyping Tool version 2.0 http://dbpartners.stanford.edu/RegaSubtyping/ and Recombinant Identification Program: RIP http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html

3.2.6 Phylogenetic analysis

In cases inter-subtype recombinant and the different subtype from CRF01_AE and subtype B, the nucleotide sequences of HIV-1 *gag-pol* and *env* gp120 C2-V3-C3 regions were compared with the *pol* and *env* regions of 158 HIV-1 subtype references. The 158 reference sequences which represented subtypes A1-A2, B, C, D, F1-F2, G, H, J, K, and CRF01_AE to CRF49_cpx, were obtained from the HIV databases at Los Alamos National Laboratory and were used to determine the HIV-1 subtype of the inter-subtype recombination.

These sequences were aligned with Clustal W [Thompson *et al.*, 1994] and adjusted to optimize the alignment. Neighbor-joining trees were constructed with Neighbour-joining method (MEGA version 5.0 programs [154]. The branch significance was analyzed by bootstrap with 1,000 replicates.

3.2.7 Statistical analysis

The results from HIV-1 V3 serotype and *gag-pol* genotype were compared to conclude the HIV-1 subtype. Cohen's kappa coefficient were used to measure inter-rater agreement for two methods, since κ takes into account the agreement occurring by chance.

HIV-1 subtype distribution and intersubtype recombinants circulating in two groups, for example Thai citizens and non-Thai citizens, were compared using SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL). Frequencies were expressed as percentages and then compared using the independent t-test. A p-value < 0.05 was considered statistically significant.