# CHAPTER III

# METHODS AND STUDY POPULATIONS

## 3.1. Study samples in Thailand

This was a retrospective, observational study utilizing stored plasma samples from two different cohorts that were assayed by the standard genotype method with undetectable Y181C and M184V reverse transcriptase mutations. Plasma samples were from the *TREAT Asia* (Therapeutics, Research, Education, and AIDS training in Asia) Studies to Evaluate Resistance (*TASER-S*) surveillance cohort study for naïve patients and the HIV Netherlands Australia Thailand Research Collaboration (HIV-NAT) long-term cohort study for pretreated patients. Samples from the TASER-S study were collected between 2008 and 2010 from 104 homosexual clients attending the Thai Red Cross Anonymous Clinic who were recently infected with HIV-1, ARV-naïve and older than 18 years. Stored plasma with no major DRAMs detected by conventional genotyping from adults with recent HIV infection (i.e., positive Calypte® HIV-1 BED capture enzyme immunoassay (Calypte Biomedical Corporation, Maryland, USA) or previous negative HIV test in past year) were used for this study. Samples from HIV-NAT were obtained from 22 patients with virological failure attending the HIV-NAT clinic. Virological failure was defined as having two consecutive viral loads (VL) $\geq$ 1,000 copies/ml [31]. Blood samples were collected while the patients were on their failing regimen.

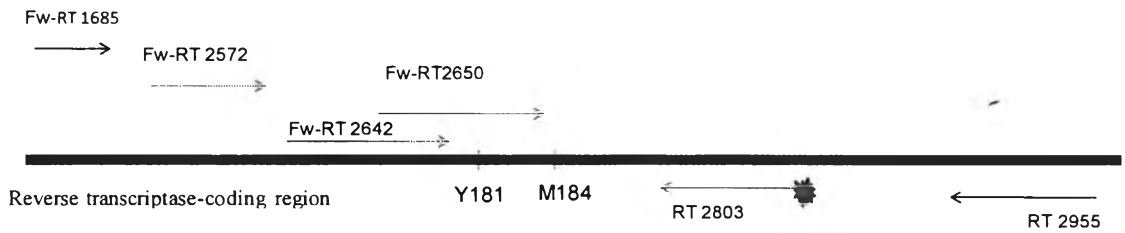
The study protocol was reviewed and approved by the Human Ethical Committee Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Thailand.

### 3.2. Pyrosequencing assay

Based on the reference sequence of CRF01_AE clade, CM240 [173], the amplification and sequencing primers for the PSQ assay were constructed by using the PyroMark Assay Design Software (Biotage Ltd.,Upsala, Sweeden). The assay was performed as previously described [174, 175]. Briefly, DNA was amplified with forward primer RT 2572 (5'-CAC AGG GAT GGA AAG GAT CAC-3') and biotin-labeled reverse primer RT 2803 (5'- AAT GGA GGT TCC TTC TGA TGC-3') to produce biotinylated PCR products. The PyroMark Vacuum Prep Workstation ™ was used to wash the biotinylated PCR products. Biotinylated 252 bp amplicons were binded on Streptavidin-coated Sepharose™ beads on a 96-well plate. The beads captured onto filter probes of a vacuum tool were washed with 70% ethanol, denaturation buffer (0.2 M NaOH) to separate the two strands of the PCR product, and washing buffer (10mM Tris-acetate pH7.6). Turning off the vacuum, beads coated with single stranded DNAs were released into a well of a PSQ™ 96- well plate containing sequencing primer and annealing buffer. The sequencing primers used to initiate PSQ were designed to stop just prior to the single nucleotide polymorphism of interest. Forward sequencing primer RT 2642 (5'-AAA AAA TCC AGA MAT RRT TAT-3') and forward sequencing primer RT 2650 (5'-CAG AAA TRR TTA TCT RTC AA-3') were used to detect Y181C and M184V, respectively (Figure 14). PSQ was performed using the PyroMark™Q96 ID system with AQ mode of SNP PSQ software.

To prepare standards, RT fragments derived from patients which contained either WT or Y181C or M184V mutants were cloned into pGEM®-T Easy Vector (Promega, Madison, USA) following the company's manual. The obtained plasmids were purified by QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA, USA), sequenced to confirm the presence of expected inserts, and quantified by NanoDrop (Thermo Fisher Scientific, Waltham, USA). Serial dilutions of plasmids harboring WT and mutant sequences were prepared and were

subjected to PSQ to identify the sensitivity and accuracy of the assays.




Amplification:

1st round PCR
F primer-PT1685 (5'-GGA ATT TTC CTC AGA GCA GAC CAG-3')
R primer-RT 2955 (5'-GCT TTA CCT TAA TCC CTG CAT AAT-3')
2nd round PCR
F primer- RT 2572 (5'-CAC AGG GAT GGA AAG GAT CAC-3')
R primer-RT 2803-biotin (5'- AAT GGA GGT TCC TTC TGA TGC-3')

Pyrosequencing:
Sequencing primer
Y181C RT 2642 (5'- AAA AAA TCC AGA MAT RRT TAT-3')
M184V RT2650 (5'- CAG AAA TRR TTA TCT RTC AA-3')

**Figure 14. Primers for pyrosequencing assays.**

### 3.3. Patients and methods for longitudinal analysis of minority N155H mutation in France

This retrospective study used plasma and whole blood samples collected at different time-points from 5 patients infected with HIV-1 subtype B who experienced virological failure with RAL-based salvage regimen at Saint-Louis Hospital, Paris, France. Informed consents were obtained from all 5 patients before proceeding. Since treatment options were limited in these patients, RAL therapy was still administrated for a period of time even when RAL-associated mutations were detected.

Plasma viral load was determined using Cobas Ampliprep/Cobas Taqman® HIV-1 (Roche, Meylan, France) and $CD4^+$T lymphocyte count was measured by flow cytometry using FACScalibur cytometer (Becton Dickinson, San Jose, California, USA).

Longitudinal stored samples before, during and after RAL-based regimen were analyzed. Plasma samples (1 ml) were ultracentrifuged at 28,100g for 2h at 4°C, and then were extracted using the QIAamp viral RNA minikit (QIAGEN Inc., Coutaboeuf, France). Total HIV-1 DNA was extracted from whole blood samples (MagnaPure, Roche Meylan, France). For standard genotyping, ANRS (Agence Nationale de Recherches sur le SIDA et les hépatites virales) consensus protocols (http://www.hivfrenchresistance.org) were used to amplify the regions of interest. Amplicons were subjected to purification (QIAquick PCR Purification Kit; QIAGEN Inc.) prior to sequencing using the BigDye Terminator Kit (Applied Biosystems, Villebon-sur-Yvette, France) on an ABI 3100 capillary genetic analyzer (Applied Biosystems). Sequence analysis was performed using Geneious software [176] with the HIV-1 subtype B HXB2 sequence as a reference. Mutations associated with nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and INIs were identified using the International AIDS Society-USA (IAS-USA) drug resistance mutations list [177]. Mixtures of WT and resistant residues were considered as resistant mutants.

Allele-specific PCR (AS-PCR) system with the detection threshold of 0.05% viral population developed by Charpentier *et al* was used for quantification of the N155H mutation in viral RNA and double-stranded DNA derived from plasma and whole blood samples, respectively. The details of assay were previously described elsewhere [152]. The AS-PCR for quantitative analysis of the N155H mutation was carried out in triplicate for each sample.

### 3.4. Samples and methods for analysis of genetic barrier to the development of resistance to INIs in HIV-1 subtypes CRF01_AE and B

It was a retrospective study with 57 stored plasma samples collected from patients in Saint Louis hospital, Paris, France. All samples were from patients who have never been exposed to INIs before the time of sampling. Genetic subtype identification was based on the sequences of reverse transcriptase and protease coding regions. Of 57 samples, 22 were collected from patients infected with HIV-1 subtype CRF01_AE and 35 were from patients infected with HIV-1 subtype B. To increase the sample size, 87 HIV-1 CRF01_AE sequences derived from samples of ARV-naïve patients from Cambodia (n=32), Vietnam (n=18), and Thailand (n=37) downloaded from database [61] were included in our study.

HIV RNA was extracted from 500 µl of plasma using QIAamp Viral RNA mini kit and a 1086 base pair fragment covering the entire IN gene was amplified using the ANRS consensus protocols as prescribed previously [178]. Sequence analysis was performed using Geneious software [176]. Using the HXB2 reference sequence, the nucleotide changes in codons for evolution from WT to drug resistance-associated substitution were assessed in 41 IN positions related to 66 RAL, EVG and DTG resistance-associated mutations which were categorized as follows: 1) RAL and EVG primary mutations (Q148H/K/R, N155H, Y143R/C, T66I, E92Q, S147G); 2) RAL and EVG secondary mutations (H51Y, T66A/K, L68V/I, V72I, L74M/A/I, E92K, T97A, Q95K, H114Y, S119R/G, F121I/Y, T125K, A128T, E138A/K, G140C/A/S, Y143H/ G, P145S, Q146K/P, Q148E/G, V151I/L, S153A, M154I, N155S/T, K156N, E157Q, K160N/D, G163K/R, V165I, E170A, V201I, I203M, T206S, S230R, N232D, V249I, R263K, C280Y); 3) DTG mutations (E92Q, L101I, A124T, Q148H/R, S153F/Y, G193E) [50, 51, 59, 60]. The genetic barrier was calculated for every substitution using the method published previously [54]. Briefly, transitions (ts) defined by the replacement of a purine by another purine (A ↔ G) or of a pyrimidine by another pyrimidine

(C↔T) occur on average 2.5 times more frequently than transversion (tv) defined by the replacement of a purine by a pyrimidine and vice versa (A↔C, A↔T, G↔T, G↔C). The smallest number of ts (scored as 1) and tv (scored as 2.5) needed for evolution from WT codon to mutated codon were calculated and the genetic barrier was taken as the sum of the scores for each amino acid position.