CHAPTER III

MATERIALS AND METHODS

PART I : DEVELOPMENT OF DUPLEX SELECTIVE PCR FOR GENOTYPIC ANALYSIS

1.1 Control

The controls for evaluation of the duplex selective PCR are divided into two groups :

- Wild type positive controls : wild type (WT) HIV-1 (III_B) virions and wild type plasmid, both of which have the WT amino acid at codon 103(K), 181(Y), 151 (Q), and 215(T).

- Mutant positive controls : 151(M) mutant plasmid at codon 151(M) and sequencing analysis proven isolates for MT amino acid at codon 103(N), 181(C), and 215(Y/F).

The wild type plasmid and 151(M) plasmid were kindly provided by Dr. Hiroaki Mitsuya (NIH, Bethesela, Maryland, U.S.A.).

1.2 Primer Design

According to the published drug resistance mutation in Los Alamos HIV Drug Resistance Database, this study has focused on the most common and crucial mutations (codon 103, 181, 151, and 215) for designing the primers. All oligonucleotide primers were designed with the primer analysis software OLIGO version 4.1 (National Biosciences Inc, Plymouth, MN) and the published sequence HIV-1 genotype A/E strain CM240 (Accession No. U54771) information and were synthesized by GENSET Singapore Biotech, Pte Ltd. (Appendix I).

Duplex Selective PCR involves two rounds of PCR. The primer OU (outer upper) and OL (outer lower) primers which encompassed the *RT* gene from position

1969 to 2796 (according to U54771) were used for first round PCR (Appendix I, Table XI).

For the second round PCR (duplex selective amplification), duplex selective primers was designed by the principle of the Amplification Refractory Mutation System (ARMS) to increase the specificity of the PCR. This technique improves the specificity of primers by introducing additional mismatches close to the 3'-terminal nucleotide in either WT or MT primers. The primers for different codons of interest were designed to yield different PCR product sizes and to allow identical conditions in all PCR reactions.

1.3 Optimization of duplex selective PCR condition

Duplex Selective PCR

Duplex selective PCR was done by using a "double" PCR procedure and using WT plasmid (as described in 1.1) as a control for optimization of duplex selective PCR condition.

In the first round PCR (pre-amplification), the conserve region of RT gene (1969-2796) was amplified by OU and OL primers that anneal to conserved sequences. The reaction mixture contains 1x PCR-buffer, 1.25 mM $MgCl_2$, 0.4 mM each dNTPs, 25 pmol of each primer and 1.25 Units *Taq* polymerase (Promega). The volume is corrected to 45 μL using nuclease-free water (Gibco/BRL) and 5 μL of plasmid is added. The PCR conditions include pre-heat at 94°C for 5 min, then 40 cycles of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C, with a final extension step at 72°C for 7 min. All reactions were performed in a GeneAmp PCR system 9700 (Perkin-Elmer, Norwalk Conn., U.S.A.).

In the second round PCR (duplex selective amplification), separate selective PCR were done for analysis of individual primer sets of each codons. If individual primer sets worked correctly (no false positive), the primers were then combined into duplex selective PCR reaction. If did not work correctly (false positive was observed as a result of non-specific priming), the primers were redesigned and the PCR condition were re-optimized.

In this study, duplex selective PCR was divided into two groups by different optimal condition as follow :

1. K103N / Y181C duplex selective PCR
2. Q151M / T215Y/F duplex selective PCR

Details on two groups of duplex selective PCR are given in Table V and VI. Briefly, three microliter of first-PCR product was used in each duplex selective PCR amplification. The PCR products of 141, 281, 374, and 468 bp (Figure 1) were obtained from amplification codon 103, 181, 151, and 215, respectively.

Table IV : Conditions of K103N/Y181C and Q151M/T215Y/F duplex selective PCR

PCR	Primers	PCR conditions	Cycling Conditions
1. K103N/Y181C duplex selective PCR	IU, 103WT[a] and 181WT[c] or IU, 103MT[b] and 181MT[d]	1X-PCR buffer 1.25 mM $MgCl_2$ 0.4 mM each dNTPs 10 pmol primer IU 15 pmol primer 103WT or MT 5 pmol primer 181 WT or MT 1.25 Units *Taq* polymerase	Gene Amp PCR system 9700 pre-denature at 94°C for 5 min. 30 cycles of 94°C for 30 sec 48°C for 30 sec 72°C for 30 sec final extension at 72°C for 7 min.
2. Q151M/T215Y/F duplex selective PCR	IU, 151WT[e] and 215WT[g] or IU, 151MT[f] and 215MT[h]	1X-PCR buffer 1 mM $MgCl_2$ 0.4 mM each dNTPs 10 pmol primer IU 5 pmol primer 151WT or MT 12 pmol primer 215WT or MT 1.25 Units *Taq* polymerase	Gene Amp PCR system 9700 pre-denature at 94°C for 5 min. 30 cycles of 94°C for 30 sec 50.5°C for 30 sec 72°C for 30 sec final extension at 72°C for 7 min.

a for amplifying fragment carrying sequence AAA (amino acid K) at codon 103

b for amplifying fragment carrying sequence AAC (amino acid N) at codon 103

c for amplifying fragment carrying sequence TAT (amino acid Y) at codon 181

d for amplifying fragment carrying sequence TGT (amino acid C) at codon 181

e for amplifying fragment carrying sequence CAG (amino acid Q) at codon 151

f for amplifying fragment carrying sequence ATG (amino acid M) at codon 151

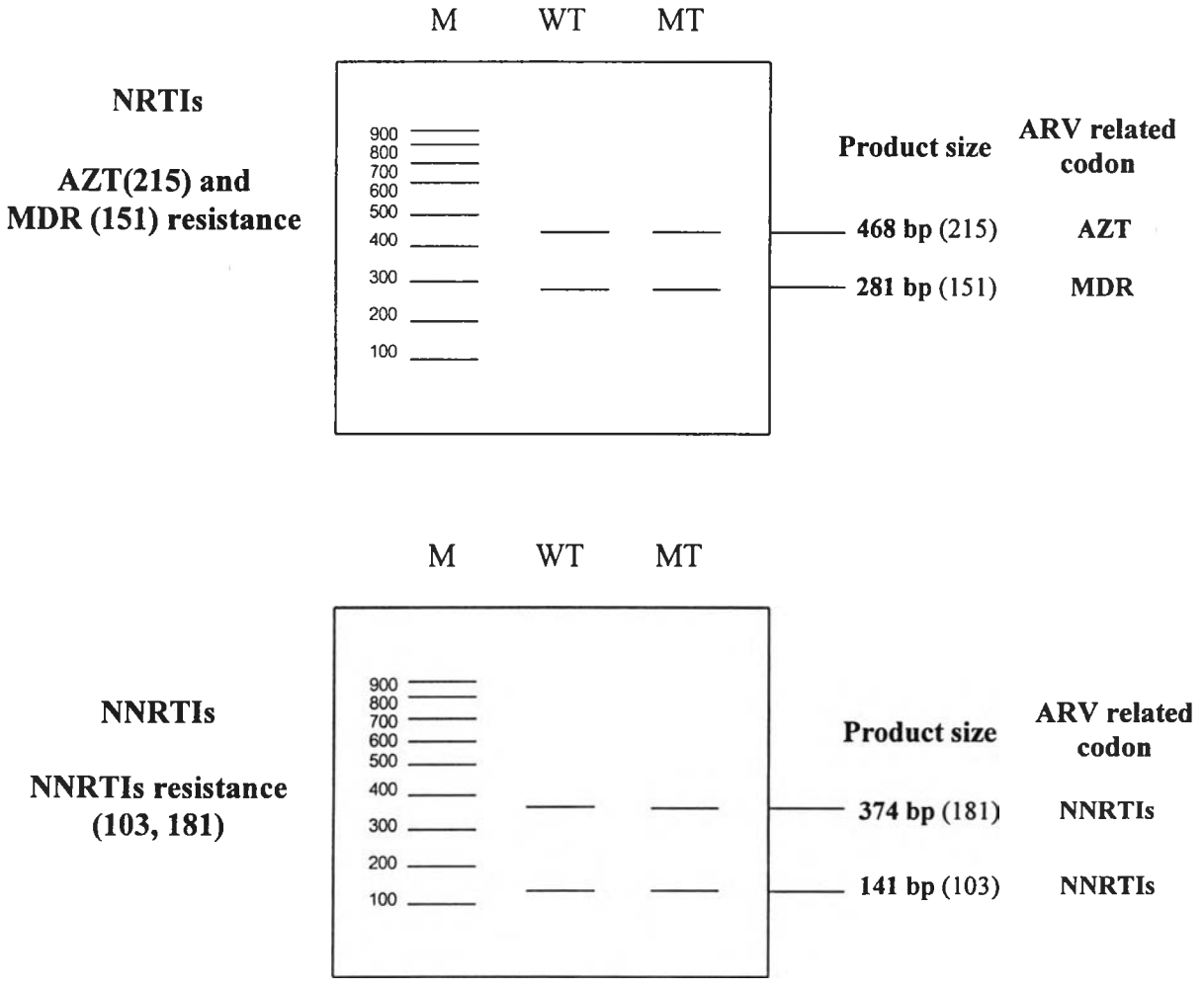
g for amplifying fragment carrying sequence ACT (amino acid T) at codon 215

h for amplifying fragment carrying sequence TTT(amino acid F) and TAT(amino acid Y) at codon 215

Analysis of Amplification products

PCR products were analyzed by agarose gel electrophoresis. The duplex selective PCR product (10 μL) was mixed with 2 μL 6X loading buffer (bromphenol blue indicator) and applied to the wells of a 1.5% agarose gel with 0.3 mg/mL ethidium bromide in Tris-borate-EDTA-buffer with a 100 bp DNA marker ladder (Bio-Rad). A voltage of 60 V was applied for 1 hour, and then the gel was visualized using a UV light transilluminator. The image was captured with a Polaroid-camera with black and white print film (Sigma). The genotype of each codon was read directly from the gel by measuring the product band sizes.

Figure 1 : Diagram for the duplex selective PCR patterns in agarose gel electrophoresis



1.4 Specificity and sensitivity of the duplex selective PCR

To indicate the sensitivity and specificity of the duplex selective PCR, the primer pairs were tested before and after combining into the duplex selective PCR by using serial dilutions of WT plasmid (vary from 500 to 10^6 copies) and WT HIV-1 (III_B) virus stocks (vary from 500 to 500,000 copies/mL).

PART II : GENOTYPIC RESISTANCE STUDY IN HIV-1 INFECTED PATIENTS

2.1 Study group

Forty-five HIV-1 infected patients from the Immune Clinic of King Chulalongkorn Memorial Hospital and Anonymous Clinic, Thai Red Cross AIDS Research Centre were recruited into this study by the basis of the following inclusion criteria :

- Confirmed anti-HIV seropositive
- Plasma HIV-1 RNA level of more than 1,000 copies/mL.

This study protocol was approved by the Human Ethical Committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from all study patients.

The patients were divided into 2 groups

Group I : Antiretroviral-naïve patients who had no prior treatment with antiretroviral drugs **(ART-naïve)** (n=20)

Group II : Antiretroviral-experienced patients who had received to at least two nucleoside analogues and/or non-nucleoside analogues for longer than 6 months **(ART-experienced)** (n=25)

2.2 Specimen Collection

Five mililiters of blood was collected from each subject by venepuncture into EDTA tubes. Plasma was separated by centrifugation at 3,000 g for 10 min within 3 hours after collection. The plasma samples were immediately frozen at -70°C until they were ready for processing and analyzed.

2.3 Plasma HIV RNA Preparation for PCR Analysis

One milliliter EDTA plasma is centrifuged at 17,000 rpm for 1 hr at 4°C. The supernatant is carefully discarded. Viral RNA is extracted from the viral pellet by adding 600 μL of lysis buffer (Guanidium isothiocyanate) then incubated for 10 min at room temperature. The RNA is precipitated with 600 μL of isopropanol, centrifuged at 14,000 rpm for 15 min at room temperature. The pellet is washed again with 1 mL of 70% ethanol and centrifuged at 14,000 rpm for 5 min at room temperature. The RNA pellet was resuspended in 20 μL of sample diluent or DEPC-treated water. RNA sample can be stored at -70°C until amplification.

2.4 cDNA Synthesis

Reverse transcriptase reaction was carried out as a single tube reaction with uninterrupted thermal cycling by using the GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, Conn.) as follow. Reaction solution was prepared and dispensed in 20 μL aliquotes to thin-walled MicroAmp tube. The final reaction mixture contained 5 μL of RNA sample, 25 pmol of primer OL, 1X RT-buffer, 5 mM $MgCl_2$, and 2 mM dNTPs. Added with 100 U of recombinant Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase and 80 U of RNase Inhibitor (Promega). The reaction was incubated at 37°C for 30 min. cDNA sample were stored at -20°C until amplification.

2.5 PCR Amplication

For analysis of genotypic resistance of the RT gene (Codon 103, 181, 151, and 215), the RNA was reverse transcribed to cDNA. Then cDNA was amplified by using "double" PCR procedure as described in 1.1.3 procedures. The WT plasmid (as described in 1.1) was used as positive controls, and nuclease-free water was used as a negative control.

2.6 Analysis of Amplification Product

Ten microliters of the PCR product was analyzed as described in 1.1.3 procedure. A sample was scored WT when PCR with WT primer gave the fragment

of highest intensity, a sample was considered to consist of mutant forms when the band with highest intensity was observed with mutant primer. A sample was judged to constitute a mixture when two fragments of similar intensity were seen and that sample was included in the mutant group in our statistical analysis.

2.7 Evaluation of duplex selective PCR results in HIV-1 infected patients

Sequencing analysis was applied for detection of drug resistance mutations in ten of the 20 ART-naïve patients and all of the ART-experienced (25) patients for evaluating the duplex selective PCR results.