CHAPTER III

MATERIALS AND METHODS

1. Study populations

A total of 19 HEPS persons and 17 of their HIV-1 seropositive sex partners (2 of the HEPS HIV-1 seropositive sex partners died before enrolling to the study) from Sanpatong Hospital, Chiang Mai, had their blood samples collected during 2001 to 2002. The ethnicity of the subjects was carefully identified and approved by the ethics committee of the Faculty of Associated Medical Sciences, Chiang Mai University. The inclusion criteria for HEPS were those who continuously had had unprotected sexual intercourse with HIV-1/2 seropositive sex partners for at least 1 year, and were persistently HIV-1/2 seronegative after testing by two anti-HIV screening tests. Blood samples were tested from 15 healthy adults, whit low risk of HIV-1/2 infections, by two anti-HIV screening tests.

2. Reagents

Reagents used in activation

Brefeldin A (BFA), was purchased from Sigma (Catalog No. B7651), and reconstituted with DMSO at 5 mg/ml. Small aliquots were stored at -20^{o}C, without refreezing after thawing. The stored Stock Brefeldin A was diluted 1:10 in sterile PBS (without sodium azide) for each assay, and BFA at 10 µl/ml of cell suspension was used for the last 4 to 5 hours of activation.

Phorbol 12-Myristate 13-Acetate (PMA), was purchased from Sigma (Catalog No. P-8139), and reconstituted with DMSO at 0.1 mg/ml. Small aliquots were stored at -20^{o}C, without refreezing after thawing. The stored PMA was diluted 1:100 in sterile PBS (without sodium azide) for each assay, and PMA at 25 ng/ml of cell suspension was used for activation.

Ionomycin was purchased from Sigma (Catalog No. I-0634), and reconstituted with EtOH at 0.5 mg/ml. Small aliquots were stored at -20^{o}C. The stored Ionomycin was diluted 1:10 in sterile PBS (without sodium azide) for each assay, and Ionomycin at 1 µg/ml of cell suspension was used for activation.

Reagents for immunophenotype and intracellular cytokine staining:

Monoclonal antibody (mAb) conjugates were used for surface staining, i.e. anti-CD3 PC5 (mouse IgG1 isotype), anti-CD4 FITC (mouse IgG1 isotype), and anti-CD69 PE (mouse IgG2b isotype), which were purchased from Immunotech A Coulter Company.

The intracellular cytokine detection monoclonal antibodies used were anti-human IL-2 PE (mouse IgG2a), anti-human IL-4 PE (mouse IgG1), and anti-human IFN-γ PE (mouse IgG1a). Isotype specific antimouse IgG1, antimouse IgG2a, and antimouse IgG2b also were purchased from Immunotech A Coulter Company.

An IntraPrep™ Permeabilization Reagent kit was used to fix and permeabilize white blood cells according to the manufacturer's directions.

3. Specimen Collection and Preparation

Ten ml of blood were each drawn into a 5 ml heparin and 5 ml EDTA tube. The tubes were mixed well and the specimen kept at room temperature at all times prior to the assay for intracellular cytokine staining (heparin tubes), and before separating EDTA whole blood for PCR and plasma for HIV antibody detection. Lithium heparin was not recommended as an anticoagulant. Whole blood activation for intracellular cytokine staining from the heparin tube should assay within 6 hours of collection. EDTA whole blood was aliquoted into micro centrifuge tubes at 200 µl per tube and stored at -20°C for DNA extraction. Plasma was separated from the EDTA tubes to micro centrifuge tubes and stored at -20°C for HIV-antibody and HIV-antigen detection. The schematic diagram of the study is shown in **Figure 5.**

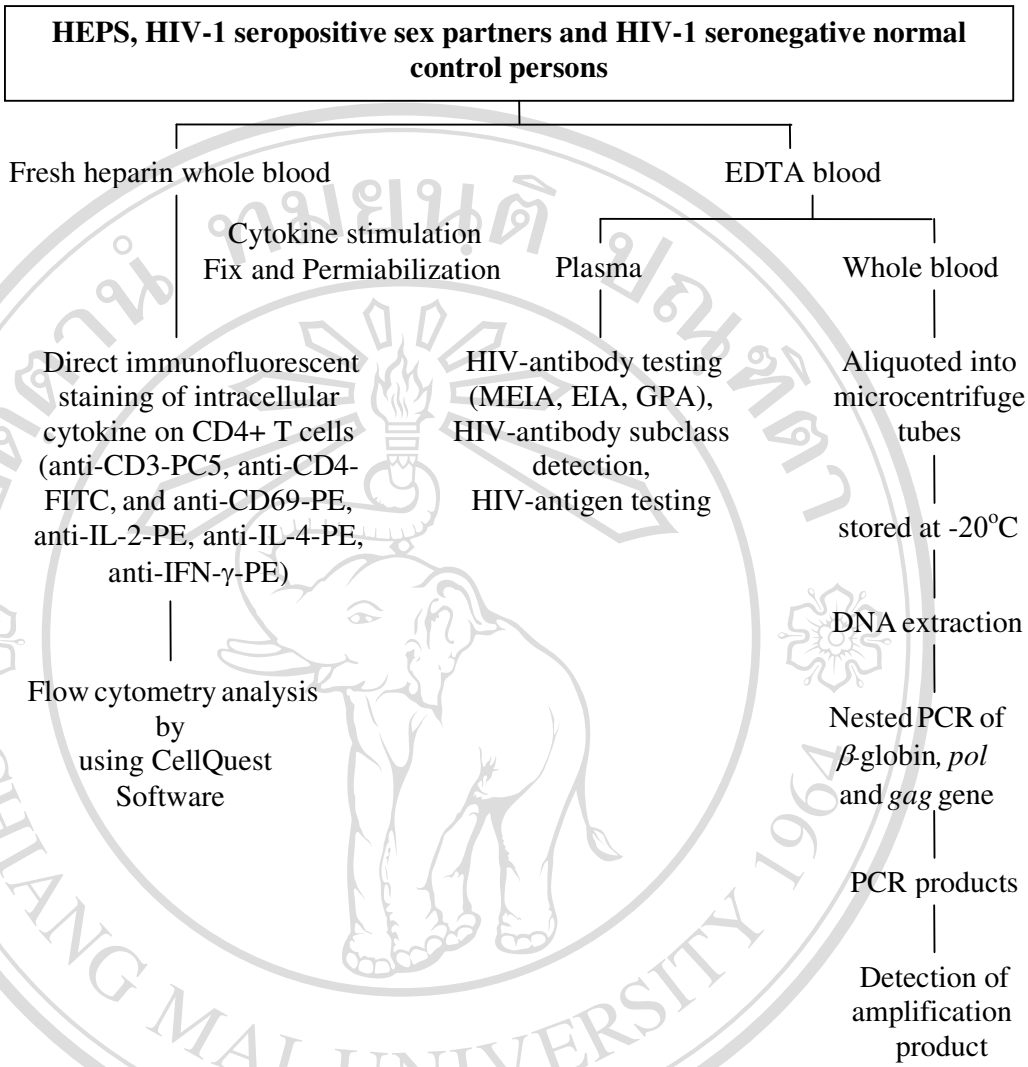

Figure 5. The schematic diagram presenting the research design of the study

4. Laboratory Testing

4.1 HIV Antibody assay

HIV-1 and HIV-2 serology was performed on all subjects by Microparticle EIA (MEIA; Abbott Laboratories, IL) or HIV-1/HIV-2 3rd Gen Plus EIA (Abbott Laboratories, IL), and Serodia HIV-1/2 (Fujirebio, Japan).

4.1.1 Microparticle EIA (MEIA; Abbott Laboratories, IL)

The MEIA test, based on the Abbott IMX® (Abbott Lab., Abbott Park, IL, USA) analyser, is a semi-automated immunoassay with a manual pre-treatment step. The specimen, serum or plasma, was poured into the sample well and the IMX HIV reagents were added by the probe/electrode assembly to the incubation well in the following sequence.

Antigen coated microparticles (HIV-1 envelope, HIV-1 core, and HIV-2 envelope) were sampler to preincubation well by the probe/electrode assembly of the instrument. Antibodies in the specimen were bound with antigen on microparticles and formed antigen-antibody complexe. They were subsequently transferred to a glass fiber matrix. The microparticles were bonded with the glass fiber matrix and washed to remove unbound material. Biotinylated recombinant antigens (HIV-1 envelope, HIV-1 core, and HIV-2 envelope) and synthetic peptides were dispensed onto the matrix, and anti-biotin:alkaline phosphatase conjugate was subsequently added onto the matrix and bound to the antigen-antibody-antigen complex.

An aliquot of the reaction mixture containing the specimen, and the 'antigen-antibody-antigen alkaline phosphatase' complexes bound to the microparticles were transferred to the glass fiber matrix. The microparticles bound irreversibly to the glass fiber matrix, which was washed to remove unbound materials. The substrate, 4-methylumbelliferyl phosphate, was added to the matrix and the fluorescent product was measured by MEIA optical assembly. Two controls (negative control and positive control) were supplied with the kit.

The rates of fluorescent product formation to the cutoff were compared to determine the presence or absence of antibodies to HIV-1 and/or HIV-2. Cutoff was calculated from the MODE 1 Calibrator rate. Anti-HIV was considered reactive when the rate of the specimen was greater than or equal to the cutoff.

4.1.2 HIV-1/HIV-2 3rd Gen Plus (EIA; Abbott Laboratories, IL, USA)

The Abbott recombinant HIV-1/HIV-2 3rd generation plus enzyme immunoassay (EIA) test was used for screening the normal group. One hundred and fifty microliters of specimen and 50 µl of specimen diluent were added into each well of macroplate and antigen coated macrobeads (HIV-1 envelope, HIV-1 core, and HIV-2 envelope) were subsequently added. After 30 minutes rotation at 40±2°C, the macrobeads were washed 2 times with the COMMANDER Parallel Processing Center (PPC) to remove unbound materials. HIV-1/HIV-2 antigen (HIV-1 envelope, HIV-1 core, and HIV-2 envelope) horseradish peroxidase (HRPO) conjugate at 200 µl was added and incubated for 30 minutes rotation at 40±2°C. The macrobeads were again washed 2 times with the COMMANDER Parallel Processing Center (PPC) again to remove unbound materials, and 300 µl of chromogen, o-Phenylenediamine (OPD)

was added for 30 minutes incubation at room temperature. This reaction was stopped by adding 300 µl of 1 *N* sulfuric-acid and the absorbance in each well was read at wavelength 492 nm. The instrument was blanked with the valid substrate blank.

The colour development was proportional to the amount of HIV-1/HIV-2 antibody present in the sample. In addition to the blood samples, five internal controls (three negative and two positive) were mandatory. All of the reagents were stored at 4°C.

4.1.3 Serodia HIV-1/2 particle agglutination (PA) test (Fujirebio, Japan)

The Serodia-HIV-1/2 particle agglutination (PA) test was carried out according to the manufacturer's directions. Sample diluent was added to each of four wells in a round-bottom microtiter plate. Seventy five microliters were added to the first well, and 25 µl to wells 2 through 4. Next, 25 µl of serum sample were added to the first well, making it a 1:4 initial dilution of the sample. The contents of the first well were mixed, and 25 µl was transferred to the second well. This procedure was continued through well 4, with 25 µl being discarded from the fourth well. Twenty-five microliters of unsensitized particles were added to the second well, making a 1:16 dilution, 25 µl of HIV-1 sensitized particles were added to the third and fourth well, making a 1:32 dilution of serum, and 25 µl of HIV-2 sensitized particles were added to the fourth well, making a 1:64 dilution of serum. The final serum dilutions were 1:16 for the unsensitized control well, 1:32 for the HIV-1 test well and 1:64 for the HIV-2 test well. The contents of the wells were mixed thoroughly using a vibrating shaker. The plates were covered and left at room temperature for 2 hours. Reactive and nonreactive controls were included in each run. A sample was considered reactive if a mat of particles covered the bottom of the well. A 1+ reactive sample had a diffuse ring of particles around the periphery of the mat of particles, while a 2+ reactive sample lacked this ring. A sample with a button of particles in the bottom of the well was considered nonreactive.

4.2. Detection of HIV antibodies (IgG, IgA and IgM) in plasma by Flow Cytometric Immunofluorescence Assay (FIFA)

In this study, diluted FITC-conjugated anti-human IgG was 1:20 with PBS, whereas FITC-conjugated anti-human IgA and FITC-conjugated anti-human IgM was diluted at 1:5.

Three microcentrifuge tubes were prepared and labeled for each sample (for IgG, IgA and IgM detection). HIV-coated latex beads were added at 5 µl into each microcentrifuge tube with diluted plasma (with PBS at 1:10) at 25 µl, mixed by vortex and incubated for 30 minutes at room temperature (18-25^{o}C). Washing to remove unbound material was carried out done for 2 times by adding 1 ml of PBS into each micro centrifuge tube and vigorously vortexing tube by tube, centrifuging for 5 minutes at 10,000 rpm at 4^{o}C and discarding supernatant by aspiration.

FITC-conjugated anti-human IgG was diluted with PBS to 1:20, and FITC-conjugated anti-human IgA and FITC-conjugated anti-human IgM were diluted with PBS to 1:5. Diluted FITC-conjugated anti-human Ig (IgG, IgA and IgM) was added at

10 µl into each class Ig assay micro centrifuge tube, then mixed by vortex and incubated for 30 minutes at room temperature (18-25^{o}C) in the dark. Washing to remove unbound material was performed by adding 1 ml of PBS into each micro centrifuge tube and vigorously vortexing tube by tube, centrifuging for 5 minutes at 10,000 rpm at 4^{o}C and discarding supernatant by aspiration. The latex beads were resuspended in 0.5 ml of PBS, mixed by vortex and aspirated to 12x75 mm. polystyrene tubes, for flow cytometry analysis.

4.3 HIV Antigen assay

The Coulter HIV antigen test was used to detect HIV-1 antigen (p24). The Coulter HIV Ag assay utilizes microtiter plates coated with monoclonal antibodies (anti-p24) for the capture of HIV antigen in test samples. The sample (200 µl) and 20 µl of lyse buffer were incubated simultaneously with anti-p24 monoclonal antibody-coated plates at 37±2^{o}C for 60±5 minutes followed by five buffer washes (with a soaking time of 25 seconds). After washing to remove unbound material, 200 µl of biotinylated anti-p24 polyclonal antibody were incubated at 37±2^{o}C for 60±5 minutes. The p24 antigen in the sample bound to the monoclonal anti-p24 antibody coated in microtiter plates and was recognized by biotinylated anti-p24 polyclonal antibody. Following the washing step, streptavidin coupled to horse radish peroxidase (HRPO) was added to the well, which bound to the biotin, and was incubated at 37±2^{o}C for 30±2 minutes, followed by five buffer washes. In the next step, the amount of conjugate that bound was determined by incubation with 200 µl of hydrogen peroxide–3,3', 5,5'-tetramethylbenzidine (TMB) substrate solution for 37±2^{o}C minutes. The incubation was terminated with 50 µl of H_2SO_4, and the absorbance measured at wavelength 450 nm, with a reference set to 570 nm. All the steps performed were automated.

The detection of HIV p24 antigen in a sample was achieved based on the binding of the antigen to the solid phase and its proportion to horse radish peroxidase (HRPO) activity. The cutoff was calculated as the mean of negative control values plus 0.055. The overall mean had to be less than 0.055 for the run to be valid.

4.4 Detection of proviral DNA from whole blood by Multiplex Nested PCR (National HIV Repository and Bioinformatic Center -Thailand)

4.4.1 Specimens

Frozen EDTA blood in microcentrifuge tubes, 200 µl in each tube, was stored at 0^{o}C for at least 1 hour.

4.4.2 DNA preparation

DNA extraction was prepared from frozen whole blood. Solution I was added at 200 µl into thawed EDTA blood in microcentrifuge tubes; vortexed briefly, and incubated on ice for 10 minutes. Red blood cells were lysed in this step. Samples were pelletted by centrifugation at 10,000 rpm for 2 minutes and the supernatant was subsequently discarded by careful aspiration. The pellets were resuspended in 200 µl

of Solution II and vortexed briefly to clear the supernatant of debris. Samples were pelletted by centrifugation at 10,000 rpm for 2 minutes and the supernatant was subsequently discarded by careful aspiration. The pellets were resuspended in 200 µl of Solution II and vortexed briefly to clear the supernatant of debris. Cells were pelletted by centrifugation at 10,000 rpm for 2 minutes and the supernatant was subsequently discard by careful aspiration before lysing by proteinase K.

Diluted proteinase K was prepared; 10 µl of 10 mg/ml proteinase K to 1 ml of lysis buffer and subsequently vortexed.

The diluted proteinase K was added at 50 µl into each micro centrifuge tube and vortexed untill the pellets were lost and then lysed by incubation at 56^{o}C for 1 hour. After the incubation, proteinase K was inactivated at 95-100^{o}C for 10 minutes, for DNA amplification.

4.4.3 DNA storage

Frozen DNA was stored f at -20^{o}C untill testing.

4.4.4 DNA Amplification

A primary PCR tube, containing 1 pair of beta-globin gene, 1 pair of outer primer *gag* gene (SK 380, SK 390) and 1 pair of outer primer *pol* gene (POL O1, POL O2) primers (**Table.3**) was checked to ensure that the cap was closed firmly and the contents had thawed. The reaction of primary PCR was spun down before use. Extract DNA was added at 10 µl into the primary PCR tube, which was checked to ensure the cap was closed firmly, mixed by vortex and spun down then proceed to thermal cycle program (Gene Amp PCR System 2700, Perkin-Elmer, USA). One tube was used for only one sample and carried out with a negative and positive control simultaneously with PCR. The PCR was performed in a thermocycler as shown below. The following cycling parametrics were programmed for 35 cycles:

PCR condition for outer primer amplification

35 cycles of denaturing: at 94^{o}C for 60 sec (outer primer);
annealing: at 55^{o}C for 60 sec (outer primer);
extentding: at 72^{o}C for 60 sec (outer primer);
and 72^{o}C for 10 minutes

After the last cycle, an additional 10 min elongation step was carried out.

4.4.5 Nested PCR

Nested PCR reactions (50 µl) were performed using 5µl of the primary PCR product. The reaction mixture contained 10x PCR buffer, 10 mM of each dNTP, 25 mM MgCl2, primer of beta-globin gene (10 pmol/µl PCO4 primer, 10 pmol/µl GH20 primer), *pol* gene (10 pmol/µl PolO1primer, 10 pmol/µl PolO2 primer), *gag* gene (10 pmol/µl SK380 primer, 10 pmol/µl SK390 primer) and 5U/µl *Taq* DNA pol. The primary PCR product of the negative and positive control had to be run with the samples. The PCR was performed in a thermocycler as shown below.

PCR condition for inner primer amplification

35 cycles of denaturing: at 94°C for 60 sec (inner primer);
annealing: at 55°C for 60 sec (inner primer);
extentding: at 72°C for 60 sec (inner primer);
and 72°C for 10 minutes

After the last cycle, an additional 10 min elongation step was carried out.

4.4.7 Detection of amplification product

The reaction products were analysed by electrophoresis in 2% agarose gel in a TAE buffer, stained with ethidium bromide and visualised under UV light. Ten µl of PCR product were loaded from Nested PCR on 2% (wt/vol) agarose gel and horizontal electrophoresis was performed in TBE buffer at 220 volts for 90 minutes. The gel in the 5 µg/ml of ethidium bromide solution was stained for 15 minutes and destained in distilled water for 15 minutes. The DNA fragment was visualized on a UV transilluminator and photographed with a camera.

4.4.8 Interpretation of the results

Multiplex Nested PCR composed of 3 pairs of primers for amplification of segments of the *β*-globin gene, *pol* gene and *gag* gene. The size of the 3 products was 260, 180 and 115 base pair, respectively.

The *β*- globin gene was used as an internal control for PCR condition.

The *Pol* and/or *gag* gene products indicated whether the HIV-1 DNA positive.

4.5 Intracellular Cytokine Staining; ICCS

4.5.1 Cell Activation

Each of two 15 ml conical polypropylene tubes (with caps) was labeled per sample: Unstimulated and Activated were labeled. Sodium heparinized blood was aliquoted into each tube at 0.5 ml per tube. Then, 0.5 ml RPMI was added into each tube and mixed. Phorbal 12-myritate 13-acetate (PMA) (25 µl/ml) was added to diluted whole blood samples: at 25 ng/µl in the Activated tube and Ionomycin (20 µl) was subsequently added at 0.5 mg/µl to stimulate in vitro production of interferon-gamma(IFN-γ), interleukin-2 (IL-2) (type 1 cytokines) and interleukin-4 (IL-4) (type 2 cytokine). The secretion inhibitor, Brefeldin A (BFA) (10 µl), was included to block the transport of cytokines to the cell surface, in both the Unstimulated and Activated tube. Sterile PBS, as a negative control (unstimulated), was added at 45 µl to the Unstimulated tube, which was capped tubes loosely and vortexed. The culture tubes were incubated upright in a humidified 37°C, 5% CO_2 incubator for a total of 4-6 hours. At 4-6 hours, 50 µl of 20 mM EDTA (for a final concentration of 2mM EDTA) were added directly to the whole blood cultures. Sample tubes were vortexed vigorously and incubated for 15 minutes at 22°C (room temperature) to remove adherent cells.

4.5.2 Cell Staining

4.5.2.1 Cell surface staining

Nine of the polystyrene tubes (12x75 mm.) were labeled as shown in **Table 3**. Unstimulated or activated whole blood was aliquoted into staining tubes at 50 µl for each sample in both the assay and control tube. Membrane-specific conjugated monoclonal antibodies (Anti-CD3-PC5 and Anti-CD4-FITC) were added at 5 µl into each tube to stain cells. Each tube was vortexed vigorously and incubated for 15 minutes at 22°C (room temperature) in the dark.

4.5.2.2 Fixed white cells

White blood cells were fixed in 5.5% formaldehyde (Intraprep reagent 1, Immunotech). IntraPrep Reagent 1 was added at 100 µl into each tube, and vigorously vortexed tube by tube before incubating for 15 minutes at room temperature (18-25°C) in the dark. The fixed cells were washed in 4 ml of PBS and subsequently mixed with autopipette, centrifuged for 5 minutes at 300xg at 18-25°C (room temperature) and the supernatant was discarded by aspiration.

4.5.2.3 Red blood cell lysis and permeabilized white cells

IntraPrep Reagent 2 (Immunotech, France) was added at 100 µl into each tube and mixed without vortexing. Red blood cells were lysed and white blood cells permeabilized. Staining tubes were incubated for 5 minutes at room temperature (18-25°C) without vortexing and subsequently agitated (manually) gently for 1 to 2 seconds.

4.5.2.4 Intracellular cytokine staining

Cells were stained in the dark for 15 minutes with PE-conjugated antibody (anti-IL-2, anti- IFN-γ, anti-IL-4 or anti-CD69). Isotype matched control antibodies (PE-conjugated mouse isotype control; Immunotech) were included to detect non-specific binding to cells. Anti-CD69-PE was used to detect activated T cells. Intracellular conjugated specific antibodies were added at 5 µl to each assay tube and intracellular conjugated isotype control was added at 5 µl into each control tube (as shown in **Table 3**) before gently vortexing tube by tube and incubating for 15 minutes at room temperature (18-25°C) in the dark. After intracellular cytokine staining, the cells were washed with 4 ml of PBS and mixed with autopipette by pipetting up and down. Cells were pelleted by centrifugation for 5 minutes at 300xg at room temperature (18-25°C) and the supernatant was discarded by aspiration. The cells were subsequently resuspended in 0.5 ml of PBS, containing 0.5% formaldehyde before flow cytometry analysis. The specimen had to be analyzed within two hours of IntraPrep treatment when stored at 18-25°C. Otherwise, fixed preparations should be stored at 2-8°C in the dark and analysed within 24 hours.

4.5.2.5 Flow cytometric analysis

Flow cytometric analysis was performed on a FACSCalibur ™ flow cytometer (BDIS). Data were acquired using CellQuest™ software (Becton-

Dickinson, USA.), typically collecting 3,000 gated CD3+ events using FL3 (PC5) as a fluorescent trigger.

Data were displayed as two-color dot plots (FL1 vs. FL2) in CellQuest™ software (BDIS) to measure the proportion of the double positive (cytokine+/CD4+) cells. CD69 staining was included to confirm that cells were activated in the system. CD3+ cells or FL3 (PC5) versus side scatter gating was employed in data analysis to exclude any CD4+ monocytes. Cells staining positively for cytokines were expressed as a percentage of the CD4+ T cells.

4.5.2.6 Statistical analysis

Flow cytometric data were analysed using a non-parametric Man Withney U test to compare cytokine responses in each subject group and the same groups were compared by analyzing with the Wilcoxon matched pairs test. *P* values of <0.05 were considered significant. Mean values were plus or minus standard errors, where appropriate.

Table 3. Primers of HIV-1 PCR

Primer	Gene	Sequence (5'-3")	Location
Outer primers	SK 380	gag GAGAACCAAGGGGAAGTGACATAGCAGG	684-712
	SK 390	gag TAGAACCGGTCTACATAGTCTCTAAGGGG	874-903
	POL O1	pol CCCTACAATCCCCAAGTCAAG	4652-4674
	POL O2	pol TACTGCCCCTTCACCTTTCCA	4955-4975
Inner primers	SK 38	gag ATAACCACCTATCCCAGTAGGAGAAT	759-785
	SK 39	gag TTTGTTCCTTGTCTTATGTCCAGAATG	840-867
	POL I1	pol TTAGACAGCAGTACAAATGGCAG	4744-4766
	POL I2	pol GCTGTCCCTGTAATAACCCG	4898-4918

Table 4. Intracellular Cytokine Staining method

Reagents	Tube No.								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Unstimulated whole blood (50 µl)	+	+	+						
Activated whole blood (50 µl)				+	+	+	+	+	+
Anti-CD3-PC5 (5 µl)	+	+	+	+	+	+	+	+	+
Anti-CD4-FITC (5 µl)	+	+	+	+	+	+	+	+	+
Mixed by vortex and incubated for 15 minutes at room temperature (18-25°C) in the dark.									
Intraprep Reagent 1 (100 µl)	+	+	+	+	+	+	+	+	+
Mixed by vortex and incubated for 15 minutes at room temperature (18-25°C) in the dark.									
PBS (4 ml)	+	+	+	+	+	+	+	+	+
Centrifuged for 5 minutes at 300xg at room temperature (18-25°C). Discarded supernatant by aspiration.									
Intraprep Reagent 2 (100 µl)	+	+	+	+	+	+	+	+	+
Mixed without vortexing. Incubated for 5 minutes at room temperature (18-25°C) without vortexing. Gently agitating (manually) for 1 to 2 seconds.									
Anti-IL2 –PE (IgG2a) (5 µl)	+					+			
Anti-IL-4 –PE (IgG1-mouse) (5 µl)		+					+		
Anti-IFN-γ-PE (IgG1-mouse) (5 µl)			+					+	
Isotype IgG2a mouse-PE (5 µl)				+					
Isotype IgG1 mouse-PE (5 µl)					+				
Anti-CD69-PE (5 µl)									+
Gently vortexed tube by tube. Incubated for 15 minutes at room temperature (18-25°C) in the dark.									
PBS (4 ml)	+	+	+	+	+	+	+	+	+
Centrifuged for 5 minutes at 300xg at room temperature (18-25°C). Discarded supernatant by aspiration.									
0.5 % formaldehyde in PBS (500 µl)	+	+	+	+	+	+	+	+	+
Proceed to flow cytometry analysis									