CHAPTER IV

RESEARCH DESIGN, MATERIALS AND METHODS

4.1 Research designs

The OLA target for this study is the M184V mutation of HIV-1 CRF01_AE which is associated with resistance to lamivudine (2',3'-dideoxy-3'-thiacytidine: 3TC) and emtricitabine (FTC). First, the primers and probes were designed based on the HIV-1 sequences of Thai patients published in the public database. Subsequently, the OLA condition was optimized and clinical performance was evaluated using archival plasma from HIV-1 infected patients who failed to respond to the first-line antiretroviral drugs with nevirapine-based treatment (N = 40). These specimens have been tested for the presence of HIV-drug resistance by standard genotypic sequencing, which is regarded as the gold standard of this study (Praparattanapan et al., 2011).

4.2 Materials and Methods

In this study, we developed, optimized and evaluated the performance of a probe-based Oligonucleotide Ligation Assay (OLA) for the detection of the M184V point mutation in the HIV-1 CRF01_AE reverse transcriptase enzyme that is associated with resistance to Lamivudine (3TC) and emtricitabine (FTC). The procedures were as follows.

4.2.1 Collection of clinical specimens

The archival plasma specimens from HIV-1 infected patients who were diagnosed with treatment failure (focusing on the M184V mutation) during the period of 2007-2009 (N = 40) served as specimens in the evaluation of clinical performance. Furthermore, the plasma from patients collected before 2005 was used for the construction of reference plasmids containing drug-resistant variants. The HIV in these specimens have been previously genotyped by standard sequencing methods as part of HIV-1/AIDS clinical care under the Thai national policy guidelines at the HIV-1 drug resistance laboratory, Division of Infectious Disease, Department of Internal Medicine, Faculty of Medicine, Chiang Mai University. This study was reviewed and approved by the Research Ethics Committee (REC), Faculty of Medicine, Chiang Mai University and by the Human Experimentation Committee (HEC) of the Research Institute for Health Sciences, Chiang Mai University, Chiang Mai, Thailand.

4.2.2 Collection of culture supernatant containing virus

The clinical isolate of HIV-1 subtype CRF01_AE (strain HM-12) (Utaipat, 2002) and the laboratory-adapted HIV-1 BaL of subtype B (Dr. Renu Lal, Centers for Disease Control and Prevention, USA, 1998) were used to construct the wild-type reference. HM-12 virus was propagated in the PHA-activated HIV-1 negative PBMC and BaL was propagated using the H9 cell line. The culture supernatant was harvested and stored at -70 ^{o}C until used for RNA extraction.

4.2.3 Designing of primers to generate PCR product for OLA

The primers for the *pol* region of HIV-1 circulating in Thailand were developed according to the previously described OLA with some modifications (Beck et al., 2008). These primers were designed based on multiple sequences of HIV-1 subtype CRF01_AE (N = 101) which were retrieved from the Los Alamos National Laboratory website (lanl Database, http:// www. hiv. lanl. gov) (accessed date 6.10.2007). The multiple sequences were aligned with CLUSTAL W2 software (http:// www. ebi. ac. uk/ Tools/ msa/ clustalw2/) then transferred to BioEdit Sequence Alignment Editor software (version 7.0.5.2) (Hall, 1999) for visual inspection. The modifications of primers were made by incorporating degenerate bases to accommodate polymorphisms encountered in the target sequences obtained from the CRF01_AE database.

4.2.4 Designing of OLA probes for M184V detection

The designation of the probe with substantial capacity to hybridize with HIV-1 sequences at high frequency rely on pre-existing information on HIV-1 genetic sequences. The synthetic oligonucleotide probes for OLA to detect M184V of HIV-1 subtype CRF01_AE in this study were designed based on the public HIV-1 sequence database (accessed via the internet) together with our own sequence database (accessed in 2007) of antiretroviral treatment failure patients. Multiple sequences (N=101 with accessed date 6.10.2007) of 45-bp regions encompassing 23-bp upstream and 22-bp downstream from the point mutation of M184V were aligned. The probe sequences were derived from the consensus in which the degenerate bases were incorporated at nucleotide variations positions. The detector probes (23-bp in

length) for wild-type or mutant variants were annealed at the point mutation toward the 5' direction, where the probe annealing downstream was used as a common probe. Because the principle of the OLA is probe ligation, the wild-type and the mutant variants can be distinguished by ligation between the detector probe, for the wild-type or the mutant, and the common probe. In addition, the probes specific for wild-type and mutant variants were labeled at the 5'end with digoxigenin and fluorescein (Operon Biotechnologies GmbH, Cologne, Germany), respectively. The common probe was biotinylated at the 3' end and phosphorylated at the 5' end (Bio Basic, Inc., Ontario, Canada).

4.2.5 Specimen preparation

4.2.5.1 RNA extraction

HIV-1 RNA extraction was performed using QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, the specimen was first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Purification of RNA was performed by adsorption onto the QIAamp silica-gel membrane. Wash conditions ensured complete removal of any residual contaminants without affecting RNA binding. Purified RNA was eluted from the silica-gel membrane by elution buffer. The extraction procedure is described as follows.

First, 560 µl of prepared buffer AVL containing carrier RNA was added into a 1.5 ml microcentrifuge tube. Next, 140 µl of plasma was added to the buffer AVL-carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 seconds. After that, the solution was incubated for 10 minutes at room temperature

(15-25 ^{o}C). Then, the tube was briefly centrifuged to remove drops from inside of the lid and 560 µl of ethanol (96-100%) was added to the sample. The solution was again mixed by pulse-vortex for 15 seconds. The tube was then briefly centrifuged and 630 µl of the solution was carefully transferred onto the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed and the tube was centrifuged at 6,000 g (8,000 rpm) for 1 minute. The QIAamp spin column was placed into a clean 2 ml collection tube and the tube containing the filtrate was discarded.

Afterwards, the QIAamp Mini spin column was opened and 630 µl of the solution was again applied onto the QIAamp Mini spin column and the cap closed. Then, the spin column was centrifuged at 6,000 g for 1 minute and the QIAamp spin column was placed into a clean 2 ml collection tube. The tube containing the filtrate was discarded. After that, the QIAamp Mini spin column was carefully opened, 500 µl of buffer AW1 was added and the cap was closed. The spin column was centrifuged at 6,000 g for 1 minute and the QIAamp spin column was placed into a clean 2 ml collection tube. The tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and 500 µl of buffer AW2 was added. Then, the cap was closed and the solution was centrifuged at full speed (20,000 g or 14,000 rpm) for 3 minutes.

To eliminate any chance of possible buffer AW2 carryover, the QIAamp spin column was placed into a new 2 ml collection tube and the old collection tube containing the filtrate was discarded. After the solution was centrifuged at full speed for 1 minute, the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and the old collection tube containing the filtrate was discarded. Then, the

QIAamp spin column was carefully opened and 40 µl of buffer AVE was added. The cap was closed and the solution was left to incubate at room temperature for 1 minute. Afterwards, the solution was centrifuged at 6,000 g for 1 minute, the QIAamp spin column was opened and another 40 µl of buffer AVE was added. The cap was closed. Then, the sample was left to incubate at room temperature for 1 minute and subsequently centrifuged at 6,000 g for 1 minute. Finally, the filtrate was collected in a clean 1.5 ml microcentrifuge tube and all of the filtrate was transferred into a new, clean 1.5 ml microcentrifuge tube. The purified RNA was used as the template of Reverse – Transcription Polymerase Chain Reaction (RT-PCR) which can either be used immediately or stored at -70^{o}C.

4.2.5.2 Reverse – transcription polymerase chain reaction (RT-PCR)

The HIV-1 RNA was reverse transcribed and amplified simultaneously using the One-Step RT-PCR kit (QIAGEN). The region of the first round PCR product starts from HIV-1 gene base position 1209 and continues to 2529 of AY713423 (or corresponds to 2011 to 3328 of HIV-1 HXB2 reference strain). The outer primers set included the UU1L_pol (5’ CCT AGR AAA AAG GGY TGT TGG AAA TGT GG 3’) and the UU1R_pol primers (5’ AAY TTY TGT ATG TCA TYG ACA GTC CA 3’). The RT-PCR was carried out in a 50 µl reaction mixture containing 10 µl HIV-1 RNA, 1X PCR buffer, 0.4 mM deoxynucloside triphosphates mix (dNTPs mix), 0.6 uM of the primers UU1L_pol, UU1R_pol and 2.5 U of One Step RT-PCR enzyme Mix and 10 U of RNase Inhibitor. The thermal profile for the One-Step RT-PCR was as follows: 50^{o}C for 30 minutes, 95^{o}C for 15 minutes, followed by 40 cycles of denaturation at 94^{o}C for 20 seconds, annealing at 55^{o}C for 20 seconds and extension

at 72°C for 2 minutes and the final extension of 72°C for 10 minutes to ensure the completion of amplification.

4.2.5.3 Nested polymerase chain reaction (Nested PCR)

The nested or the second round of PCR was performed using the inner primer set, the UU2L_pol (5' ACT GAR AGA CAG GCT AAT TTT TTA GGG A 3') and the UU2R_pol primers (5' CAT YTG TCA GGR TGG AGT TCA TA 3'). These primers amplify approximately a 1,200 bp fragment spanning from the 3' end of HIV-1 gag to codon 260 of the RT enzyme. The second round PCR was carried out in a 50 µl reaction mixture containing 0.2 mM each of deoxynucleotide triphosphate (dNTPs), 1X PCR buffer minus Mg^{2+} (50 mM KCl, 10 mM Tris-HCl, pH 8.8), 4.0 mM of $MgCl_2$, 0.4 uM of each UU2L_pol and UU2R_pol, and 0.1 U/µl of Platinum Tag DNA polymerase (Invitrogen, Brazil) and 2 µl of first round PCR products. The thermo cycling conditions were as follows: 94°C for 5 minutes, then 35 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 1 minute and the final step of 72°C for 7 minutes to ensure the completion of amplification. The 1,200 bp-amplified products were visualized by electrophoresis in 1% agarose gel and ethidium bromide staining. These amplified products were stored at -70°C and used as the starting material in the OLA.

4.2.5.4 DNA concentration measurement

To determine the amount of amplified DNA, the DNA concentration was measured using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Oregon, USA). The PicoGreen reagent is a proprietary, asymmetrical cyanine dye. Free dye

does not fluoresce, but upon binding to dsDNA it exhibits >1000-fold fluorescence enhancement. PicoGreen is 10,000-fold more sensitive than UV absorbance methods, and highly selective for dsDNA over ssDNA and RNA. The PicoGreen® dsDNA assay procedure is described as follows.

First, 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) was prepared from the 20X TE stock buffer, which was supplied in the PicoGreen kit. To make 50 ml (sufficient for 250 assays), 2.5 ml of 20X TE buffer was added into 47.5 ml sterile distilled DNase-free water. Next, the DNA standards were diluted from 100 µg/ml to 2 µg/ml using 1X TE buffer. The 600 µl of 2 µg/ml DNA standard was prepared by adding 12 µl of the 100 µg/ml stock DNA standard to 588 µl 1X TE. Then, the microplate was prepared and solutions were added into the wells according to the following Table 2.

Table 2 Microplate preparation.

Plate Well	Final [DNA] (ng/mL)	Vol. (µl) 2 µg/ml DNA standard	Vol. (µl) 1X TE buffer	Final Total DNA (ng)/Well
A1 & B1	25	2.5	97.5	5
C1 & D1	50	5	95	10
E1 & F1	100	10	90	20
G1 & H1	200	20	80	40
A2 & B2	300	30	70	60
C2 & D2	400	40	60	80
E2 & F2	500	50	50	100
G2 & H2	1000	100	0	200
A3 & B3	1200	120	0	240

For each unknown sample, 1 µl of PCR sample was added to 99 µl of 1X TE buffer in the microplate well and mixed by pipetting up and down. Then, a 1:200 dilution of the PicoGreen reagent in 1X TE buffer was prepared. The PicoGreen reagent is light sensitive, therefore, it was kept wrapped in foil while thawing and in the diluted state. For each standard and each unknown sample, a volume of 100 µl was needed. 100 µl of diluted PicoGreen was added to every standard and sample. Afterwards, the solution was mixed by pipetting up and down. The microplate was covered with foil and the solution was allowed to incubate at room temperature for 2-5 minutes. Afterwards, the microplate was read with excitation wavelength/ bandwidth at ~ 485 nm/ 20nm and detected emission wavelength/bandwidth at ~ 530 nm/ 25nm. Finally, a standard curve was generated using the average values of the standard s and the concentrations of DNA in the unknown samples were determined from the standard curve.

4.2.6 Construction of reference plasmids

The reference plasmids with known HIV-1 M184V genotype were constructed. Briefly, the PCR products derived from clinical specimens known to contain the wild-type or mutant genotypes were cloned into the pCR 2.1 TOPO vector (TOPO TA Cloning®, Invitrogen, CA, USA) and transformed into chemically competent Top 10 cells (*E.coli* competent cells) according to the manufacturer's instructions. The plasmid DNA was extracted and purified with the QIAGEN® Plasmid Purification kit (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions. Purified plasmid DNA was sequenced using M13 Forward primers (5' GTA AAA CGA CGG CCA G 3'), M13 Reverse primers (5'

CAG GAA ACA GCT ATG AC 3') and HXB2_2395 primers (5' GGG GAA TTG GAG GTT TTA TC 3'). All plasmids were sequenced with fluorescence-labeled dideoxy chain terminators (Big dye version 3.1, Applied Biosystems) on the ABI 3130 Genetic Analyzer, Applied Biosystems/ HITACHI) in order to confirm the presence of the M184 or 184V mutation in the HIV-1 *pol* gene. The plasmid controls preparation procedures are described as follows.

4.2.6.1 Transformation of recombinant plasmid DNA

The PCR products derived from clinical specimens known to contain the HIV-1 M184V wild-type or mutant genotypes were cloned into the plasmid vector to serve as the control for the OLA. To achieve this purpose, the second round PCR products were inserted into the pCR 2.1 TOPO vector (TOPO TA Cloning®, Invitrogen) and transformed into chemically competent Top 10 cells (E.coli competent cells) according to the manufacturer's instructions. The transformation was carried out by adding 1 μl of PCR product to competent cells in a screw cap tube and put on ice for 10 minutes. The cells were heat shocked for 30 seconds in a water bath at 42°C. Then, the tubes were returned immediately to the ice bath. 250 μl of S.O.C. medium was added into the reaction tube and incubated for 1 hour at 37°C with shaking at the rate of around 225 rpm. After that, 150 μl of transformation culture was plated onto an ampicillin X-galactose plate. The transformed bacteria were spread onto LB agar plate containing ampicillin and X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), which produces a blue/white color as an indicator for the colonies of interest. After incubation at 37°C for 16-18 hours, hundreds of bacterial colonies, mostly white, were typically observed. About 5-10 white colonies were randomly

picked to grow onto the new ampicillin-LB agar plate and the clones with the plasmid insert were screened by PCR.

4.2.6.2 Propagation and preparation of recombinant plasmid DNA

The clones with positive PCR were cultured in 3 ml of ampicillin-LB broth at 37^{o}C for 12-16 hours in order to propagate amplify the recombinant plasmid. The plasmids' mini-preparation were purified using QIAGEN® Plasmid Purification kit according to the procedure recommended by the manufacturer. Briefly, 3 ml of transformed bacteria was spun down. The supernatant was subsequently discarded. Then, the pellet was re-suspended and mixed using 300 μl of buffer P1. After 300 μl lysis buffer P2 was added, mixed and incubated at room temperature (RT) for 5 minutes. Afterward, 300 μl of buffer P3 was added, mixed by inversion, and incubated on ice for 5 minutes. Then, the sample was centrifuged at 14,000 rpm for 10 minutes at RT. During this time, the column was equilibrated with 1 ml of buffer QBT. Next, the supernatant was loaded onto the column. After buffer QC 2 ml was added into the column, the eluent was eluted with 800 μl of buffer QF. Then, isopropanol was added in the eluent at 0.7 times the volume of eluent. The eluent was centrifuged at 15,000 rpm for 40 minutes at RT. The supernatant was subsequently discarded. The pellet was washed with 1 ml of 70% Ethyl alcohol, mixed by pulse-vortex and centrifuged at 14,000 rpm for 10 minutes at RT. The supernatant was again discarded. After the ethanol was removed, the pellet was dried at 65^{o}C for 5 minutes. The plasmids were re-suspended with 50 ul of DNase/RNase-free water and kept at 4^{o}C until analysis.

4.2.6.3 Quantitation of the purified plasmid DNA

The plasmid DNA concentration was determined using spectrophotometry. The purity of the DNA sample indicated by the OD 260/280 ratio was observed. The DNA was diluted in TE buffer at a dilution of 1:5 and the Optical Density (OD) was measured for nucleic acid and protein at the wavelengths of 260 nm and 280 nm using the UV spectrophotometer. The purity of the DNA sample was determined by the OD 260/280 ratio. The concentration of DNA was calculated using the following equation:

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

4.2.6.4 Confirmation of wild-type and mutant genotypes

The plasmid DNA products were sequenced in order to identify the presence of the 184M (wild-type) or 184V (mutant) in the HIV-1 *pol* gene insertion. The DNA sequencing procedures were described as follows.

4.2.6.4.1 Preparation of DNA sequencing reaction

Approximately 120 ng of purified template was mixed with 4.0 µl of dye terminator premix containing A-dye T, C-dye T, G-dye T, T-dye T, dNTPs, Tris-HCl pH 9, MgCl2, AmpliTag DNA Polymerase. The sequencing primers (M13For, M13Rev and HXB2-2395) were added in a concentration of 3.2 pmol and adjusted to the total volume of 20 µl with DNase/RNase-free water. The mixture was mixed and placed in the thermocycler (MJ Research, Inc.). The reaction was run for 25 cycles. Each cycle was performed in 3 steps: denaturation at 95^{o}C for 10 seconds, primer annealing at 50^{o}C for 5 seconds and extension at 60^{o}C for 4 minutes.

4.2.6.4.2 Purification of sequencing products

The unincorporated nucleotide was removed from the reaction mixture using ethanol precipitation. Eight microlitres of 7M Ammonium acetate pH and 71.2 µl of 95% ethanol were added, and incubated at RT for 15 minutes (mixed by tapping every 3 minutes). Afterwards, the product was centrifuged at 15,000 rpm for 40 minutes at RT. The supernatant was discarded and the pellet was washed once with 70% ethanol. After the ethanol was removed, the pellet was dried at 65^{o}C for 5 minutes and kept at -20^{o}C until sequence analysis.

4.2.6.4.3 Identification of DNA sequences

The pellet of purified sequencing product was re-suspended in 20 µl of HiDi Formarmide. The mixture was mixed briefly by pulse-vortex ~200 times. The mixture was then centrifuged briefly and heated at 95^{o}C for 5 minutes before being brought to sequence analysis. All plasmids were sequenced with fluorescence-labeled dideoxy chain terminators (Big dye version 3.1, Applied Biosystems) on the ABI 3130 Genetic Analyzer (Applied Biosystems/HITACHI). The nucleotide sequencing data was shown as an electrophoregram. The multiple nucleotide sequence alignments between the samples and reference serotypes were obtained using computer programs.

4.2.7 Optimization of the OLA condition

OLA was performed following the protocol of the genotyping kits for the detection of HIV-1 *pol* drug-resistance mutations made available from the NIH AIDS research and reference reagent program (www.aidsreagent.org). Three important

components - the thermostable DNA ligase enzyme (ampligase DNA ligase, Epicentre Biotechnologies, Madison, WI, USA), the probes, and the template in the OLA reaction were verified in order to obtain the condition in which the wild-type and mutant variants can be optimally distinguished. The amplified PCR products (amplicons) derived from the plasmid controls were used as the template of the OLA. The variable concentration of the thermostable DNA ligase enzyme, probes and templates were optimized and are shown as follows.

DNA ligase enzyme concentration at 0.04, 0.08, 0.17, 0.34, 0.68 U

Probes concentration at 0.06, 0.12, 0.36, 1.08, 2.16 pmol

Templates volume at 0, 2.5, 5, 10, 0.05, 0.1, 1 µl

Templates concentration at 0, 2.5, 5, 10, 25, 50, 100 ng/µl

4.2.7.1 Oligonucleotide ligation assay (OLA)

The OLA for the detection of a HIV-drug-resistance mutation utilized two genotype specific oligonucleotides probes (the mutant-specific labeled with fluorescein, and the wild-type-specific labeled with digoxigenin) and a biotinylated oligonucleotide common to both genotypes. During ligation, the probes annealed to their complementary sequence in the PCR products and the genotype-specific oligonucleotide became covalently linked to the adjacent common probe. The biotinylated ligation products were captured on streptavidin-coated microtiter wells and an ELISA was performed with alkaline phosphatase (AP) labeled anti-fluorescein antibodies and horse-radish peroxidase (POD) labeled anti-digoxigenin antibodies. Sequential addition of the AP and the POD substrates allowed for detection of both genotypes in a single well. Otherwise, the clinical specimens and controls were

assayed in the same run and all samples were tested in duplicate. The procedures of the OLA are described follows.

4.2.7.2 Ligation reaction

The ligation reaction was set up in a thin-wall 0.2 ml tube. The amplified PCR products, the mixture of detector probes and common probe, and the ampligase DNA ligase enzyme were added in the presence of 20 mM Tris-HCl pH 8.3 at 25^{o}C), 10 mM $MgCl_2$, 25 mM KCl, 0.5mM NAD, 0.1% Triton X-100 in a 24-µl final volume. The reaction tube was placed in a thermo-cycler machine with the thermal profile set as follows; 10 cycles of 93^{o}C for 30 second and 37^{o}C for 4 minutes. Within 10 minutes of completion of the last cycle, 10 µl of 0.1 M EDTA / 0.1% Triton X-100 was added to stop the ligation reaction.

4.2.7.3 Detection of the ligation products

Detection of the ligation products was performed in a 96-well microtiterplate coated with streptavidin (Roche Diagnostics, Indianapolis, USA). The biotinylated-products from ligation were allowed to bind to streptavidin in the plate for 1 hour at room temperature. Any unbound products on the plate were washed off twice using 1X NaOH wash and 1X Tris wash. The washed plate was then blotted on a paper towel. 50 µl of the mixture of anti-digoxigenin conjugated with peroxidase and anti-fluorescein conjugated with alkaline phosphatase (Roche Applied Science, IN, USA) in BSA blocking buffer were added to the plate at 1:1000 dilution and incubated for 30 minutes at room temperature. Next, the plate was washed off 6 times by 1X Tris wash and then blotted again on a paper towel. The substrates to each enzyme were

serially added to reveal the presence of wild-type or mutant genotype in the template. The mutant was revealed first, 25 µl of GIBCO substrate reagent (Alkaline phosphatase amplification system, Invitrogen, California, USA) was added to each well. After incubation for 10 minutes at room temperature, 25 µl of GIBCO amplifier reagent (Alkaline phosphatase amplification system, Invitrogen, California, USA) was added into each well. Next, the plates were incubated for 10 minutes at room temperature or until the color in the positive controls turned magenta compared to the background color in the wild-type control wells, which remained light colored. The optical density (OD) of the magenta color products were measured at wavelength 492 nm by a spectrophotometer (TECAN: SUNRISE, Grodig, Austria). The wild-type genotype was revealed next. After the magenta products were washed-off 6 times by 1X Tris wash, 50 µl of TMB substrate reagent (Promega Corporation, Madison, WI, USA) was added to each well and incubated at room temperature for 10 minutes. The bound anti-digoxigenin conjugated with peroxidase and indicated the presence of the wild-type variant. This was demonstrated using TMB substrate, which turned the sample color blue. To stop the reaction, 50 µl of the acid solution (H_2SO_4) was added to each well. The blue color product was turned into a yellow color. The OD signal was measured at wavelength 450 nm. For the interpretation of OLA, the O.D. value above 0.2 was considered positive for mutant and wild-type detection.

4.2.8 Evaluation of the lower limit of the mutant variant detection by OLA

The known genotype plasmid controls for HIV-1 mutant (184V) and wild-type (184M) were used as the template in the OLA. The total DNA concentration 25 ng/µl of amplified PCR products derived from the wild-type (WT) plasmid were mixed with

increasing amounts of the amplified PCR products derived from the mutant (MT) plasmid from 1%, 3%, 5%, 10%, 20%, 30%, 40%, and 50%. These mixtures of amplified PCR products were analyzed using the optimal condition of OLA in parallel with 100% WT and 100% MT. The lower limit of mutant detection was evaluated. Then, the mixture of the amplified PCR products of wild-type plasmid control with increasing amount of 1%, 3% and 5% of the amplified PCR product of mutant plasmid control were tested by OLA for 5-10 replicated reaction to compare with 0% mutant control containing 100% wild-type.

Table 3 Proportion of WT and MT for determination of lower limit of mutant detection of OLA.

% MT	DNA concentration of MT (ng/µl)	% WT	DNA concentration of WT (ng/µl)
0	0	**100**	25
1	0.25	**99**	24.75
3	0.75	**97**	24.25
5	1.25	**95**	23.75
10	2.5	**90**	22.5
20	5	**80**	20
30	7.5	**70**	17.5
40	10	**60**	15
50	12.5	**50**	12.5
100	25	**0**	0

4.2.9 Evaluation of the performance of the OLA with a small set of plasmid controls and clinical specimens

The performance of M184V detection by OLA in a small set of plasmid controls and 40 clinical specimens were assessed using archival plasma specimens from HIV-1 infected patients who were diagnosed as having treatment failure focusing on M184V mutation between 2007 and 2009 at the Maharaj Nakorn Chiang Mai Hospital. These specimens were evaluated for HIV-DR by standard genotypic sequencing which was the gold standard method at the HIV-1 drug resistance laboratory (Praparattanapan et al., 2011). The OLA condition obtained after optimization was used in the performance evaluation. The performance of OLA in detecting M184V was compared with the genotypic sequencing, and the results were evaluated by statistical analysis. Any cases with indeterminate results by OLA, required further analysis of nucleotide sequences.

4.2.10 Identification of indeterminate results

Nucleotide sequencing of specimens with indeterminate results by OLA was carried-out. Afterwards, the residual PCR primers and dNTPs in PCR products of these specimens were removed using the PCR clean-up, NucleoSpin® Extract II kit (MACHEREY-NAGEL, Germany). The purified-PCR was directly sequenced by the DNA sequencing method described below.

4.2.10.1 Purification of amplified PCR products

The purification of amplified PCR products was done following the manufacturer's instructions for PCR clean up using the NucleoSpin® Extract II kit

(MACHEREY-NAGEL, Germany). The PCR clean up procedure is described as follows.

First, DNA binding conditions were adjusted by mixing 1 volume of sample with 2 volumes of buffer NT. Then, DNA was bound by placing a NucleoSpin® Extract II Column into a Collection Tube (2ml). After loading the sample, it was centrifuged for 1 minute at 11,000 g. The flow-through was discarded and the NucleoSpin® Extract II Column was placed back into the collection tube. Next, the silica membrane was washed by adding 600 µl Buffer NT3. Afterwards, the sample was centrifuged for 1 minute at 11,000 g, flow-through was discarded and the NucleoSpin® Extract II Column was placed back into the collection tube.

Dry silica membrane was centrifuged for 2 minutes at 11,000 g to remove Buffer NT3 quantitatively. It was ensured that the spin column was not exposed to direct contact with the flow-through while it was removed from the centrifuge and the collection tube. Flow-through was again discarded and the NucleoSpin® Extract II Column was placed back into the new collection tube. To elute DNA, the NucleoSpin® Extract II Column was placed into a clean 1.5 ml microcentrifuge tube. Then, 15-50 µl Elution Buffer NE was added and incubated at room temperature for 1 minute to increase the yield of eluted DNA. Finally, after the sample was centrifuged for 1 minute at 11,000 g, the eluent was collected and used as the template for the nucleotide analysis. This eluent can be used immediately or stored at -20 ^{o}C.

4.2.10.2 Nucleotide sequencing method

Cleaned PCR products were then directly sequenced using UU2L_pol, UU2R_pol primers and HXB2_2395 primers (5’ GGG GAA TTG GAG GTT TTA

TC 3'). All cleaned PCR products were sequenced with fluorescence-labeled dideoxy chain terminators (Big dye version 3.1, Applied Biosystems) on the ABI 3130 Genetic Analyzer (Applied Biosystems/ HITACHI). The nucleotide sequencing data was shown as an electrophoregram. The multiple nucleotide sequence alignments between the samples and reference serotypes were obtained by using computer programs. The specimens' nucleotide sequence were analyzed and evaluated for the indeterminate results.

4.2.11 Data analysis

The sensitivity and specificity of the OLA to detect HIV-1 M184V drug-resistant mutation were calculated by compared with the in-house genotyping results, the gold standard method. The sensitivity of OLA relative to sequencing is the proportion of samples positive by OLA among all positive samples. Sensitivity = [number of true positive samples / (number of true positive samples + number of false negative samples) x 100]. Specificity = [(number of false negative samples / number of true negative samples) x 100]. The concordance analysis was performed by summing the number of samples correctly identified as wild-type or mutant by OLA in the numerator, and dividing by the sum of the samples genotyped by bulk sequencing. Data management and statistical analysis was performed using SPSS software version 13.0.