CHAPTER III

RESEARCH DESIGN, MATERIALS AND METHODS

1. Research design

EDTA blood samples were collected from HIV-1 serodiscordant couples and HIV-1 seronegative subjects. All subjects were enrolled in Chiang Mai province. The samples were examined for the CCR5 genotypes including the nucleotide polymorphisms in the promoter region, the CCR5Δ32, and the CCR5-m303. Determination of the density of CCR5 protein expressed on the surface of CD4+ T lymphocytes and monocytes was also included. For genotyping, PBMCs were isolated from the EDTA blood by Ficoll-Hypaque density gradient centrifugation method. Genomic DNA was then extracted from the PBMCs and used as a template for PCR. In order to investigate the genetic polymorphisms, the approximately 1,190 bp of the CCR5 promoter fragment was amplified by using PCR technique. Then, the amplified product was cloned into the pGEM®-T Easy vector (Promega, USA) and subsequently sequenced by the cycles sequencing technique using the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, England). The products were analyzed by the Long-Read Tower Sequencer (Visible Genetic, USA) and the GeneObjects™ software (Visible Genetic, USA), which is commercially available oligonucleotide analysis software programs. Finally, the nucleotide sequence of the CCR5 promoter region of each sample was analyzed by the BioEdit Sequence Alignment Editor version 5.0.9 software and comparing with the reported sequences obtains from the GenBank database (the NIH genetic sequence database at NCBI; http://www.ncbi.nlm.nih.gov/Entrez/). The investigation of CCR5Δ32 genotype was performed by PCR amplification using specific primers flanking the Δ32 deletion region of the CCR5 and analyzed by size differences of the amplified product. The CCR5-m303 genotype determination was carried out by using nested-PCR and restriction enzyme analysis (REA) technique. The amplified product was digested with *Hinc*II restriction enzyme, where the wild type allele was digested while the mutant was not. Quantification of CCR5 molecules on the cell surface was

determined by using flow cytometry. Fresh EDTA blood was directly stained with the dye-labeled mAbs that bind specifically to the antigens: CD4, CD14, and CCR5 on the surface of white blood cells and then analyzed by flow cytometer. The standard microbead coated with known amount of fluorochrome (QuantiBRITE-PE; Becton Dickinson, USA) was used to generate the calibration curve in order to calculate the geometric mean fluorescence intensity generated from the stained-CCR5 molecules. All steps of the study design are shown in Figure 5.

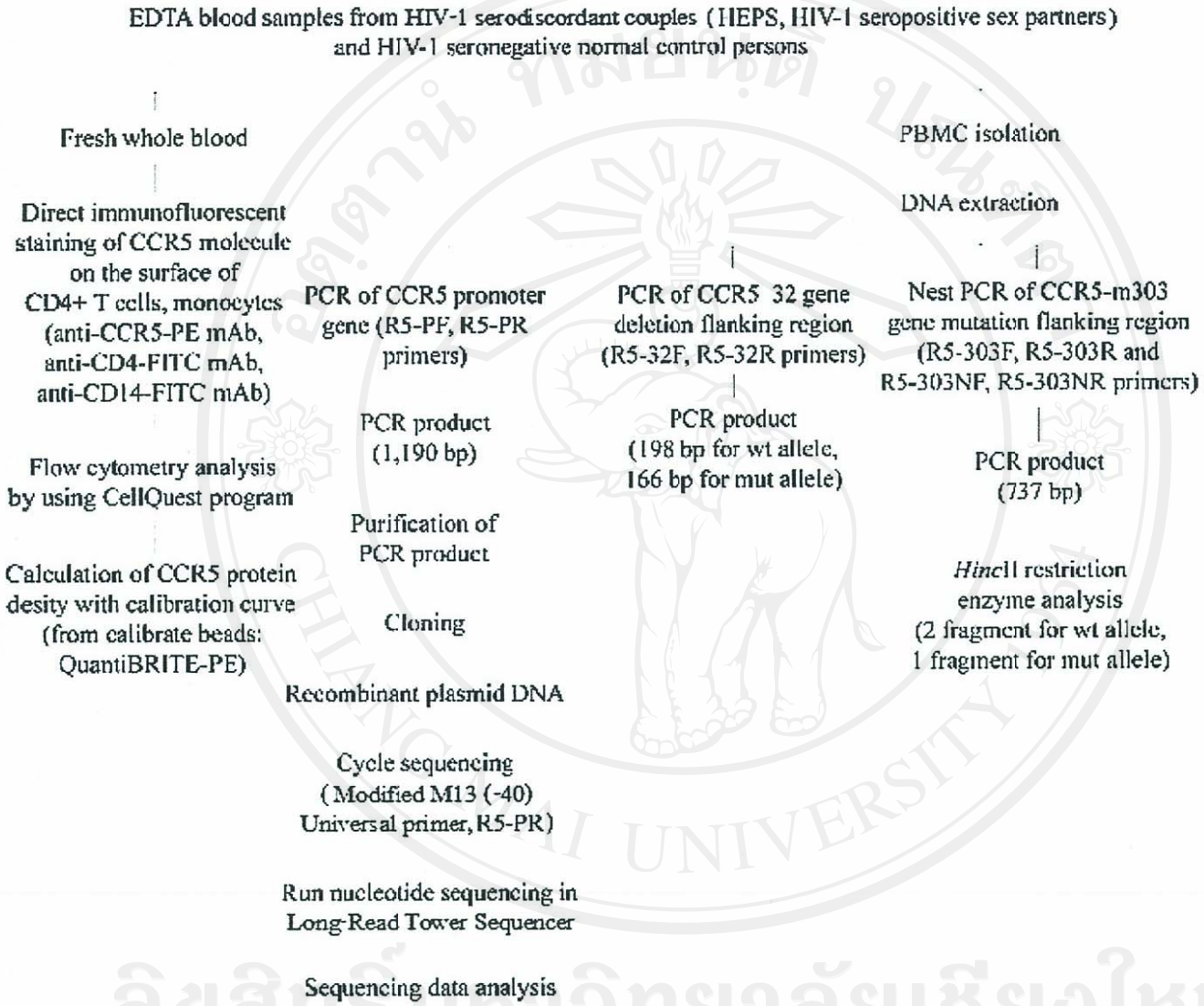


Figure 5 The schematic diagram presenting the research design of the study.

2. Materials

2.1 Study populations

Twenty HIV-1 serodiscordant couples who were attended at Sunpatong Hospital and Doisaket Hospital, Chiang Mai province during 2001 to 2002. The HIV-1 serodiscordant couple was defined as one person showed HIV-1 seropositive, while the matched couple showed HIV-1 seronegative. By the way, 20 HIV-1 seronegative individuals were classified as HEPS through their HIV-1 seropositive spouses. The inclusion criteria for the HEPS were those who have continuously unprotected sexual intercourse (without condoms) with HIV-1 seropositive spouse for at least 1 year and have seronegative as diagnosed for HIV-1/2 antibodies by ELISA at enrolment and remained lacking of HIV-1 DNA as measures by PCR using *gag* and *pol* primers for longer than 3 months. Twenty HIV-1 seropositive spouses were enrolled with their HEPS matched. All HIV-1 seropositive individuals were diagnosed and confirmed seropositive by HIV-1/2 screening assay and Western blotting using commercial kits respectively for longer than 3 months. Ten HIV-1 seronegative control groups were randomly selected from Thai individuals who had low risk for HIV-1 infection. All subjects were informed, and the study was approved by the ethics committee from the faculty of Associated Medical Sciences, Chiang Mai University.

2.2 Oligonucleotide primers

Nine oligonucleotide primers were used in this study. These included R5-32F, R5-32R, R5-303F, and R5-303R, which were selected from the published study (Quillent *et al*, 1998). The modified M13 universal primer was provided in the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (Amersham Bioscience, England). Where the R5-PF, R5-PR, R5-303NF, and R5-303NR primers were designed by using the Primer Premier version 5.0 software (PREMIER Biosoft International, CA, USA) based on the complete sequence of the CCR5 gene reported in the GenBank accession number U95626. The designed primers were further analyzed for the CCR5 specificity using the web-based BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). All of them were shown to have specificity only to the intended targets. Lists and nucleotide sequences of all primers used are shown in Table 3. The location of those primers in the CCR5 gene is shown in Figure 6. The modified M13 universal primer location is shown in Appendix C.

Table 3 Primers information

Name	Sequence (5' to 3')	Length	Position[1]
R5-32F[2]	GTCGGCAGGACACCTGCAGCTC	22 bp	62,000 – 62,022
R5-32R[2]	GTGAAGATAAGCCTCACAGCC	21 bp	62,177 – 62,198
R5-303F[2]	CCAGAGGGCATCTTGTGGCTCGGG	24 bp	61,474 – 61,494
R5-303R[2]	AGCCTCGTCGCAGACTGTGACCCTT	25 bp	62,527 – 62,549
R5-303NF	CCAGAGGGCATCTTGTGGCTCGGG	24 bp	61,579 – 61,596
R5-303NR	AGCCTCGTCGCAGACTGTGACCCTT	25 bp	62,298 – 62,315
R5-PF	CCAGAGGGCATCTTGTGGCTCGGG	24 bp	58,548 - 58,571
R5-PR	AGCCTCGTCGCAGACTGTGACCCTT	25 bp	59,713 - 59,737
Modified M13 Universal primer	GGTAACGCCAGGGTTTTCC	19 bp	2,944-2,962[3]

[1] The positions are based on the numbering system according to GenBank accession number U95626

[2] Quillent *et al*, 1998.

[3] The binding site of Modified M13 Universal primer on pGEM®-T Easy Vector (see Appendix C).

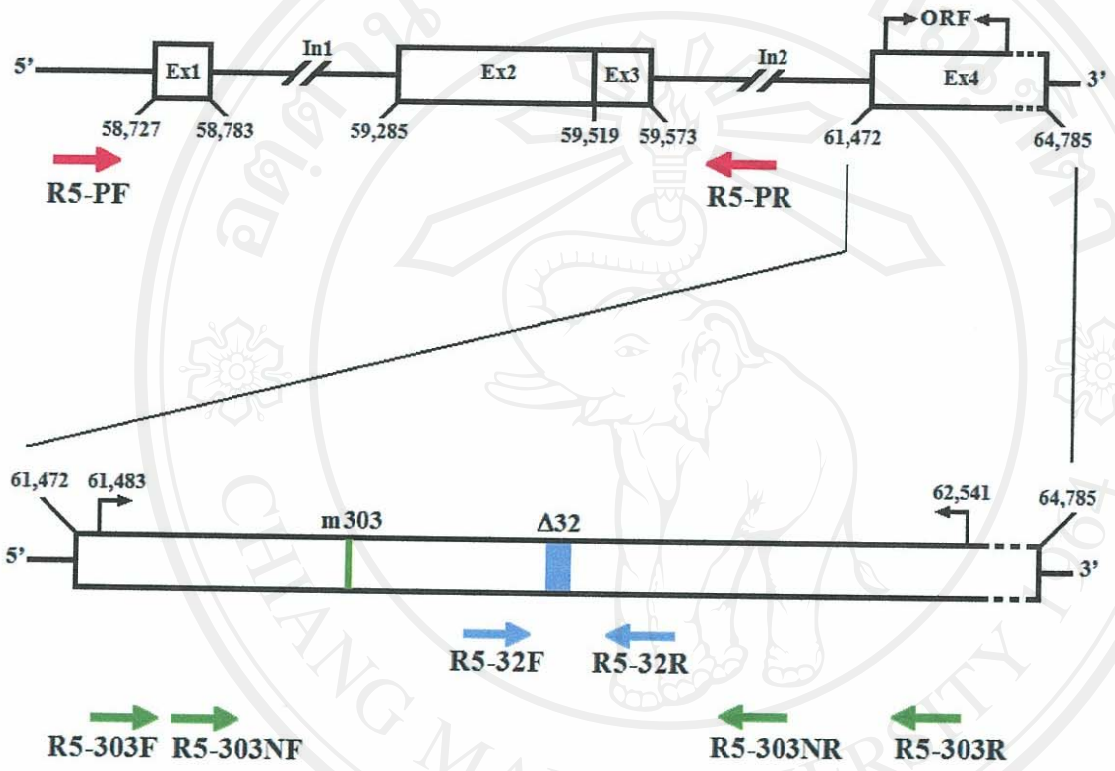


Figure 6 The location of R5-PF, R5-PR, R5-32F, R5-32R, R5-303F, and R5-303R primers in the CCR5 gene (not to scale). The CCR5 gene numbering is based on the numbering system according to GenBank accession number U95626.

2.3 Antibodies

All of the monoclonal antibodies using for the immunofluorescent staining were purchased from Becton Dickinson (San Jose, CA, USA). Lists and characteristics of those antibodies are shown as follows.

- Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD4 mAb (clone SK3). The antibody recognizes a CD4 antigen expressed primarily on helper/inducer T lymphocytes subset (CD3+, CD4+). The CD4 antigen is also the receptor for the HIV entry into the cells.
- FITC-conjugated mouse anti-human CD14 mAb (clone M5E2). CD14 is a 55 kDa glycoprotein expressed on peripheral blood monocytes and macrophages. It is also present on pleural phagocyte cells and reticular dendritic cells.
- Phycoerythrin (PE)-conjugated mouse anti-human CCR5 mAb (clone 2D7). CCR5 molecule is a seven transmembrane-spanning G protein-associated molecule. It is expressed on a subset of T lymphocytes (CD3+, CD45RO+, CD95+). The CCR5 regulates lymphocyte chemotaxis activation and transendothelial migration during inflammation and it has been found to be a co-receptor for macrophage-tropic HIV-1 on CD4+ cells.
- PE-conjugated mouse IgG2a, κ isotype control immunoglobulin (clone G155-178). G155-178 immunoglobulin is a mouse myeloma protein. It was selected as an isotype control following screening for low background on a variety of mouse and human tissues.

3. Methods

3.1 Sample collection and storage

Seven microliters of peripheral blood was drawn by venipuncture from each subjects in an Ethylene Diamine Tetraacetic Acid (EDTA) tubes at the hospitals. After collecting, the blood was kept at room temperature and transferred to the laboratory. When arriving, approximately 500 µl of the blood was taken and determined for cell surface CCR5 protein

density. The remainder was then centrifuged at 1,600 x g for 20 minutes at room temperature. Plasma was separated with a sterile pipette in a sterile 1.5 ml microcentrifuge tube and stored at -70°C for further investigations. White blood cell layer (buffy coat) was collected for peripheral blood mononuclear cells (PBMCs) isolation. All assays were performed within 6 hours after the blood drawn.

3.2 DNA preparation

3.2.1 Isolation of the PBMCs

The PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation using Isoprep (Robbins Scientific, CA, USA) according to the method indicated by the manufacturer. Briefly, the blood was diluted by addition of an equal volume of PBS pH 7.2. Six microliters of the diluted blood was carefully laid over 3 ml of Isoprep in a 15 ml centrifuge tube and avoid mixing of the blood with solution. Then, the tube was centrifuged at 800 x g for 20 minutes at room temperature. After centrifugation, the mononuclear cells (lymphocytes and monocytes) formed a distinct layer at the sample/Isoprep interface. The cells were collected from the interface using a sterile pipette without removing the upper layer. To lysis contaminated red blood cells, 5 ml of TE buffer (pH 8.0) was added and centrifuged at 500 x g for 10 minutes. The harvested mononuclear cells were washed 3 times with PBS pH 7.2 by centrifugation for 10 minutes at 250 x g. The cells pellet was collected and stored at -20°C until extraction of DNA.

3.2.2 Extraction of the genomic DNA

The genomic DNA was extracted and purified from the PBMCs by using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany). The extraction was performed as the procedure recommended by the manufacture. In brief, the PBMCs pellet was thawed and suspended in 200 µl of PBS pH 7.2. Two hundred microliters of Binding Buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100 (v/v), pH 4.4) and 40 µl of proteinase K were added into the cell suspension and incubated at 72°C for 10 minutes. One hundred microliters of isopropanol was added and then applied the mixture into the High Pure filter tube. The nucleic acids were bound to the glass fibers pre-packed in the filter

tube. After centrifugation at 8,000 x g for 1 minute, the bound nucleic acids were washed with 500 µl of Inhibitor Removal Buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6) to get rid of PCR inhibitory residuals. Then, the proteins, salts and other cellular contents were washed out by passing twice with 500 µl of Wash Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) through the filter tube. Finally, 200 µl of prewarmed (70°C) Elution Buffer (10 mM Tris, pH 8.5) was added and centrifugation at 8,000 x g for 1 minute to collect the DNA. The purified genomic DNA was stored at -70°C for further analysis.

3.2.3 Quantitation of the purified genomic DNA

The concentration of the purified genomic DNA was determined by using the spectrophotometry method. The DNA was diluted in distilled water at an appropriate dilution and then the optical density (OD) was measured for nucleic acid and protein at wavelength 260 nm and 280 nm respectively using UV spectrophotometer. The purity of the DNA sample was determined by the OD 260/280 ratio. The quantity of DNA was calculated by using the following equation:

Quantity of DNA (ng/µl) = OD at 260 nm x dilution factor x 50

3.3 Determination of nucleotide polymorphisms in the promoter region of the CCR5 gene

3.3.1 Amplification of the CCR5 promoter region by PCR

The approximate 1,190 bp fragment of the CCR5 promoter region at positions 58,548 to 59,737 was amplified by PCR using primers R5-PF and R5-PR. The reaction mixture contained 40 µM of each primer, 200 µM of each deoxynucleotide triphosphate (dNTPs; dATP, dTTP, dGTP, and dCTP), 2.5 units of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, USA), PCR buffer (600 mM Tris-SO_4 pH 8.9, 180 mM Ammonium Sulfate), 3.5 mM of $MgSO_4$. Fifty to one hundred nanograms (5-10 µl) of target DNA was added to the mixture. The final volume of the reaction was 50 µl.

The amplification was performed in an oil-free thermal cycler with a hot-start at 94°C for 1 minute and followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 68°C for 80 seconds. After the last cycle, the reaction was incubated for another 7 minutes at 68°C to ensure that the extension was completed.

The product of the amplification was separated on 1% agarose gel electrophoresis in a TAE buffer at 100 volts for 1 hour. It was then stained with 2 µg/ml of ethidium bromide for 15 minutes, destained with distilled water for 15 minutes, visualized, and photographed under ultraviolet light using an ultraviolet transilluminator. The DNA fragment size was determined by using standard DNA size marker, 1 Kb Plus DNA Ladder (Gibco BRL, USA).

3.3.2 Cloning of the CCR5 promoter region

To keep the DNA of the CCR5 promoter region for repeated analysis or further investigations, the PCR product of the CCR5 promoter region from each subject was cloned into a cloning vector as follows.

3.3.2.1 Purification of the PCR product

The amplified product of the CCR5 promoter region was purified by using the MinElute Gel Extraction Kit (Qiagen, Germany). The procedure of purification was performed as the procedures recommended in the manufacturer's instruction manual. Briefly, the amplified DNA fragment was separated on 1% agarose gel electrophoresis and the DNA band was excised by using a sterile scalpel under ultraviolet light. The gel slice was incubated in 3 gel volumes (weight by volume) of Buffer QG at 50°C for 10 minutes. After the gel slice was dissolved completely, 1 gel volume of isopropanol was added and then applied the mixture to MinElute column. The column was centrifuged at 10,000 x g for 1 minute and discarded the supernatant. Then, 500 µl of Buffer QG was added into the column and centrifuged for 1 minute and the supernatant was then removed, as before. The DNA was adsorbed to the silica-gel membrane of the MinElute column in the presence of high salt while contaminants pass through the column. After that, the other impurities were washed out with 750 µl of Buffer PE. Finally, 10 µl of Buffer EB was added to elute the DNA.

3.3.2.2 Ligation of the purified CCR5 promoter fragment

The purified CCR5 promoter fragment was cloned into vector by using the pGEM®-T Easy Vector Kit (Promega, USA). The ligation of CCR5 DNA fragment into the vector was performed according to the protocol recommended by the manufacture. In brief, an appropriate amount of the purified DNA was added to the ligation reaction mixture containing the Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG), 50 ng of the pGEM®-T Easy Vector and 3 Weiss units of T4 DNA ligase at final volume of 10 µl. After mixing, the reaction was incubated at 4°C overnight.

3.3.2.3 Transformation of the recombinant plasmid DNA

The recombinant plasmid from 3.3.2.2 was transformed into *E.coli* competent cells (JM109 strain; Promega, USA). The transformation was done, as the protocol recommended by the manufacturer. Briefly, 2 µl of each ligation reaction were added into 50 µl of competent cells in a sterile 1.5 ml microcentrifuge tube on ice, flicked the tubes to mix and placed them on ice for 20 minutes. Then, the cells were heat shocked for 50 seconds in a water bath at temperature exactly 42°C and the tubes were returned immediately to the ice bath for 2 minutes. Nine hundred and fifty microliters of room temperature SOC medium were added into the reaction tubes containing cells transformed with ligation reaction and incubated for 1.5 hours at 37°C with slowly shaking (~150 rpm). One hundred microliters of transformation culture was plated onto LB plate containing 100 µg/ml of ampicillin and incubated at 37°C overnight. After incubation, the bacterial colonies were screened for the presence of recombinant plasmid.

3.3.2.4 Screening for the recombinant plasmid DNA in the transformed bacteria

The plasmid DNA was extracted from the bacterial colonies by mini-preparation with alkaline lysis method. Briefly, a single bacterial colony was cultured in 2 ml of LB medium containing 100 µg/ml of ampicillin in a loosely capped 15 ml tube and incubated at 37°C for 24 hours with vigorous shaking. After 24 hours incubation, the culture was transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged at 12,000 x g for 30 seconds at 4°C. The supernatant was removed and the bacterial pellet was suspended in 100 µl of ice-cold Solution I and vortex shortly. Then, 200 µl of Solution II was added and mixed the contents by inverting the tube and

incubated on ice for 5 minutes. One hundred and fifty microliters of ice-cold Solution III was added and mix by gentle vortexing, then incubated on ice for 5 minutes. After centrifuged at 12,000 x g for 5 minutes at 4°C, the supernatant was collected and precipitated the DNA with 2 volumes of 95% ethanol, then centrifuged as above. The supernatant was removed by gentle aspiration. To ensure that the impurities was removed, 0.5 volumes of 7.5 M ammonium acetate was add, stored at -70°C for 10 minutes, and centrifuged to collect the supernatant. Two volumes of 95% ethanol were added again to precipitate the DNA. The DNA pellet was rinsed with 1 ml of 70% ethanol and allows the DNA pellet to dry in air for 10 minutes. Finally, the DNA was dissolved in 30 µl of distilled water and stored at -20°C until further examination.

The recombinant plasmid was quick screened by running on 1% agarose gel electrophoresis as described before. The analysis was based on the size differences between the inserted and non-inserted vectors, the pGEM®-T Easy Vector when inserted with CCR5 promoter fragment, the size was increased to approximately 4.2 kb, while the non-inserted one was only 3.0 kb. The colonies containing 4.2 kb plasmid DNA were selected and confirmed for the presence of inserted DNA by PCR technique using R5-PF and R5-PR primers.

3.3.3 Sequencing of the CCR5 promoter region

The purified recombinant plasmid DNA was sequenced by using the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (Amersham Bioscience, England). The sequencing products were resolved on the Long-Read Tower Sequencer (Visible Genetic, USA).

3.3.3.1 Purification of the plasmid DNA

The plasmid DNA was purified from the transformed *E. coli* by using the High Pure Plasmid Isolation Kit (Roche Molecular Biochemicals Mannheim, Germany) according to the procedure recommended by the manufacture. Briefly, a single transformed bacterial colony was cultured in 2 ml of LB medium containing 100 µg/ml of ampicillin in a loosely capped 15 ml tube and incubated at 37°C for 24 hours with vigorous shaking. After 24 hours incubation, 1.5 ml of the culture was transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged at 12,000 x g for 30 seconds at room temperature. The supernatant was removed and the bacterial pellet was resuspended in 250 µl RNase A/Suspension Buffer (RNase A in 50 mM Tris-HCl, 10 mM EDTA,

pH 8.0) and vortex shortly. Then, 250 µl of Lysis Buffer (0.2 M NaOH, 1% SDS) was added and mixed the contents by inverting the tube, then incubated for 5 minutes at room temperature. After incubation, 350 µl of chilled Binding Buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2) was added and mixed by gentle vortexing and then incubated on ice for 5 minutes. After centrifuged at 12,000 x g for 10 minutes, the supernatant was collected and applied to the High Pure filter tube. The plasmid DNA was bound to the glass fiber pre-paged in the High Pure filter tube. The bound plasmid DNA was washed twice with Wash Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) to remove the protein and other cellular contents and the plasmid DNA was eluted with 100 µl of Elution Buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C.

3.3.3.2 Quantity of purified recombinant plasmid DNA

The quantity of recombinant plasmid DNA was determined by using the spectrophotometry as described in 3.2.3. Only the sample that the ratio of the OD at 260/280 nm greater than 1.8 was used for the sequencing experiment.

3.3.3.3 Dye terminator cycle sequencing

The nucleotide sequence of the CCR5 promoter region was determined by dye terminator cycle sequencing method using the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (Amersham Bioscience, England). The Kit contained all sufficient reagents for dideoxy cycle sequencing reaction using Thermo Sequenase I DNA polymerase. In the reaction, the DNA template was combined with a single unlabelled primer, a thermostable polymerase, Cy5-labelled ddNTPs and dNTPs. This mixture was then subjected to repeated cycles of thermal denaturation and polymerization to generate products that terminate with specific Cy5 dye-labeled ddNTPs. The amount of chain terminated product was increased in a linear fashion in each round of denaturation and polymerization. The sequencing reaction was prepared as the procedure indicated in the instruction manual. Briefly, each of the sequencing reaction mixture was performed in a total volume of 8 µl containing 2 µl of the dNTPs/Cy5 ddATP mix, dNTPs/Cy5 ddCTP mix, dNTPs/Cy5 ddGTP mix or dNTPs/Cy5 ddTTP mix, 500 ng of the purified DNA template, 4 pmol of Modified M13 Universal primer or R5-PR primer, reaction buffer (150 mM Tris-HCl, pH 9.5, 67 mM $MgCl_2$) and 10 U of Thermo Sequenase I DNA

polymerase. The mixture was placed in the thermal cycler and running for 35 cycles. The cycling conditions were at 95°C for 30 seconds, 60°C for 45 seconds and 72°C for 80 seconds. After completion of the cycling program, the tubes were briefly centrifuged to collect any condensation and placed them on ice.

3.3.3.4 Purification of the sequencing products

The unincorporated dye terminators were removed from the sequencing reaction by using ethanol precipitation. Two microliters of each 7.5 mM ammonium acetate, Glycogen solution (Amersham Bioscience, England) and 30 µl of chilled absolute ethanol were added into the tubes and placed them on ice for 20 minutes to precipitate the DNA. To collect the precipitate, the tubes were then centrifuged at 14,000 x g at 4°C for 20 minutes. The supernatant was removed and the pellet was washed once with 200 µl of 70% ethanol. After the ethanol was removed, the pellet was dried at 37°C for approximately 3 minutes. Finally, each of the pellets was dissolved in 6 µl of Formamide loading dye (Amersham Bioscience, England) and mixed by vigorous vortexing to ensure that they were completely dissolved.

3.3.3.5 Long-Read Tower™ sequencer analysis

Long-Read Tower System version 3.1 (Visible Genetic, USA) was used to analyze the nucleotide sequence of CCR5 promoter regions. The system included Long-Read Tower™ sequencer, which is an automated fluorescence-based electrophoresis unit, GeneObjects™ software, Gel Toaster™ unit, SureFill™ Injector, and all essential instruments for acrylamide gel electrophoresis. Prior to load the samples onto the gel, the SureFill™ 6% Sequencing gel (Visible Genetic, USA) was casted in MicroCel 700 Cassette (Visible Genetic, USA) using SureFill Injector. The gel was then polymerized in the Gel Toaster unit for 3 minutes. The samples from 3.3.3.4 were heated at 70°C for 3 minutes to denature and then immediately placed on ice. Two microliter of each sample was loaded into the appropriate well of the MicroCel 700 Cassette and ran in a TBE buffer at 2,000 volts for 180 minutes.

3.3.3.6 Analysis of the nucleotide polymorphism of CCR5 promoter sequences

BioEdit Sequence Alignment Editor version 5.0.9 software (Hall, 1999) was used to analyze and compared the nucleotide sequences with together reported sequences obtain from GenBank database.

3.4 Determination of the CCR5Δ32 genotype

For determination of the 32 bp deletion of the CCR5 ORF (CCR5Δ32), the CCR5 ORF was amplified by PCR technique with primers flanking the deletion region and analyzed by fragment size differences.

3.4.1 Amplification of the CCR5 ORF by PCR

The PCR reaction mixture was performed in a total volume of 50 µl containing 40 µM of each primers, R5-32F and R5-32R, reported by Quillent *et al*, 1998, 200 µM of each dNTPs, 1.25 units of *Taq* DNA polymerase (Promega, USA), PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100), 1.5 mM of $MgCl_2$, and 50-100 ng of genomic DNA.

The reaction was amplified for 30 cycles in the thermal cycler. Each cycle consisted of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. After the last cycle, the extension step was extended for another 7 minutes to ensure that the extension was completed.

The amplified product was subjected to 2% agarose gel electrophoresis in TAE buffer at 100 volts for 90 minutes. After electrophoresis, the gel was stained with 2 µg/ml of ethidium bromide, destained with distill water, visualized, and photographed under UV light. The DNA fragment size was determined by comparison with a 1 Kb Plus DNA Ladder. A single band of DNA fragment approximately 198 bp indicate a homozygous CCR5 wild-type allele while the homozygous CCR5Δ32 allele was166 bp. The heterozygous type was shown to have both 198 and 166 bp fragments.

3.5 Determination of the CCR5-m303 genotype

For determination of the single point mutation (T to A substitution) at position 303 of the CCR5 ORF (CCR5-m303), the Nested-PCR and restriction enzyme analysis (REA) were

used. The test was performed according to the protocol modified from those described by Quillent *et al*, 1998.

3.5.1 Amplification of the CCR5 ORF by Nested-PCR

The CCR5-m303 flanking region was amplified by Nested-PCR with the two sets of primer. First round PCR reaction mixture was performed with a total volume of 50 µl. The final reaction mixture contained 40 µM of each R5-32F and R5-32R primers, 200 µM of each dNTPs, 1.25 units of *Taq* DNA polymerase (Promega, USA), PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100), 2.5 mM $MgCl_2$, and 50-100 ng of genomic DNA.

The reaction was amplified for 30 cycles in the thermal cycler. Each cycle consisted of denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 1 minute for 30 cycles. After the last cycle, the extension step was extended for another 7 minutes.

The second round PCR was performed as the protocol described above except for the R5-303NF and R5-303NR primers were used. Two microliters of the first round PCR product was added as the DNA template. The PCR cycling conditions were denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute for 30 cycles. After the last cycle, the extension step was extended for another 7 minutes to ensure complete extension.

The amplified product was analyzed by using 1% agarose gel electrophoresis in TAE buffer at 100 volts for 1 hour. After electrophoresis, the gel was stained with 2 µg/ml of ethidium bromide, destained in distill water, visualized and photographed under UV light. The DNA fragment size was determined by comparison with a 1 Kb Plus DNA Ladder marker.

3.5.2 Detection of the CCR5-m303 genotype by Restriction Enzyme Analysis (REA)

The amplified product of the CCR5 ORF from 3.5.2 was then digested with *Hinc*II restriction endonuclease (Promega, USA). The reaction mixture contained 5 U of *Hinc*II , RE Buffer B (60 mM Tris-HCl pH 7.5, 500 mM NaCl, 60 mM $MgCl_2$ and 10 mM DTT), 0.1 mg/ml of Acetylated BSA and 1 µl of second round amplified product in a total volume of 20 µl. The mixture was incubated in water bath at 37°C for 4 hours.

The wild-type CCR5 ORF amplicon was digested into 2 fragments of approximately 528 and 209 bp, whereas the mutant, CCR5-m303 was not (Figure 7). The DNA fragments were analyzed in 3% agarose gel electrophoresis at 100 volts for 1 hour.

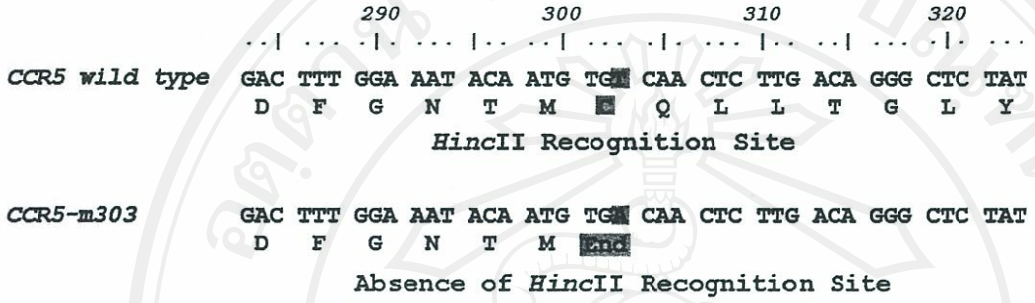


Figure 7 The nucleotide sequences and *Hinc*II recognition site (GTY˅RAC) of wild type and mutant allele.

3.6 Determination of the CCR5 protein density on surface of CD4+ lymphocytes and monocytes

3.6.1 Determination of CCR5 on the surface of CD4+ T lymphocytes

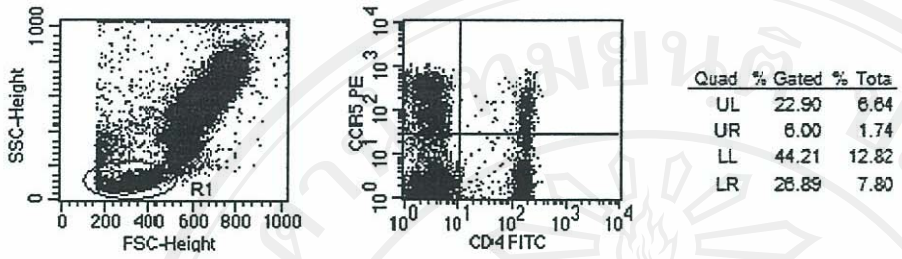
The expression of CD4 molecules and CCR5 molecules on white blood cells were determined by a direct immunofluorescent technique. In brief, 50 µl of fresh EDTA whole blood was stained with 10 µl of PE-conjugated anti-CCR5 mAb or PE-conjugated IgG2a isotype control for 15 minutes in the dark at room temperature. Then, 10 µl of FITC-conjugated anti-CD4 mAb was added and incubated as before. To lysis red blood cells, 450 µl of FACS Lysing Solution (Becton Dickinson, CA, USA) was added and incubated for 10 minutes in the dark at room temperature and centrifuged at 500 x g for 5 minutes. The cell pellet was collected and washed once with PBS containing 0.01% sodium azide. Cells were then fixed in 1% paraformaldehyde in PBS.

3.6.2 Determination of CD14 and CCR5 molecules on the surface of white blood cells

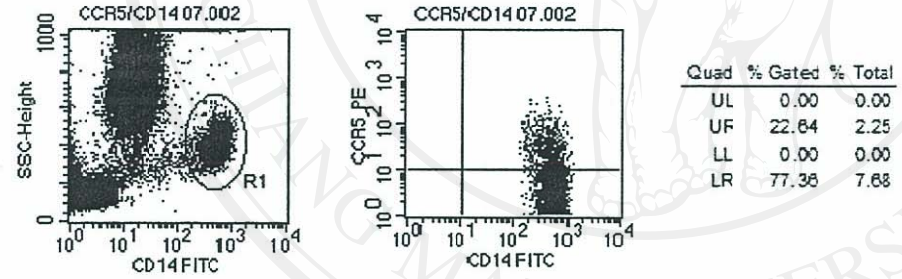
The expression of CD14 and CCR5 molecules on the surface of white blood cells were determined by the direct immunofluorescent technique with the same procedure as described in 3.6.1. Except for blocking the Fc-receptors, the sample was incubated on ice with 150 µl of an AB serum for 30 minutes before being incubated with PE-conjugated anti-CCR5 mAb or PE-conjugated IgG2a isotype control. The FITC-conjugated anti-CD14 mAb was then added into both tubes.

3.6.3 Flow cytometric analysis

Two-color flow cytometric analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) by using CellQuest software (Becton Dickinson, CA, USA). For determination of the membrane CCR5 on CD4+ T lymphocytes, exactly 10,000 events of lymphocytes were counted for each stain. The CCR5 expression of CD4+ lymphocytes was obtained after gating for lymphocytes based on forward scatter (FSC) and side scatter (SSC) and then gating on CD4 positive cells (Figure 8A). For determination of the membrane CCR5 on monocytes (CD14+), exactly 6,000 events of monocytes were acquired. The CCR5 expression of monocytes was obtained after gating on CD14 positive cells and side scatter and then gating on CD14 positive cells (Figure 8B).



A



B

Figure 8 Illustration of EDTA whole blood stained with the specific antibodies; (**A**) Anti-CCR5 PE and anti-CD4 FITC gated on lymphocytes (R1) and then gated on CD4+ cells, (**B**) Anti-CCR5 PE and anti-CD14 FITC gated on CD14+ monocytes (R1) and then gated on CD14+ cells.

3.6.4 Quantitative determination of the CCR5 protein density on the surface of CD4+ lymphocytes and monocytes

The CCR5 protein density on the surface of CD4+ T lymphocytes and monocytes (CD14+) was calculated by converting the fluorescence intensity from the flow cytometric analysis into antibody-binding capacity using standard microbeads conjugated with known amount of PE (QuantiBRITE™PE; Becton Dickinson). These microbeads were preformed by adding 500 µl of PBS with azide plus 0.5% BSA into a lyophilized pellet of QuantiBRITE™PE, vortices and analyzed by flow cytometry using the same software as used in the assay. The geometric means fluorescence intensities of the four sets of conjugated microbeads were used for construction of a calibration curve (mean fluorescence intensity against number of PE molecules per bead). After subtract the geometric mean fluorescence for the isotype control from the geometric mean fluorescence of the CCR5-PE positive cells, the number of PE molecules bound per cell (CD4+ lymphocyte and CD14+ monocytes) as the CCR5 density was calculated according to the geometric means fluorescence intensity of the cell sample by interpolation on the calibration curve.

3.6.5 Statistical analysis

The Mann-Whitney U test was used to compare similar parameters between two separate groups of individuals. *P* values of <0.05 were considered to be statistically significant.