การพัฒนาการตรวจวิเคราะห์การดื้อยาของเชื้อไวรัสเอชไอวี-1 โดยวิธีจีโนไทป์ที่มีต้นทุนต่ำ

นายสยมภู สงวนสิทธิอนันต์

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ปีการศึกษา 2555

THE DEVELOPMENT OF HIV-1 GENOTYPIC DRUG RESISTANCE TESTING FOR A LOW-COST ALTERNATIVE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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สยมภู สงวนสิทธิอนันต์ : การพัฒนาการตรวจวิเคราะห์การดื้อยาของเชื้อไวรัสเอชไอวี-1 โดยวิธีจีโนไทป์ที่มีต้นทุนต่ำ. (THE DEVELOPMENT OF HIV-1 GENOTYPIC DRUG RESISTANCE TESTING FOR A LOW-COST ALTERNATIVE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.ปาลนี อัมรานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : อ.ดร. นาวิน ห่อทองคำ, 100 หน้า.

การรักษาด้วยยาต้านไวรัส แบบ Highly Active Antiretroviral Therapy (HAART) เป็นทางเลือกที่ดีที่สุดในการควบคุม การเพิ่มปริมาณของไวรัสเอชไอวีในกระแสเลือด การตรวจการดื้อยาของไวรัส โดยวิธีจีโนไทป์จึงเป็นการตรวจที่ มีบทบาทสำคัญต่อประสิทธิภาพการ ดูแลรักษาผู้ติดเชื้อเอชไอวี ในปัจจุบันชุดตรวจสำเร็จรูปที่ใช้ในการตรวจการดื้อยาของไวรัส เอชไอวีได้มีการค้นคว้าพัฒนามาอย่างต่อเนื่อง แต่อย่างไรก็ตามชุดตรวจเหล่านั้นถูกออกแบบมาให้มีความจำเพาะกับเชื้อไวรัสเอชไอวี-1 สายพันธุ์ย่อย B ซึ่งเป็นสายพันธุ์ที่แพร่ระบาดในทวีปยุโรปและอเมริกา ซึ่งในแต่ละปี ประเทศไทยใช้งบประมาณจำนวนมากในการดูแลผู้ติดเชื้อเอชไอวี ดังเหตุผลที่กล่าวมาเพื่อลดต้นทุนค่าใช้จ่าย การศึกษาครั้งนี้จึงมีวัตถุประสงค์ที่จะออกแบบ และพัฒนาการตรวจการดื้อยาสำหรับ ไวรัสเอชไอวี-1 ที่ไม่ใช่สายพันธุ์ย่อย B ตัวอย่างเลือดจากผู้ติดเชื้อเอชไอวี 99 ราย ได้ถูกนำมาตรวจ การดื้อยาโดยวิธีที่เรา ออกแบบขึ้นและนำไปเปรียบเทียบกับชุดตรวจสำเร็จรูป พบว่าความสัมพันธ์ทางสถิติของผลการดื้อยาระหว่างชุดตรวจสำเร็จรูปและวิธีที่ทำการศึกษาให้ผลที่ ไม่แตกต่างกัน ($p>0.05$) ในยาต้านไวรัสทุกชนิด การศึกษานี้จึงเป็นอีกหนึ่งทางเลือกในการตรวจการดื้อยาของไวรัสเอชไอวีสำหรับประเทศ

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สาขาวิชา ชีวเคมีคลินิกและอณูทางการแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5277214337 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE

KEYWORDS : GENOTYPE / DRUG RESISTANCE / IN-HOUSE ASSAY / HIV-1

SAYOMPOO SANGUANSITTIANANT : THE DEVELOPMENT OF HIV-1 GENOTYPIC DRUG RESISTANCE TESTING FOR A LOW-COST ALTERNATIVE. ADVISOR : ASST. PROF. PALANEE AMMARANOND, Ph.D., CO-ADVISOR : NAVIN HORTHONGKHAM, Ph.D., 100 pp.

Since Highly Active Antiretroviral Therapy (HAART) becomes the most active way to control HIV replication, genotypic drug resistance testing has played an important role for HIV medication. Commercial test kits have been invented and developed continuously. However, all of those commercial kits are especially designed for HIV-1 subtype B which is the major strain of Europe and America continent. In each year, Thailand National Health Control expenses massively in HIV-infected individuals. To reduce cost of expenditure, we developed methods for genotyping drug resistance testing. In-house genotypic drug resistance assay was designed and developed for HIV-1 non-B subtype assay. 99 plasma HIV-infected samples were performed by in-house method and compared with commercial kits. There was no statistically different in antiretroviral resistance result comparison between commercial kit assay and in-house assay ($p>0.05$). This should be an alternative method for HIV drug resistance testing in resource-limited country.

Department : Clinical Chemistry Student's Signature .....

Field of Study : Clinical Biochemistry and Molecular Medicine Advisor's Signature .....

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INTRODUCTION

1.1 Background and problem

Human Immunodeficiency Virus or HIV is the virus in the family of *Retroviridae* and causes acquired immunodeficiency syndrome (AIDS). In 1984, Human Immunodeficiency Virus was first found in Thai homosexual man. According to the report of Department of Disease Control, Ministry of Public Health, from 1984 to September 2012, there were 276,947 AIDS patients and more than 1.2 million HIV-infected people in Thailand and there was increasing rate for worldwide. It became serious situation because of the increased demands for medical care and social support. According to the National Health Security Office (NHSO) report, the expenditure of health care for HIV infection and AIDS was 2,770.85 million Baht in 2010 and 2,997.74 million Baht in 2011.

The long term therapy and follow up testing are required for HIV infected and AIDS patient to control disease progression and virulence. Antiretroviral therapy is used to treat patient for these purposes by HIV viral load decreasing and CD4+ T lymphocyte count increasing. There are five main groups of antiretroviral drug for HIV. The first group is Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) which interfere with HIV-1 replication by competitively inhibiting transcriptase, leading to chain termination of HIV-1 proviral DNA. The second group is Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs). Their structure can bind at a position distance from active site, resulting in a conformational change at active site with a resultant inhibition of reverse transcriptase. The Protease Inhibitors

(PIs). is the third group. It binds to active site and inhibit maturation step resulting in abortive infectious particles. The fourth is Fusion Inhibitors. It binds to gp41 transmembrane protein on HIV envelope which is the important part for HIV fusion mechanism. The last group is Integrase Inhibitors. It blocks the action of HIV integrase enzyme which is important for integration of viral genome into the DNA of the host cell (1).

There are many factors involved in HIV drug resist emergence, such as a high replication rates and rapid turnover which is around 10^{10}–10^{11} virions being generated daily, high error rates of reverse transcriptase (1:10^4 nucleotide), frequency of spontaneous mutation ($3x10^{-5}$ base pairs per replication cycle), several recombination between the diploid genomes in each virion and incomplete suppression of viral under therapy create HIV variant associated drug resistance (2).

In Thailand, antiretroviral therapy medicates HIV infected individuals in combination regimen, taken two or more type of antiretroviral drugs. This treatment is known as Highly Active Antiretroviral Therapy, or HAART. The combination antiretroviral therapy's purpose is highly effective treatment and avoidance of antiretroviral resistance of HIV. According to Thai national guidelines for antiretroviral therapy in HIV-1 infected adults and adolescents 2010, it is recommend starting antiretroviral therapy at CD4+ T lymphocyte count <350 cells/mm^3 (2-7).

Before and after antiretroviral treatment, there are three important assays to monitor the treatment, which are CD4+ T lymphocyte count, HIV RNA viral load and HIV drug resistance testing (antiretroviral testing). CD4+ T lymphocyte count and HIV RNA viral load are standard markers of disease progression and patient staging in HIV infection. The guideline recommends taking CD4+ T lymphocyte count every

6 months in HIV infected individuals. HIV RNA viral load is recommended for taking every 6 months before antiretroviral therapy start and in the first year antiretroviral therapy start, 12 months after the first year and taking every time before drug regimen change or when treatment failure (8). For HIV drug resistance assays, there are two assays which are phenotyping and genotyping assay. In Thailand, genotyping assay is the first choice because of cost and convenience of procedure. The genotypic drug resistance testing is based on nucleotide mutation of HIV genome. World Health Organization (WHO) recommends to take this test when the treatment failure or clinical symptoms occurrence.

According to The Comptroller General's Department, medical fee of genotypic drug resistance testing in Thailand cost 8,000 Baht. It is a high cost for patients in developing country to access this test even there are some supportive from government.

In Southeast Asian country including Thailand, The circulating recombinant form AE (CRF01_AE) is the most prevalent strain circulating (9-12). The commercial HIV-1 genotypic drug resistance test kits are developed and designed for HIV-1 subtype B, the majority of subtype in America and Europe (13). Although there are studies showed that commercial HIV-1 genotyping systems can be performed to various HIV-1 subtypes, some problems with non-B subtypes have been reported (14-16).

1.2 Objective

Therefore, the development of HIV-1 genotypic drug resistance testing for lower cost and HIV-1 CRF01_AE is necessary in Thai HIV infected patient. This

study aims to respond to this demand by designing specific primers for CRF01_AE and developing assay for the lower cost.

1.3 **Keywords :** genotype, drug resistance, in-house assay, HIV-1

CHAPTER II

LITERRATURE REVIEW

Human Immunodeficiency Virus (HIV) is believed to have originated from zoonotic infection. It cross-species infected and evolved from Simian Immunodeficiency Virus (SIV), virus that infects non-human primate. HIV infection leads to reduction in number of CD4+ T Lymphocyte that decreases the Efficiency of immune system and causes acquired immunodeficiency syndrome (AIDs). In 1984, HIV was first found in Thailand in homosexual man. According to the report of Department of Disease Control, Ministry of Public Health, from 1984 to September 2012, there were 276,947 AIDS patients and more than 1.2 million HIV-infected people in Thailand (17). In December 2010 as World Health Organization (WHO) data and statistic report of global HIV/AIDS epidemic, the total number of people living with HIV is 34.0 million (Figure 1), people newly infected HIV in 2011 is 2.5 million and AIDS death in 2011 is 1.7 million. There was increasing rate for worldwide (12).

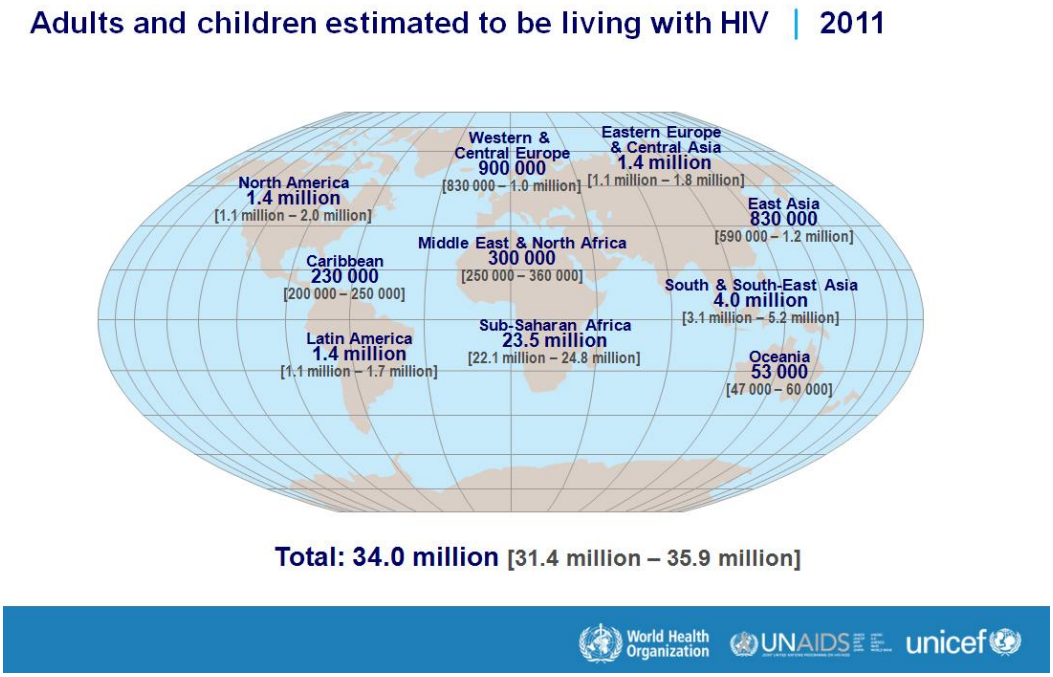


Figure 1 shown the number of people living with HIV in each area in 2011 (copied from *http://www.who.int/entity/hiv/data/2012_epi_core_en.pps*)

2.1 HIV classification

Human Immunodeficiency Virus is single-stranded RNA virus in a member of the *Retroviridae* family, *Lentivirinae* subfamily, and genus *Lentivirus* (18, 19). HIV has been characterized into two types: HIV-1 and HIV-2. HIV-1 is more virulent, more infective and is the cause of the majority of global HIV infection, whereas HIV-2 is largely found in West Africa. The sequence derived from HIV-1 can be classified into group and subtype.

HIV-1 can be divided into 3 groups: M (major or main), O (outlier) and N (new or non-M/non-O). The major group (M) has been subdivided further into nine major subtypes or clades: A-D, F-H, J and K. HIV genome recombination can occur in co-infection with two different but related strains. This combination creates new viral strain called circulating recombinant forms (CRFs) (9, 11, 20-22).

HIV-1 subtype B is the most commonly found in America and West Europe. In South East Asia including Thailand, the majority strain prevalence is the recombinant A/E or CRF_A/E (9-11, 20). The HIV-1 pandemic distributes different strain in different part of the world (Figure 2).

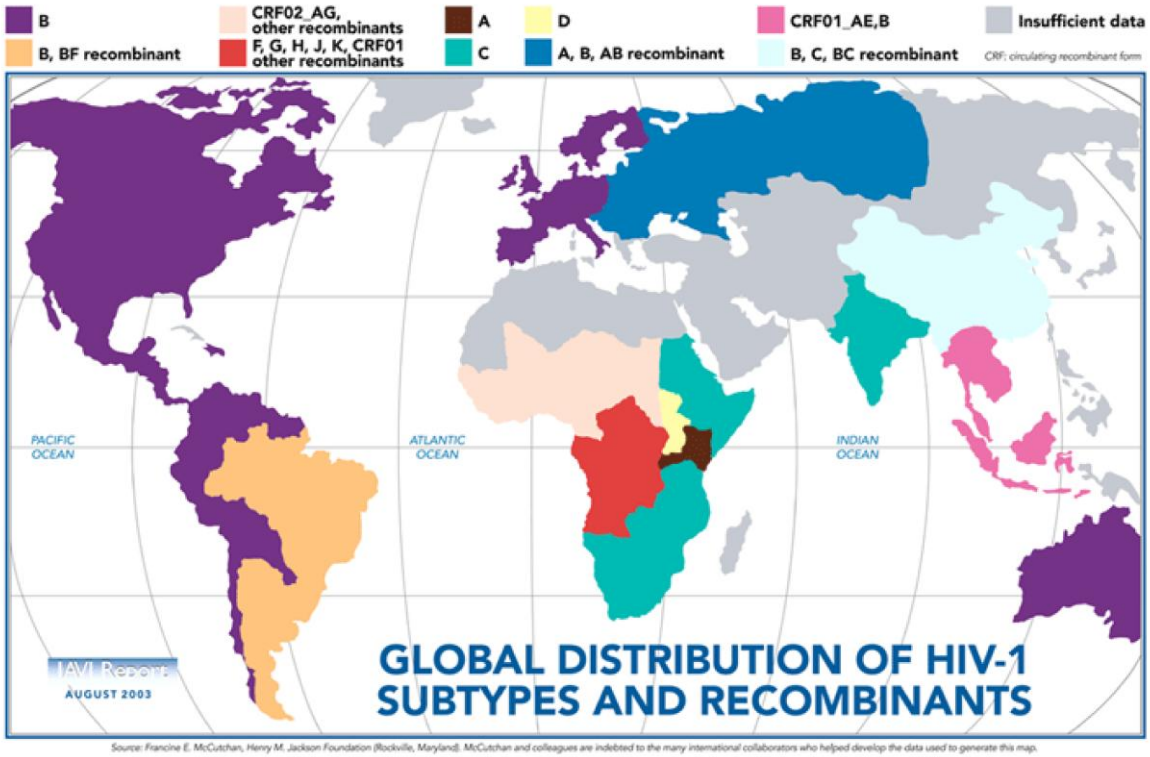


Figure 2 shown global distribution of HIV-1 subtype and recombinants (copied from *http://pathmicro.med.sc.edu/images/global.gif*)

2.2 HIV structure

The HIV-1 virion has approximately 100-120 nm in diameter. It carries two copies of a single-stranded RNA genome, structural proteins and replication enzymes in their core enclosed by capsid consisted of core protein, called p24. There is Matrix protein (p17) surrounding the capsid core protein layer. The envelope is lipid bilayer containing the envelope proteins, gp120 and gp 41 (Figure 3) (1-3).

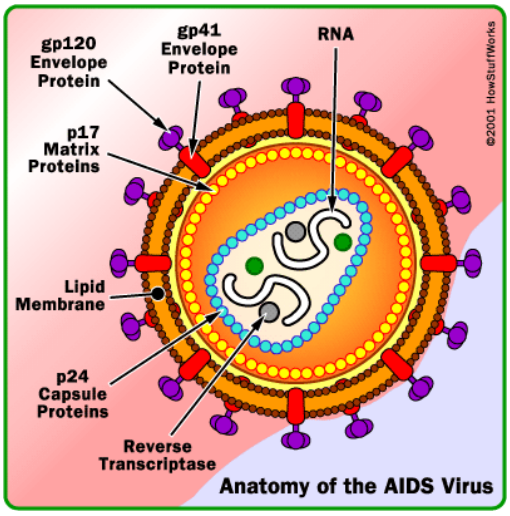


Figure 3 HIV-1 structure (copied from *http://www.helpaids.worldmedic.com/datacenter/howtocure.htm*)

The HIV RNA genome is approximately 10 Kb in length. The genome contains several structural genes that similar to other retroviruses. Structural genes consist of three major regions coding for structural proteins: core (*gag*), polymerase (*pol*), and envelope (*env*) genes.

GAG: The genomic region encoding the capsid proteins (group specific antigens). The precursor is the p55 myristoylated protein, which is processed to p17 (matrix), p24 (capsid), p7 (nucleocapsid), and p6 proteins, by the viral protease.

POL: The genomic region encoding the viral enzymes such as protease, reverse transcriptase, and integrase. These enzymes are produced as a Gag-Pol precursor polyprotein, which is processed by the viral protease.

ENV: Viral glycoproteins produced as a precursor (gp160), which is processed to give a non-covalent complex of the external glycoprotein gp120 and the transmembrane glycoprotein gp41. The mature gp120-gp41 proteins are bound by non-covalent interactions and are associated as a trimer on the cell surface (3).

In HIV genome, there are regulatory and accessory genes that unlike other retroviruses. These genes code for proteins that play the important role in transcriptional regulation and HIV pathogenesis. At the 3' and 5' ends of the genome, non-coding sequences, the long terminal repeats (LTRs) contain the promotor regions, a polyadenylation site, and recognition sites for cellular transcriptional factors (Figure 4).

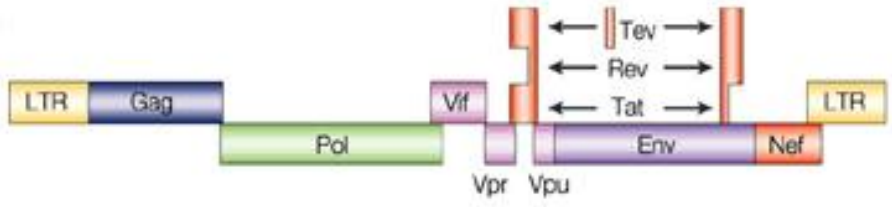


Figure 4 the structure of gene region on HIV genome (copied from *https://www.cs.ualberta.ca/system/files/images/bioinfo_hiv_genome.jpg*)

HIV infected macrophages and CD4+ T Lymphocyte by the specifical binding of viral envelope glycoproteins (gp120) on its surface to the chemokine receptor (CCR5 and CXCR4) on the target cell followed by fusion of the viral envelope with the cellular membrane and the release of the HIV capsid into the cell.

The first step in fusion involves the high-affinity attachment of the CD4 binding domains of gp120 to CD4 (3, 4, 6). When gp120 is bound with the CD4 protein, the envelope complex goes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor. This causes gp41 penetration to the cell membrane and forming the collapse of the extracellular portion of gp41 into a hairpin. This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and entry of the viral capsid. After entry of HIV, by its enzyme, the viral single-strand RNA genome

is transcribed into double-strand DNA and integrated into a host chromosome (Figure 5).

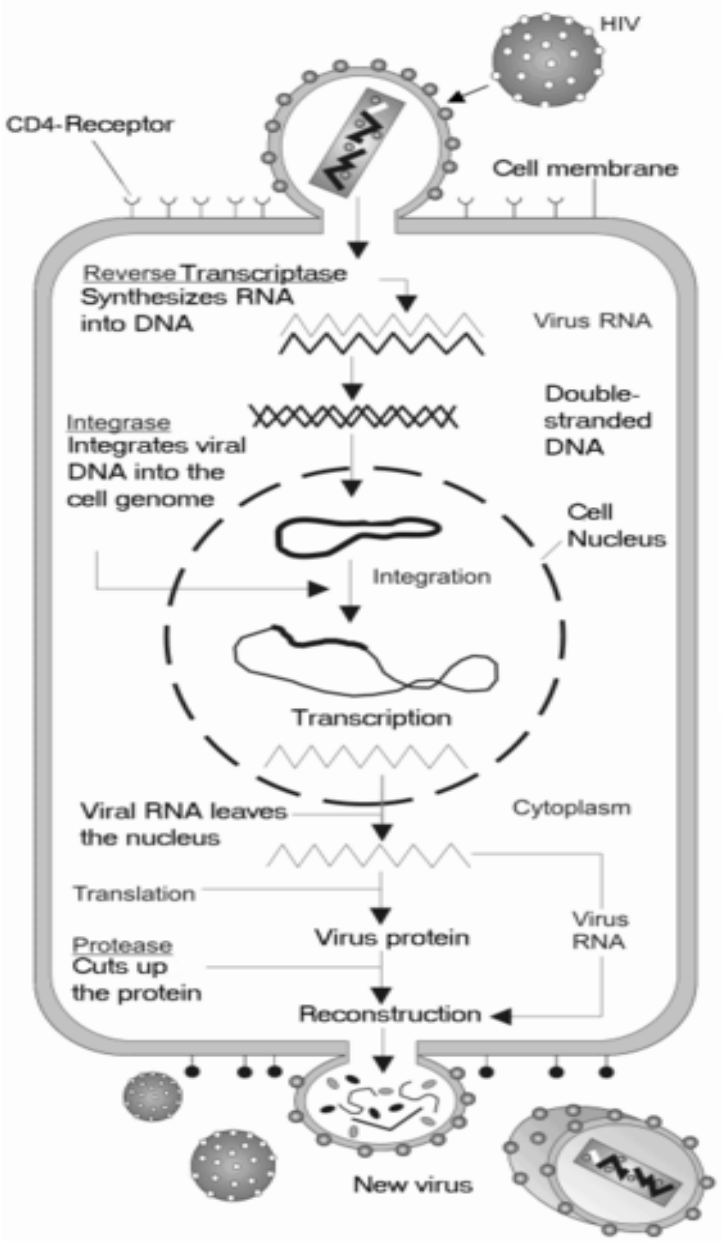


Figure 5 the HIV-1 replication (copied from *http://en.wikipedia.org/wiki/File:HIV_gross_cycle_only.png*)

2.3 Antiretroviral Therapy

Antiretroviral (ARV) drugs play an important role in HIV infection treatment. The aim of using ART is to inhibit the replication of HIV. It protects the immunity destruction, especially CD4+ T Lymphocyte, from HIV pathogenesis (3-7, 23). This recovers patient's immunity and reduces mortality rate and Morbidity rate in HIV infected patient. Antiretroviral drugs which commonly use in Thailand can be classified into five groups by the phase of the retrovirus life-cycle that the drug inhibits (1, 24-26).

2.3.1 Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs)

Nucleoside and nucleotide reverse transcriptase inhibitors, which inhibit the viral reverse transcriptase, have structure similar to the natural deoxynucleoside which is the important substrate for DNA synthesis. Therefore, the NRTIs compete with normal nucleotides in binding to the dNTP binding site resulting in DNA synthesis interference (27, 28). Drugs in this class: Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Abacavir, Emtricitabine, Tenofovir.

2.3.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Non-Nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcriptase by non-competitively binding to an allosteric site of the enzyme. It results in a conformational change in the reverse transcriptase, inhibiting polymerization (25). Drugs in this class: Efavirenz, Nevirapine, Delavirdine, Etravirine, Rilpivirine

2.3.3 Protease inhibitors (PIs)

Protease inhibitors (PIs) prevent viral replication by inhibiting the activity of proteases. It results in protein cleavage and makes HIV cannot complete the viral particle production. Drugs in this class: Saquinavir, Ritonavir, Indinavir, Nelfinavir, Amprenavir, Lopinavir, Atazanavir, Fosamprenavir, Tipranavir, Darunavir

2.3.4 Fusion Inhibitors

Fusion Inhibitors bind to gp41 transmembrane protein on HIV envelope which is the important part for HIV fusion mechanism. Drugs in this class: enfuvirtide

2.3.5 Integrase Inhibitors

Integrase Inhibitors blocks the action of HIV integrase enzyme which inserts the viral genome into the DNA of the host cell. Drugs in this class: Raltegravir

2.4 Highly Active Antiretroviral Therapy (HAART)

HIV has high replication rates estimated 10^{10}–10^{11} virions being generated daily. RNA virus polymerases have high error rates that are not subject to host cell proofreading mechanisms. RNA viruses, including HIV, thus average ~1 mutation per genome per replication cycle. The genetic diversity of HIV is also facilitated by several recombination events per cycle between the diploid genomes in each virion. Because of high replication rates, mutation recombination, there can be occurrence of mutation associated drug resistance.

The treatment of HIV/AIDS has evolved since the beginning of the epidemic from no treatment to treatment with a single drug (AZT) to dual-drug therapy and, now, to highly active antiretroviral therapy (HAART). HAART is defined as treatment with at least three active antiretroviral medications, typically two nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs) plus a non-nucleoside reverse

transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). HAART is also called the drug "cocktail" or triple-therapy.

The combination antiretroviral therapy's purpose is highly effective treatment and avoidance of antiretroviral resistance of HIV. According to Thai national guidelines for antiretroviral therapy in HIV-1 infected adults and adolescents 2010 (8), it is recommended starting antiretroviral therapy as follow:

Table 1 antiretroviral therapy start condition

Clinical Symptom	**CD4 level (cells/mm^3)**	**Suggestion**
AIDS-defining illness	Any level	ART start
Symptomatic	Any level	ART start
Asymptomatic	<350	ART start
Asymptomatic	≥350	follow the CD4 level every 6 months
Pregnancy	Any level	Start ART until birth if ≥350 cells/mm^3 of CD4 before treatment

Table 2 Thailand antiretroviral therapy regimens

	2NRTIs		**NNRTIs**		**PIs**
Preferred	AZT + 3TC TDF + 3TC / FTC	**+**	NVP EFV	**or**	LPV/r
Alternative	ABC + 3TC D4T + 3TC ddI + 3TC				ATV/r DRV/r SQV/r

2.5 Diagnosis and monitoring test for HIV infection

2.5.1 Diagnosis testing

According to Thai national guidelines for antiretroviral therapy in HIV-1 infected adults and adolescents 2010, the determination of HIV infection is required 3 different tests for confirmation. Testing performed in determination can be divided in 2 assays, anti-HIV detection and viral testing. Anti-HIV detection test is easy to use and spend a little time such as ELISA, Gelatin Particle Agglutination and immunochromatographic rapid test. Viral testing is the direct viral particle detection such as P24 detection and nucleic acid amplification test (8).

2.5.2 Monitoring testing

Monitoring tests are required to HIV infected individuals for decision of antiretroviral therapy start and following up treatment effect. There are three important tests to monitor the treatment, consisting of CD4+ T lymphocyte count, HIV RNA viral load and HIV drug resistance testing (antiretroviral testing).

2.5.2.1 CD4+ T lymphocyte count

The CD4+ T lymphocyte count is considered the laboratory marker of progression of HIV infection. It is performed as the important parameter for determining the stage of disease and the beginning of antiretroviral therapy. The number of CD4+ T lymphocyte is commonly count using flow cytometry. The guideline recommends taking CD4+ T lymphocyte count twice a year in HIV infected individuals. HIV infected individuals who have CD4+ T lymphocyte count between 350 to 500 cells/mm^3 are recommend to follow the CD4+ T lymphocyte count every 3 months. Antiretroviral drug should be started for below 350 cells/mm^3 patient and opportunistic drug should be started for below 200 cells/mm^3 patient (3-8).

Principle of flow cytometry in CD4+ T lymphocyte count

Flow cytometry is most often used in the clinical laboratory for the purpose of immunophenotyping. The antibodies labeled with different fluorescences consist of CD3/CD4/CD45 antibodies (CD3 found mainly in mature T cell, CD4 in helper T cell and CD45 in leukocyte). They are bound to CD4+ T cell surface receptors. Their presence on the cell is most often defined in terms of positive or negative by flow cytometer detection, with a cutoff set relative to a non-staining control population. CD4+ T cell is measured into percentage. The absolute CD4+ T cell is calculated by %CD4 and by white blood cell count and %Lymphocyte from complete blood count (CBC).

$$\text{Absolute CD4} = \frac{\%\text{CD4} \times \text{WBC} \times \%\ \text{Lymphocyte}}{10{,}000}$$

2.5.2.2 HIV RNA viral load

The HIV RNA viral load test is a quantitative measurement of HIV nucleic acid (RNA). It is also important markers of disease progression and patient staging in HIV infection. This test aims to monitor the status of HIV disease, to guide recommendations for therapy and to predict the future course of HIV. Keeping the viral load levels as low as possible for as long as possible decreases the complications of HIV disease, slows the progression from HIV infection to AIDS, and prolongs life. On the other hand, the high level of HIV viral load reflects to the failure of antiretroviral drug treatment.

In the recent, there are three commonly used principles for HIV RNA viral load test consisted of Reverse Transcriptase Polymerase Reaction (RT-PCR), Nucleic

Acid Sequence Based Amplification (NASBA) and Branched DNA (b-DNA) signal amplification.

2.5.2.3 HIV drug resistance testing

HIV has high replication rates estimated 10^{10}–10^{11} virions being generated daily. RNA virus polymerases have high error rates that are not subject to host cell proofreading mechanisms. RNA viruses, including HIV, thus average ~1 mutation per genome per replication cycle. The genetic diversity of HIV is also facilitated by several recombination events per cycle between the diploid genomes in each virion. Because of high replication rates, mutation recombination, there can be occurrence of mutation associated drug resistance (1).

HIV drug resistance testing or antiretroviral testing can detect HIV resistance and help identification which medications are suitable against the mutated virus. There are two major assays for HIV drug resistance testing, phenotyping assay and genotyping assay (8, 13, 29-32).

2.5.2.3.1 Phenotypic drug resistance testing

Phenotypic drug resistance testing measures the extent to which an antiretroviral drug inhibits virus replication in vitro. Similar to bacteriologic methods, this is typically performed by demonstrating an increase in the inhibitory concentration (IC) that is required to inhibit in vitro growth by 50 percent (IC50) compared with virus replication in the absence of drug. Results are reported as a fold-change in drug susceptibility of the patient sample compared with a laboratory reference strain.

2.5.2.3.2 Genotypic drug resistance testing

Genotypic drug resistance testing is the detection of genetic mutation in HIV genome. This test sequences the Reverse transcriptase and Protease regions on the *pol* gene and compares that genomic sequence to HIV wild type genome. The antiretroviral drug resistance-associated mutations are analyzed and interpreted into the antiretroviral drug resistance. This assay is commonly used because it is lowering cost and easier than phenotypic assay.

CHAPTER III

MATERIALS AND METHODS

3.1 Study Population and selection criteria

In this study, 99 samples were obtained from HIV-1 infected patients attending Health Science Service Unit, HIV Laboratory, Faculty of Allied Health Sciences, Chulalongkorn University, during November 2010 to February 2012.

The experiment was approved by The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University, Bangkok, Thailand (COA No. 080/2555).

HIV-RNA from all plasma samples were extracted and quantified by using COBAS® Ampliprep/COBAS® Taqman® HIV-1 test (version1.0). HIV-1 plasma samples which were quantified and shown values more than 2,000 copies/ml would be collected for next extraction by the COBAS® AmpliPrep Total Nucleic Acid Isolation Kit (TNAI) in order to perform antiretroviral drug resistance assay (TRUGENE HIV-1 Genotyping Kit).

Ninety nine samples which found mutation in reverse transcriptase (RT) region of polymerase (pol) gene and resistance to Nucleoside Reverse Transcriptase Inhibitors (NRTIs) or Non- Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) were selected for in-house PCR and direct sequencing.

3.2 Specimen Collection, Preparation, and Storage

Six millitres of peripheral blood was collected from HIV infected patients using standard techniques with ethylenediaminetetraacetic acid or EDTA (lavender top) anticoagulant blood collection tube. Plasma separation is performed within 6

hours after blood collection by centrifugation for 10 minutes at 1500 rpm. One millitre plasma was aspirated to each 2 ml sterile tubes and stored at -70°C.

3.3 HIV-1 RNA quantitation

One tube of EDTA plasma was thawed at 4^{o}C and centrifuged at 13,000 rpm for 1 minute. 850 µL of EDTA plasma was analyzed for HIV-1 viral load by COBAS® Ampliprep/ COBAS® Taqman® HIV-1 test using COBAS® Ampliprep/COBAS® Taqman® instrument.

This quantitative measurement of HIV in human plasma is based on three major processes: (1) specimen preparation to isolate HIV-1 RNA, (2) reverse transcription of the target RNA to generate complementary DNA (cDNA), and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide probe specific to the target. COBAS® Ampliprep instrument was performed for automated specimen processing. COBAS® Taqman® 48 Analyzer was performed for automated amplification and detection.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test utilizes automated specimen preparation on the COBAS® AmpliPrep Instrument by a generic silica-based capture technique. The HIV-1 virus particles are lysed by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HIV-1 RNA from RNases in plasma. Protease is introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture is incubated and the HIV-1 RNA is bound to the surface of the magnetic glass particles. Unbound substances, such as salts, proteins and other cellular impurities, are removed by washing the magnetic glass particles. After separating the magnetic glass particles and completing the washing steps, the

adsorbed nucleic acids are eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HIV-1 RNA, is added to the amplification mixture and transferred to the COBAS® TaqMan® Analyzer. HIV-1 RNA is then reverse transcribed, amplified and simultaneously detected by cleavage of a target-specific dual-labeled oligonucleotide probe.

The reverse transcription and PCR amplification reaction is performed with the thermostable recombinant enzyme *Thermus specie* DNA Polymerase. Processed specimens are added to the amplification mixture in amplification tubes (K-tubes) in which both reverse transcription and PCR amplification occur. The reaction mixture is heated to allow a downstream primer to anneal specifically to the HIV-1 target RNA. Polymerase extends the annealed primers along the target template to produce a double-stranded DNA molecule termed an amplicon.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test utilizes real-time PCR technology. The use of dual-labeled fluorescent probes allows for real-time detection of PCR product accumulation by monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HIV-1 and HIV-1 QS-specific oligonucleotide probes with a reporter dye and a quencher dye. In the COBAS® AmpliPrep/ COBAS® TaqMan® HIV-1 Test, the HIV-1 probes are labeled with different fluorescent reporter dyes. When these probes are intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and is cleaved by the 5' → 3' nuclease activity of the thermostable DNA polymerase. Once the reporter and quencher dyes are released and

separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplification of HIV-1 RNA is measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HIV-1 RNA.

3.4 Extraction of viral HIV-1 RNA for HIV-1 drug resistance

Isolation of the viral RNA from the 650 μL plasma sample was performed by the COBAS® AmpliPrep Total Nucleic Acid Isolation kit on the COBAS® AmpliPrep Instrument. The COBAS® AmpliPrep Total Nucleic Acid Isolation kit utilizes automated specimen process by a generic silica-based capture technique consisted of the following five steps:

1. Protease Solution digestes proteins to facilitate the release of RNA and DNA.
2. Addition of Lysis Reagent to the sample results in a complete lysis by denaturation of proteins. RNA and DNA are released and simultaneously stabilized.
3. The released nucleic acid binds to the silica surface of the added magnetic glass particles due to the chaotropic salt conditions and the high ionic strength of the Lysis Reagent.
4. Wash Reagent removes unbound substances and impurities such as denatured proteins, cellular debris and potential PCR inhibitors such as hemoglobin, etc. and reduces the salt concentration.
5. Purified nucleic acids are eluted at elevated temperature.

After extraction step, RNA was stored at -80^{o}C until using for HIV-1 drug resistance assay.

3.5 HIV drug resistance assay (TruGene method)

3.5.1 HIV RT-PCR amplification

A discrete sequence of the reverse transcriptase and protease genes was amplified by the TRUGENE HIV-1 Genotyping Kit. The HIV-1 *pol* genes are 297 and 1,680 nucleotides in length for the protease and reverse transcriptase regions respectively and are located at positions 1835-4678 (including the RNase H region) in the *pol* region of the HIV-1_{LAV-1} genome (GenBank number K02013). Due to the highly polymorphic nature of HIV-1, a mixture of several primers targeted at the most common viral variants is used in this kit.

The reverse transcription reaction was performed with a genetically engineered version of the Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) to transcribe the HIV-1 RNA into cDNA. The amplification reaction was then performed with a thermostable DNA polymerase. Under kit reaction conditions, both reverse transcription and PCR amplification occur in the same reaction mixture.

The extracted RNA specimens were added to reaction tubes in which both reverse transcription and PCR amplification occurred. The reaction mixture was heated and then cooled to a specific temperature to allow the oligonucleotide primers to anneal specifically to the HIV-1 target RNA. In the presence of excess deoxynucleotide triphosphates (dNTPs), the RT enzyme extended the annealed primers forming a complementary DNA:RNA hybrid (cDNA:RNA).

Following reverse transcription of the HIV-1 target RNA, the reaction mixture was heated to denature the cDNA:RNA hybrid and expose the cDNA HIV-1 target sequences. As the mixture cools, the upstream primers annealed to the cDNA strand, and the polymerases catalyze the extension reaction to synthesize a second DNA

strand. This completes the first cycle of PCR, yielding a double stranded DNA copy of the target region of the HIV-1 RNA. The reaction mixture was heated again to separate the double-stranded DNA and expose the primer target sequences. As the mixture cools, the primers annealed to the target DNA. The polymerases, in the presence of excess dNTPs, extend the annealed primers along the target templates to produce an amplicon. This heating and cooling process was repeated for 36 cycles, each cycle effectively doubling the amount of amplicon. Amplification occurs only in the region of HIV-1 genome between the RT-PCR primers. The entire HIV-1 genome was not amplified.

The RT-PCR Master Mixes preparation

1. Thaw TRUGENE reagents at room temperature except for enzymes and RNase inhibitor.
2. Vortex each component for 5 seconds, microcentrifuge for 5 seconds and place on ice.
3. Remove enzymes and RNase inhibitor from the freezer and place on ice immediately before use.
4. Label six 0.2 mL individual thin-wall PCR tubes (4 patient samples plus a positive and a negative control) and place on ice.
5. Label a 0.5 mL sterile, RNase-free microcentrifuge tube for RT-PCR Master Mix I and place on ice. Prepare the RT-PCR Master Mix I by adding the following in the order listed:

Master Mix I

RT-PCR Primers	42.0	µL
dNTP Solution	10.5	µL
DTT Solution	7.0	µL
RNase-Inhibitor	3.5	µL
TOTAL	63.0	µL

6. Vortex the mix 5 seconds and microcentrifuge 5 seconds, and then aliquot 9 µL into the bottom of each 0.2 mL PCR tube on ice. Close the tube caps.
7. Place tubes in a covered ice bucket or on an ice block and put to one side.
8. Label a 0.5 mL sterile, RNase-free tube for RT-PCR Master Mix II and place on crushed ice. Prepare RT-PCR Master Mix II by adding the following in the order listed. Work quickly (< 15 min) to minimize the standing time for the enzymes.

Master Mix II

RT-PCR Buffer	70.0	µL
RNase-Inhibitor	3.5	µL
RT Enzyme	7.0	µL
DNA Polymerase	17.5	µL
TOTAL	98.0	µL

9. Vortex the mix 7-10 seconds and microcentrifuge 7-10 seconds. Keep on ice.
10. Take the ice bucket (or ice cool block) containing the 6 PCR tubes of RT-PCR Master Mix I, the tube of Master Mix II, the positive control and the negative control to the dead air fume hood in Area 3.

Performing RT-PCR

1. Set up the RT-PCR conditions on the thermocycler (GeneAmp® PCR System 9700 or MJ Mini Gradient Thermal Cycler) as follows:

RT-PCR Cycle Program (All ramp times at 1°C/second)

One cycle of:	**20 cycles of:**	**17 cycles of:**	**One cycle of:**
90°C for 2 min	94°C for 30 sec	94°C for 30 sec	68°C for 7 min
50°C for 60 min	57°C for 30 sec	60°C for 30 sec	4°C hold
94°C for 2 min	68°C for 2 min	68°C for 2.5 min	

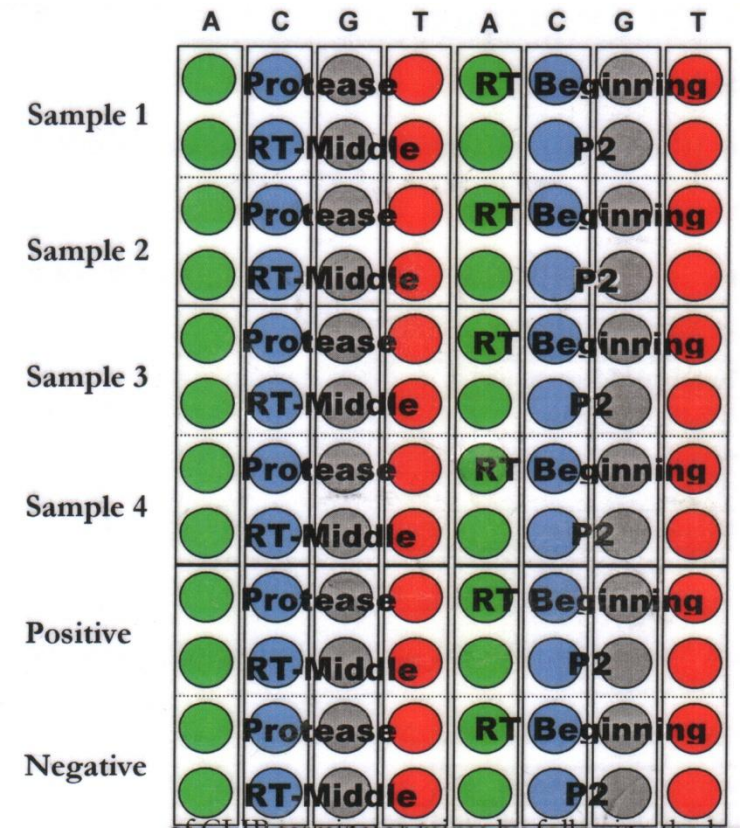
2. Preheat the thermocycler to 90°C and press "PAUSE".
3. Keeping the tubes on ice in the dead fume air hood, add 17 µL of each of the extracted RNA samples, the positive control and the negative control to the bottom of their respective PCR tubes containing Master Mix I. Mix up and down using the pipette, and change tips between each sample. Only open one tube at a time. Check that the tube caps are closed before proceeding.
4. Place the 6 PCR tubes into the thermocycler, and press "RESUME" or "PROCEED". Cycling should start with 90°C for 2 minutes, then move to 50°C.
5. After 5 minutes at 50°C, pause the thermocycler. Add 14 µL of RT-PCR Master Mix II to the bottom of each tube, opening only one tube at a time. Mix by pipetting up and down, then close the tube. Keep all tubes in the block while adding Master Mix II. Change tips between each sample. Check that the tube caps are closed before proceeding.
6. Close the lid of the thermocycler and allow the RT-PCR program to proceed.

7. The completed RT-PCR reactions may be held at 4°C in the thermocycler, for a maximum of 24 hours or stored at 2-8°C in the refrigerator, for a maximum of 24 hours.

3.5.2 CLIP Master Mix preparation

The CLIP reaction sequences both strands of the double-stranded DNA simultaneously. A reaction tube is prepared containing two or more primers. The forward (sense) and reverse (antisense) primers are each labeled with a different fluorescent dye. When hybridized to the DNA, the primers are oriented to allow chain extension towards each other (one primer on the sense strand and one on the antisense strand). The tube also contains all the reagents necessary for chain-extension along with one of the four chain-terminating dideoxynucleotide triphosphates (ddNTPs). The reaction is initiated with the addition of the sample and a thermostable DNA polymerase with a high affinity for ddNTPs. As the reaction mixture is thermally cycled, primers hybridize to template DNA and are extended, then usually terminated somewhere along the target DNA sequence. Four CLIP reactions yield both the forward and reverse sequence of the target between the two CLIP primers.

Using the 0.2 mL 8-tube strips, or using a 96 well sequencing tray specific to the thermocycler, and using the CLIP mixes provided, set the plate up as follows:



1. Load 7 µL of each terminator mix into the 8-tube strips according to the template. Load terminator mixes directly to bottom of each well to minimize cross contamination in subsequent loading steps. Change tips between each terminator mix. Keep the plate on ice (or in ice cool block).
2. Label six new 0.5 mL microcentrifuge tubes 1 to 6 for CLIP Master Mix.
3. Prepare the following CLIP Master Mix in a 1.5 mL microcentrifuge tube by adding the reagents in the order listed below. Keep the enzyme on ice and add it last.

CLIP Master Mix (6 reactions)

Molecular grade (DNase-free) water	474.0	µL
CLIP BUF (buffer)	120.0	µL
CLIP ENZ (enzyme)	22.5	µL
TOTAL	616.5	µL

4. The enzyme is supplied in glycerol; vortex the CLIP Master Mix for 7 to 10 seconds to thoroughly mix.
5. Aliquot 95 µL of the CLIP Master Mix to each of the six labeled CLIP tubes (1 to 6). Close the tube caps and keep on ice.

3.5.3 CLIP Sequencing of the Amplified DNA

1. Remove the tubes containing the RT-PCR product from the thermocycler. Keep the tubes on ice.
2. Using filtered pipette tips, add 5 µL of RT-PCR product to each of the 0.5 mL CLIP Master Mix: the RT-PCR product from sample 1 goes in tube 1, the RT-PCR product from sample 2 in tube 2, and so on. Vortex the tubes for 5 seconds, microcentrifuge 5 seconds and place on ice.
3. From each of these 6 CLIP tubes, transfer 5 µL into each well or tube of the appropriate row (16 wells) of the 96-well plate on ice. With a repeat pipette, add 5 µL onto the side of the wells at an angle, without touching the CLIP terminator mix in the bottom of the well to avoid cross contamination. If you do not have a repeat pipette, use a 20 µL pipette with a 20 µL filtered pipette tip. Close the tube caps or seal the tray with sealant and keep the tubes on ice. Ensure a secure seal on the caps or tray sealant.

4. Turn the thermocycler on and setup the CLIP sequencing cycle program.

CLIP sequencing Cycle Program (All ramp times at 1°C/second)

One cycle of:	**30 cycles of:**	**One cycle of:**
94°C for 5 min	94°C for 20 sec	70°C for 5 min
	56°C for 20 sec	4°C hold
	70°C for 1.5 min	

5. Preheat the thermocycler to 94°C and press "PAUSE".
6. Firmly tap the plate (resting on its base) on the bench once to collect reagents to the bottom of the wells or tubes.
7. Place the CLIP reaction mixes on the preheated thermocycler and press "RESUME" or "PROCEED".
8. When the thermocycler run is complete, remove the plate from the thermocycler and add 14 μL of the vortexed Stop Loading Dye to each well or tube using the repeat pipette.
9. Ensure that the wells are covered, or tubes are properly recapped. Mix the reactions thoroughly by tapping the plate (resting on its base) on the bench to collect the reagent at the bottom of the wells or tubes or vortex for 7 to 10 seconds to thoroughly mix.
10. Denature the CLIP samples by placing the 96 well sequencing plate in the thermocycler for 3 minutes at 85°C, then put the samples immediately on ice. Ensure that the tubes are well capped or the sequencing plate well covered.
11. Keep the samples on ice until ready to load onto the MicroCel 500 cassetle.
12. The CLIP sequencing samples can be stored at 2 to 8°C away from light overnight.

3.5.4 Polyacrylamide gel electrophoresis of CLIP reaction product

Following the CLIP sequencing reaction and the addition of the Stop Loading Dye, the reactions are heated to separate the double-stranded DNA fragments. A fraction of each reaction is then loaded into the top of a MicroCel™ 500 cassette containing an ultra thin vertical polymerized polyacrylamide gel that has a formed matrix of specific pore size. The polyacrylamide gel contains a high concentration of urea to maintain the DNA fragments in a single-stranded denatured state. A buffered solution maintains contact with both the top and bottom of the ultra thin gel. A high voltage electric field is applied, forcing the negatively charged fragments of DNA to migrate through the gel towards the anode. The speed of migration of the DNA fragments is related to the size of pores formed by the polyacrylamide matrix and DNA fragment size, with the smaller fragments migrating faster. Near the bottom of the polyacrylamide gel, a laser beam excites the fluorescent dye linked to the DNA fragments moving past the laser, and detectors measure the amount of light and wavelength produced by the fluorescent dye. This light measurement is then collected by the sequencer and transmitted to a workstation that stores the data. Each sequencing reaction requires four lanes, one for each of the four chain terminating dideoxynucleotides (ddATP, ddCTP, ddGTP, ddTTP).

For each template, two regions of the amplicon (protease and RT) are sequenced bi-directionally using four CLIP primer modules:

Protease: A bi-directional sequence of the protease gene (codons 10 to 99) and a sequence in at least one direction from condon 1 to 9.

P2: A bi-directional sequence of the protease gene (codons 21 to 99) and a sequence in at least one direction from codons 7 to 20.

RT Beginning: A bi-directional sequence of the beginning of the reverse transcriptase gene (condons 41 to 139) and a sequence in at least one direction from codons 140 to 142, and of codon 40.

RT Middle: A bi-directional sequence of the middle of the reverse transcriptase gene (codons 148 to 237) and a sequence in at least one direction from codons 138 to 147, and from codons 238 to 247.

Both of these regions of the HIV-1 genome are sequenced using the CLIP process, for a total of sixteen lanes of sequencing data (four reactions per CLIP primer module). The software then assembles the data, aligns the sequences and determines the DNA sequence by looking at the order of the bases.

1. Bring the SureFill 6% Sequencing Gel cartridge out of 4°C storage to room temperature. Do not use a heater or hot plate to warm the acrylamide solution to room temperature.
2. Make sure the computer is on.
3. Turn the Long-Read Tower sequencer on.
4. Start the GeneObjects™ software and select “Run Setup Wizard”. In “Test Group” window select sequencing assay type for four primer regions both 3’ and 5’ direction. Then, select “Create New Run”.
5. A new Run Worksheet will appear.
6. In “Run Information” tab, select the Long-Read Tower and click “Connect”
7. In “Sequencer Control” tab, use the following Sequencer Control settings:

Gel Temperature (°C)	60°C
Gel Voltage (V)	2000 V
Laser Power (%)	50%

Run Clock (Sampling Interval)	0.5 sec
Run Clock (Run Duration)	50 min

8. click “Heat” for warm the Long-Read Tower to 60°C
9. In “Assay Information” tab, inform the sample data.
10. Insert the splash guard into the bottom of the Fill Fixture unit.
11. Remove air from the tube connecting the SureFill injector cartridge to the Gel Toaster polymerization unit before and after connecting them.
12. Place the Softplug comb in the MicroCel 500 cassette and secure using clips.
13. Open the drawer of the Gel Toaster polymerization unit. Place the assembled MicroCel 500 cassette in the Fill Fixture unit. Tighten the thumbscrew.
14. Fill the MicroCel 500 cassette with acrylamide solution.
15. Close the drawer. Press the START button on the Gel Toaster polymerization unit cycle.
16. The cycle will run for 3 minutes. When the alarm sound on, remove the MicroCel 500 cassette from the drawer. Scrape off excess acrylamide solution, remove clips and Softplug comb from the MicroCel 500 cassette taking care to avoid damaging the wells.
17. Set up the prepared MicroCel 500 cassette in the Long-Read Tower sequencer and add 1x TBE buffer both to both the upper and lower buffer chambers.
18. Close the door and pre-run the cast gel.
19. The pre-run will be completed when both the Run and Pre-Run lights are illuminated on the Long-Read Tower sequencer
20. Load 1.5 μL of the first 8 wells of a sample into the gel in lanes 1 to 8. Close the door for 10 to 15 seconds. Load the next 8 wells of the same sample.

21. Place the buffer chamber cover on the upper reservoir of the buffer chamber and close the sequencer door.
22. Push the "Run" button on the Long-Read Tower sequencer to start data collection.

3.5.5 Data analysis

The forward and reverse CLIP sequences are combined and compared to the HIV-1_{LAV-1} with the OpenGene system software to determine the presence of all drug resistance mutations. All the bases in each of the codons known to be associated with resistance are selected by the software. The user reviews and edits the sequence as necessary. The interpretive report is based upon a defined set of criteria developed by an international panel of HIV-1 clinical and research experts and implemented in the TRUGENE HIV-1 Module for GuideLines™ Rules.

3.6 In-house PCR and direct sequencing method

3.6.1 In-house polymerase chain reaction

In this study, 904-nucleotide length of reverse transcriptase region on the HIV-1 *pol* genes was amplified by nested polymerase chain reaction. The first step is reverse transcription polymerase chain reaction (RT-PCR) to transcribe the HIV-1 RNA into cDNA by an RNA-dependent DNA polymerase, *Moloney Murine Leukemia Virus* Reverse Transcriptase (M-MLV RT). The two steps of amplification reaction, PCR and nested PCR, were then performed with a highly thermostable DNA polymerase of the thermophilic bacterium *Thermus aquaticus* (Taq DNA polymerase). The first PCR was performed to amplify cDNA from RT-PCR. The nested PCR was performed for high quantification and more specificity of DNA product.

Four In-house primers are modified according to HIV genome strain CM240 from Thailand for each step. In RT-PCR step, RT-PCR-end primer was required. In first PCR step, RT-PCR-end primer and PA3 primer were required. In first PCR step, RT-PCR-start primer and PA1 primer were required. The sequence of primers was described as follows:

RT-PCR-end (R)	5’-GCTCCCCTGAGGAGTTTACACA-3’
PA3 (F)	5’-GGAATTTTCCTCAGAGCAGACCAG-3’
PA1 (R)	5’-GCTTTACCTTAATCCCTGCATAAA-3’
RT-PCR-start (F)	5’-TTAGTAGGACCTACACCTGTCAACAT-3’

3.6.1.1 Denaturation of RNA

For denaturation of RNA secondary structure, 10 μL of extracted RNA sample is transfered to 0.2 mL PCR tubes and heated at 70°C for 5 minutes in thermocycler. After heating, RNA sample is placed on ice immediately.

3.6.1.2 In-house RT-PCR

Master Mixes preparation

1. Thaw reagents (buffer, dNTP, $MgCl_2$, primer and deionized water) at room temperature except for M-MLV RT enzyme and RNase inhibitor.
2. Vortex each component for 5 seconds, microcentrifuge for 5 seconds and place on ice.
3. Remove M-MLV RT enzyme and RNase inhibitor from the freezer and place on ice immediately before use.
4. Label a 0.5 mL sterile, RNase-free microcentrifuge tube for RT-PCR Master Mix and place on ice. Prepare the RT-PCR Master Mix by adding the following in the order listed:

RT-PCR Master Mix (for 1 reaction)

5xM-MLV RT buffer	5.0	µL
$MgCl_2$ (25 mM)	2.0	µL
dNTP (2.5 mM each)	2.0	µL
RT-PCR-end Primers (10 pmole/µL)	1.0	µL
Distilled water	4.25	µL
M-MLV RT enzyme (200U/ µL)	0.5	µL
RNase-Inhibitor (40U/ µL)	0.25	µL
TOTAL	15.0	µL

5. Mixed RT-master mix by inverting for 5 times and microcentrifuge 5 seconds, and then aliquot 15 µL into the bottom of each 0.2 mL PCR tube containing heated RNA sample. Mix up and down using the pipette, and change tips between each sample. Close the tube caps.

Performing in-house RT-PCR

Set up the RT-PCR conditions on the thermocycler (GeneAmp® PCR System 9700 or MJ Mini Gradient Thermal Cycler) as follows:

In-house RT-PCR Cycle Program (All ramp times at 1°C/second)

One cycle of:

42°C for 45 min

95°C for 5 min

4°C hold

Proceed to first PCR step

3.6.1.3 In-house 1st PCR

Master Mixes preparation

1. Thaw reagents (buffer, dNTP, $MgCl_2$, primer and deionized water) at room temperature except for Taq DNA polymerase enzyme.
2. Vortex each component for 5 seconds, microcentrifuge for 5 seconds and place on ice.
3. Remove Taq DNA polymerase enzyme from the freezer and place on ice immediately before use.
4. Label six 0.2 mL individual thin-wall PCR tubes and place on ice.
5. Label a 0.5 mL sterile, RNase-free microcentrifuge tube for 1st PCR Master Mix and place on ice. Prepare the 1st PCR Master Mix by adding the following in the order listed:

1st PCR Master Mix (for 1 reaction)

10xTaq buffer	5.0	µL
25 mM $MgCl_2$	2.0	µL
2.5 mM dNTP	4.0	µL
RT-PCR-end Primers (10 pmole/µL)	1.5	µL
PA3 Primers (10 pmole/µL)	1.5	µL
Distilled water	25.5	µL
Taq DNA polymerase (5U/ µL)	0.5	µL
TOTAL	40.0	µL

6. Mix PCR master mix by inverting for 5 times and microcentrifuge 5 seconds, and then aliquot 40 µL into the bottom of each 0.2 mL PCR tube.

7. Add 10 μL of the RT-PCR product into the bottom of each 0.2 mL PCR tube containing 1st PCR Master Mix. Mix up and down using the pipette, and change tips between each sample. Close the tube caps.

Performing in-house 1st PCR

Set up the 1st PCR conditions on the thermocycler (GeneAmp® PCR System 9700 or MJ Mini Gradient Thermal Cycler) as follows:

In-house 1st PCR Cycle Program (All ramp times at 1°C/second)

One cycle of:	**35 cycles of:**	**One cycle of:**
94°C for 4 min	94°C for 30 sec	72°C for 7 min
	55°C for 30 sec	4°C hold
	72°C for 2 min	

Proceed to nested PCR step

3.6.1.4 In-house nested PCR

Master Mixes preparation

1. Thaw reagents (buffer, dNTP, $MgCl_2$, primer and deionized water) at room temperature except for Taq DNA polymerase enzyme.
2. Vortex each component for 5 seconds, microcentrifuge for 5 seconds and place on ice.
3. Remove Taq DNA polymerase enzyme from the freezer and place on ice immediately before use.
4. Label six 0.2 mL individual thin-wall PCR tubes and place on ice.
5. Label a 0.5 mL sterile, RNase-free microcentrifuge tube for 1st PCR Master Mix and place on ice. Prepare the nested PCR Master Mix by adding the following in the order listed:

Nested PCR Master Mix (for 1 reaction)

10xTaq buffer	5.0	µL
25 mM $MgCl_2$	2.0	µL
2.5 mM dNTP	4.0	µL
RT-PCR-start Primers (10 pmole/µL)	1.5	µL
PA1 Primers (10 pmole/µL)	1.5	µL
Distilled water	30.5	µL
Taq DNA polymerase (5U/ µL)	0.5	µL
TOTAL	45.0	µL

6. Mix PCR master mix by inverting for 5 times and microcentrifuge 5 seconds, and then aliquot 45 µL into the bottom of each 0.2 mL PCR tube.
7. Add 5 µL of the 1st-PCR product into the bottom of each 0.2 mL PCR tube containing nested PCR Master Mix. Mix up and down using the pipette, and change tips between each sample. Close the tube caps.

Performing in-house nested PCR

Set up the nested PCR conditions on the thermocycler (GeneAmp® PCR System 9700 or MJ Mini Gradient Thermal Cycler) as follows:

In-house nested PCR Cycle Program (All ramp times at 1°C/second)

One cycle of:	**35 cycles of:**	**One cycle of:**
94°C for 4 min	94°C for 30 sec	72°C for 7 min
	55°C for 30 sec	4°C hold
	72°C for 2 min	

The PCR product can be stored at 4°C for short period and -20°C for longer period before next process.

3.6.2 Agarose gel electrophoresis for PCR product detection

The expected size of PCR product was 904 base pairs. PCR products were detected by agarose gel electrophoresis. Prepared 0.7% agarose gel as following

1. Weighed 0.7 grams of agarose gel and dissolved in 100 ml of 0.5X TBE.
2. Mixed well and melted until solution was cleared.
3. Left at room temperature for 10 minutes and poured on gel platform.
4. Let gel set for 30 minutes.
5. Loaded PCR product by mixing 1 µL of 6X loading dye with 5 µL PCR product.
6. Run at 100 Voltages for 1 hour.
7. Stained gel with Gel red for 10 minutes
8. Observed the expected product under UV light by comparing with 100 base pair DNA marker.

3.6.3 PCR product purification

Purification of PCR product is performed by HiYield™ Gel/PCR Fragments Extraction Kit. The method uses a chaotropic salt, guanidine thiocyanante to dissolve agarose gel and denature enzymes. DNA fragments in chaotropic salt solution bind to the glass fiber matrix of the spin column. Following washing off contaminants, the purified DNA fragments are eluted by addition of low salt elution buffer or water. Salts, enzymes and unincorporated nucleotides are effectively removed from reaction mixtures without phenol extraction or alcohol precipitation.

Sample preparation

1. Transfer up to 100 µl reaction product to a microcentrifuge tube (not provided).

2. Add 5 volumes of DF Buffer to 1 volume of the sample and mix by vortexing.

DNA Binding

1. Place a DF Column in a Collection Tube.
2. Apply the sample mixture from previous step into the DF Column.
3. Centrifuge at 6,000 xg (8,000 rpm) for 30 seconds.
4. Discard the flow-through and place the DF Column back in the Collection Tube.

Wash

1. Add 500 µl of Wash Buffer (ethanol added) into the DF column.
2. Centrifuge at 6,000 xg (8,000rpm) for 30 seconds.
3. Discard the flow-through and place the DF Column back in the Collation Tube.
4. Centrifuge again for 2 minutes at full speed (14,000rpm) to dry the column matrix.

DNA Elution

1. Transfer dried Column in a new microcentrifuge tube (not provided).
2. Add 15 µl of Elution Buffer or water into the center of the column matrix.
3. Stand for 2 minutes until Elution Buffer or water is absorbed by the matrix
4. Centrifuge for 2 minutes at full speed to elute purified DNA.

3.6.4 DNA concentration determination

Determination of DNA concentration is performed by NanoDrop™ Spectrophotometers. The spectrophotometer measures the absorbance of a nucleic acid at 260 nm and convert to concentration in ng/µL. 2 µL of purified PCR product is required in this step before sequencing.

3.6.5 Direct sequencing

The purified PCR product which had concentration over 30 ng/µL was sent to 1st BASE DNA Sequencing Services. Six in-house primers were modified according to HIV genome strain CM240 from Thailand and appropriately selected for sequencing reaction. The sequences of primers were described as follows:

RT41 (F)	5’- CCCAATTAGTCCTATTGACACTGTA -3’
RT74 (R)	5’- GATGCGGTATTCCTAATTGAACTTC -3’
RT100 (F)	5’- GGCCTGAAAATCCATACAATACTCC -3’
RT108 (R)	5’- GTACTGATATCTGATTCCTGGTGTCTCA -3’
RT181 (F)	5’- CAATGTGCTGCCACAGGGATG -3’
RT219 (R)	5’- CAGCTGTCTTTTTCTGGCAGTTCTA -3’

3.6.6 Validation, alignment and interpretation of sequencing results

Multiple DNA sequencing fragments data were edited and assembled by comparing to a reference sequence with DNA Baser Sequence Assembler (http://www.dnabaser.com). Interpretation of the sequences according to resistance was performed using the Stanford HIV drug resistance database (http://hivdb.stanford.edu/).

3.7 Statistical analysis

The antiretroviral drug resistance results from two assays were statistically analyzed for correlation by Statistics Package for the Social Sciences (SPSS).

CHAPTER IV

RESULT

4.1 Characteristics of the population

Antiretroviral resistance was examinined in 99 plasma samples from drug-treated HIV-1 infected patients. These samples consist of 37 males, 39 females, and 23 samples which not known gender. After analysis, it found that there were one B subtype and 98 CRF01_AE samples. The median viral load is 46,650 copies/mL (range 2,030-2,160,000 copies/mL). There are 49 samples which below 50,000 copies/mL of viral load (median=12,300 copies/mL). In the over 50,000 copies/mL group (median=171,000), there are 50 samples.

Table 3 characteristics of the population

Characteristics	**VL < 50,000 copies/mL**	**VL > 50,000 copies/mL**
Number of genotypic testing (N=99)	49	50
Gender		
• **Male**	19	18
• **Female**	20	19
• **unknown**	10	13
Subtype		
• **CRF01_AE**	49	49
• **B**	-	1
VL mean (range)	17,452 (2,030-42,400)	285,841 (50,900-2,160,000)

4.2 Prevalence of codon mutation

Sequences which performed by TRUGENE HIV-1 Genotyping kit and In-house assay has the frequency of resistance-associated mutation detail in table 2 and table 3. The most frequency of NRTI-resistance-associated was found at position 184, and 181 for NNRTI.

According to the prevalence of NRTI-resistance-associated codon mutation table, they show that the in-house assay can detect the mutation frequency more than the kit assay at position 44, 69, 115, and 210. But the in-house assay can detect the mutation frequency less than the kit assay at position 67, 70, 75, and 184.

At Position 44, 69, 115, and 210, the in-house assay can detect the amino acid mutation in form of the mutated amino acid or the primary amino acid such as E44D/E.

At Position 67, and 75, the kit assay can detect the amino acid mutation in form of the mutated amino acid or the primary amino acid

At Position 70, the in-house assay can detect the mutated amino acid for one sample (sample no.36) and the kit assay can detect the amino acid mutation in form of the mutated amino acid or the primary amino acid for two samples (sample no.22, and 99).

At Position 184, the kit assay can detect the mutated amino acid for one sample (sample no.84).

Table 4 prevalence of NRTI-resistance-associated codon mutation

Codon mutation	Frequency of codon mutation (n=99)	
	Kit	In-house
NRTI		
M41	14	14
E44	8	9
K65	6	6
D67	23	22
T69	12	13
K70	15	14
L74	10	10
V75	14	13
F77	2	2
Y115	1	2
F116	2	2
V118	7	7
Q151	6	6
M184	70	69
H208	1	1
L210	11	12
T215	27	26
K219	16	18

According to the prevalence of NNRTI-resistance-associated codon mutation table, they show that the in-house assay can detect the mutation frequency more than the kit assay at position 90, 138, and 227. But the in-house assay can detect the mutation frequency less than the kit assay at position 101, 103, 108, 181, 190, 221, 230, 238, and 318.

At position 318 (Sample no.138, 169), the in-house assay cannot detect because its sequence is not long enough. Nevertheless, the NNRTI resistance interpretation of these two samples is not different.

Table 5 prevalence of NNRTI-resistance-associated codon mutation

Codon mutation	**Frequency of codon mutation (n=99)**	
	Kit	**In-house**
NNRTI		
V90	5	6
A98	11	11
L100	2	2
K101	24	21
K103	40	38
V106	8	8
V108	10	9
E138	10	11
V179	8	8
Y181	47	45
Y188	2	2
G190	34	33
H221	11	9
F225	3	3
F227	0	1
M230	2	1
K238	3	1
Y318	2	0

According to the result, the in-house assay can detect one case of D67 deletion in sample no.38 and two cases of T69 insertion in sample no.18 and no.169. Compare

to the kit result, it can detect only deletion in sample no.38, detected by the Stanford HIV drug resistance database, and insertion in sample no. 18.

4.3 The antiretroviral resistance interpretation

Edited sequences from TRUGENE HIV Genotyping kit and In-house assay were analyzed and interpreted into antiretroviral resistance report. Sequence from TRUGENE HIV Genotyping kit was analyzed and interpreted by the TRUGENE HIV-1 Module for GuideLines^{TM} Rules. Sequence from in-house assay was analyzed and interpreted using the Stanford HIV drug resistance database (http://hivdb.stanford.edu/).

TRUGENE HIV-1 Module for GuideLines^{TM} Rules interprets the antiretroviral resistance result into 3 forms (No Evidence of Resistance, Possible Resistance, and Resistance) while the Stanford HIV drug resistance database interprets the antiretroviral resistance result into 5 forms (Susceptible, Potential low-level resistance, Low-level resistance, Intermediate resistance, and High-level resistance). For the convenience of interpretation, we refer “No Evidence of Resistance” as “Susceptible”, “High-level resistance” as “Resistance”, and the rest 3 result interpretation of Stanford HIV drug resistance database (Potential low-level resistance, Low-level resistance, and Intermediate resistance) as “Possible Resistance”.

The related test of the two assays was performed by The Wilcoxon Matched Pairs Signed-Ranks Test applied on Statistics Package for the Social Sciences (SPSS). In interpretation according to each assay, all drugs were each compared for 99 samples except Etravirine and Rilpivirine because of the limitation of kit interpretation.

Table 6 the correlation of each antiretroviral resistance result between the two assays

ART	Asymp. Sig. (2-tailed)	
	Kit (kit GuideLines) vs Inh	Kit (Stanford) vs Inh
Abacavir (ABC)	0.001	0.157
Didanosine (DDI)	0.001	0.317
Lamivudine (3TC)	0.206	0.317
Emtricitabine (FTC)	0.206	0.317
Stavudine (D4T)	0.061	1.000
Tenofovir (TDF)	0.670	0.083
Zidovudine (AZT)	0.144	1.000
Efavirenz (EFV)	0.021	0.414
Etravirine (ETR)	-	0.655
Nevirapine (NVP)	0.785	0.317
Rilpivirine (RPV)	-	1.000

According to the statistic comparison of the result using different interpretation, Lamivudine (3TC), Emtricitabine (FTC), Stavudine (D4T), Tenofovir (TDF), Zidovudine (AZT), Efavirenz (EFV), and Nevirapine (NVP) show the correlated result (>0.05) between two assays, while Abacavir (ABC) and Didanosine (DDI) show the significant difference (<0.05).

Table 7 frequency of "Possible resistance" difference

ART	Frequency of "Possible resistance" difference (N=99)	
	Kit (kit GuideLines) vs Inh	Kit (Stanford) vs Inh
Abacavir (ABC)	54	2
Didanosine (DDI)	18	4
Lamivudine (3TC)	6	0
Emtricitabine (FTC)	6	0
Stavudine (D4T)	23	3
Tenofovir (TDF)	22	3
Zidovudine (AZT)	23	4
Efavirenz (EFV)	12	6
Etravirine (ETR)	-	5
Nevirapine (NVP)	1	1
Rilpivirine (RPV)	-	4

Table 8 frequency of "Susceptible vs Resistance" difference

ART	Frequency of "Susceptible vs Resistance" difference (N=99)	
	Kit (kit GuideLines) vs Inh	Kit (Stanford) vs Inh
Abacavir (ABC)	1	0
Didanosine (DDI)	2	0
Lamivudine (3TC)	1	1
Emtricitabine (FTC)	1	1
Stavudine (D4T)	0	0
Tenofovir (TDF)	0	0
Zidovudine (AZT)	0	0
Efavirenz (EFV)	0	0
Etravirine (ETR)	-	0
Nevirapine (NVP)	2	0
Rilpivirine (RPV)	-	0

According to the table 7 and 8, they show the decreased difference of the antiretroviral resistance result, when interpretations are based on the same database.

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

In this study, the in-house HIV-1 genotypic drug resistance test was performed using 99 HIV-infected plasma specimens attending Health Science Service Unit, HIV Laboratory, Faculty of Allied Health Sciences, Chulalongkorn University, during November 2010 to February 2012. The overall results from the in-house assay were compared to those obtained from the commercial kit assay, TRUGENE HIV-1 Genotyping kit.

The overall samples were analyzed for HIV-1 RNA quantitation by COBAS® Ampliprep/ COBAS® Taqman® HIV-1 test using COBAS® Ampliprep/ COBAS® Taqman® instrument using Real-time RT-PCR technique. The HIV-1 viral load result range was cover variant level since 2,030 to 2,160,000 copies/mL (median was 46,650 copies/mL). All of these samples from 37 males, 39 females, and 23 samples which not known gender consisted of one B subtype samples and 98 recombinant A/E or CRF_A/E which is the majority strain of HIV-1 distributed in Thailand.

After using DNA Baser Sequence Assembler to edit the fragments of RT-region sequence from the in-house HIV genotypic drug resistance test and interpreting the sequence into antiretroviral resistance report, the frequency of resistance-associated codon mutation and the pattern of antiretroviral resistance in each sample was analyzed for the correlation between the in-house HIV genotypic drug resistance assay and the TRUGENE HIV-1 Genotyping kit assay.

According to the result, the frequency of all NRTI and NNRTI resistance-associated codon mutation looks similar between the in-house assay and the kit assay.

The frequency of antiretroviral resistance-associated codon mutation was also examined into the mutation pattern of each drug. The in-house assay can detect one case of D67 deletion in sample no.38 and two cases of T69 Serine insertion in sample no.18 and no.169, while the kit assay can detect only T69 Serine insertion in sample no. 18.

The 69 insertion complex is associated with resistance to all nRTIs currently approved by the US FDA when present with 1 or more thymidine analogue–associated mutations (TAMs) at codons 41, 210, or 215 (33-36). The in-house antiretroviral resistance interpretation from sample no.169, which can detect 69 Serine insertion, has resistance to almost NRTI and possible resistance to Lamivudine (3TC) and Emtricitabine (FTC), while the kit result which has no insertion give possible resistance to Abacavir (ABC) and Tenofovir (TDF), and give susceptible to Lamivudine (3TC) and Emtricitabine (FTC).

Deletions between codons 67 to 70 can occur with TAMs or Q151M complex. They give the result similar but weaker effects than T69ins (33, 37-40). According to sample no.38 resistance report, the kit assay, which no deletion, has resistance to only two NRTI, Stavudine (D4T) and Zidovudine (AZT); these cause of T215F mutation which is the major resistance mutation of these two drugs, while the in-house assay has no susceptibility to any NRTI.

The edited sequences from TRUGENE HIV Genotyping kit and in-house assay were analyzed and interpreted into antiretroviral resistance report. Sequence from TRUGENE HIV Genotyping kit was analyzed and interpreted by the TRUGENE HIV-1 Module for GuideLines^{TM} Rules. Sequence from in-house assay was analyzed and interpreted using the Stanford HIV drug resistance database.

All antiretroviral resistance reports from TRUGENE HIV-1 Genotyping kit assay and in-house assay except Etravirine (ETR) and Rilpivirine (RPV) were analyzed applied on Statistics Package for the Social Sciences (SPSS). The correlation of two assays was compared between the same antiretroviral drugs in each sample using The Wilcoxon Matched Pairs Signed-Ranks Test. We found the correlated result ($p>0.05$) in Lamivudine (3TC), Emtricitabine (FTC), Stavudine (D4T), Tenofovir (TDF), Zidovudine (AZT), Efavirenz (EFV), and Nevirapine (NVP). There is statistical significant difference ($p<0.05$) in Abacavir (ABC) and Didanosine (DDI).

When we examined the different resistance pattern of these three drugs in each sample, we found that the almost difference is caused by the difference between "Possible Resistance" and "Resistance" or "Susceptible". The "Possible Resistance" result can mean both "maybe resistance" and "maybe susceptible". Therefore, if we analyzed that "Possible Resistance" into form of "Resistance" or "Susceptible" depending on another assay result, the correlation can be not different.

In case of Didanosine (DDI), there are two samples (sample no.38, and 100), which give different result in "Resistance" and "Susceptible" pattern. Sample no.38 and 100 give the adverse result, "Susceptible" for the kit assay and "Resistance" for the in-house assay. In sample no.38, the in-house assay found D67 deletion with thymidine analogue–associated mutations (TAMs), caused all NRTI resistance, but the kit assay found only K70R, T215F, and K219Q. In sample no.100, the different mutation between the two assays found at position 151. The kit assay result gives Q151K mutation, while the in-house assay is Q151M, which can cause 151 Complex Multi-NRTI Resistance.

In case of Abacavir (ABC), the different result in pattern of “Resistance and Susceptible” was found in sample no.38. The cause of this result pattern is similar to the case of Didanosine (DDI), because of D67 deletion with thymidine analogue–associated mutations (TAMs).

In case of Lamivudine (3TC) and Emtricitabine (FTC), there is the correlated result, but we found the “Resistance and Susceptible” different pattern in sample no.84. M184V mutation occurred in the in-house sequence of sample no.84 caused the resistance result to Lamivudine (3TC) and Emtricitabine (FTC) but there is no mutation at this position in the kit sequence.

Another cause of the different antiretroviral resistance result is the different database of the guideline using for interpretation in each assay. The kit assay was interpreted by the TRUGENE HIV-1 Module for GuideLines^{TM} Rules, while the in-house assay was interpreted using the Stanford HIV drug resistance database. The reason, why we use the different database of the guideline, is the exactness of whole process performed in each assay. If we use the same database in antiretroviral resistance interpretation, the correlation can be more similar between the two assays.

Therefore, we exported the sequence data of the TRUGENE HIV-1 Genotyping kit assay to interpret with the Stanford HIV drug resistance database and compared the correlation of antiretroviral resistance result of two assays based on the same database. We found that there is correlated result ($p>0.05$) in all antiretroviral.

Almost different pattern between the kit assay and the in-house assay is “Possible Resistance” with “Resistance” or “Susceptible”. There is only one “Resistance and Susceptible” different pattern found in sample no. 84 Lamivudine

(3TC) and Emtricitabine (FTC) result because of M184V mutation in in-house sequence.

When we look at the cost between the two assays, we found that the in-house assay cost is lower than the TRUGENE HIV-1 Genotyping kit assay cost.

The TRUGENE HIV-1 Genotyping kit costs 240,000 Baht per 30 tests. In point of fact, the number of test per kit's box is depended on the number of test performed in each time. In each master mix preparation for PCR reaction or CLIP reaction, the exceed volume must required. Therefore, the many times of few tests performing means the more exceed volume which must be waste and it cannot perform up to 30 tests per box. The kit cost can be more than 8,000 Baht per test (only reagent cost).

When compared to the in-house assay cost, the in-house assay cost consists of the reagent cost and sequencing. The reagent such as dNTP, primers, enzyme, and PCR product purification kit cost 125 Baht per test, not over 200 Baht if we included tip and tube cost. The sequencing cost is 200 Baht per reaction. If all 6 primers must be performed, the maximum cost of sequencing is 1,200 Baht. The total cost of RT gene sequencing is not over 1,400 Baht. If we calculated PR gene sequencing in the same cost, the total cost of both RT and PR antiretroviral resistance test by this assay is 3,800 Baht.

Table 9 the in-house assay cost

List	Cost per test (Baht)
dNTP	7
MMLV RT enzyme	30
RNase-Inhibitor	27
Taq DNA polymerase	20
Primers	8
PCR product purification kit	33
Sequencing	1200
Total	**1325**

The different costs between two assays can proof that the in-house assay can reduce huge cost in HIV-1 genotypic antiretroviral resistance test. This reduced cost of HIV-1 genotypic antiretroviral resistance test can extremely save the expenditure of health care for HIV infection and AIDS.

5.2 Conclusion

Antiretroviral therapy plays an important role in HIV infection treatment. It is performed for HIV infected patient to control the HIV viral replication and maintain patient's immunity. There are many factors responsible for antiretroviral failure such as insufficient drug potency, pharmacological issue and drug resistance. Because of high replication rates and mutation recombination, there can be occurrence of mutation associated drug resistance. The sequence heterogeneity of HIV has the huge effect to the efficacy of antiretroviral therapy. The antiretroviral resistance identification might be useful to assess the correct therapeutic regimen. The

antiretroviral resistance testing can detect HIV resistance and help identification which medications are effective against the mutated virus.

The detection of genetic mutation in HIV genome or genotypic drug resistance testing is commonly used because of its lower cost and easier process when compared to the other assays such as phenotypic drug resistance testing. However, genotypic drug resistance testing still has high cost, about 8,500 Baht per one test. When the antiretroviral resistance identification is necessary and its cost still has high, the development of HIV-1 genotypic drug resistance testing for a low-cost alternative required.

In this study, we evaluated the in-house HIV genotypic drug resistance test on RT gene using 99 plasma samples from HIV infected patients. Overall samples was amplified by nested polymerase chain reaction and sequenced on RT gene using specific primers. All sequences were interpreted by its own database assay to compare the whole process result and by Stanford HIV drug resistance database to compare the difference of sequence result.

The RTI resistance results and codon mutation were compared to the commercial test kit results. There was significant statistic difference in some antiretroviral resistance interpretation, such as Abacavir (ABC) and Didanosine (DDI), when interpretation is performed by database according to each assay. Nevertheless, the correlated result was shown in all antiretroviral resistance interpretation comparison when interpretation is performed by the same database, Stanford HIV drug resistance database.

The correlation result by the same database can show that the results from two assays have no difference of sequencing efficiency. The in-house assay has sufficient efficiency and can be performed instead of another one.

According to The Comptroller General's Department, medical fee of genotypic drug resistance testing in Thailand cost 8,500 Baht. The commercial kit's reagent cost can be more than 8,000 Baht per test and the overall cost of genotypic drug resistance test using commercial kit (including disposable ware, labor cost, electricity fee, maintenance cost and etc.) can be more than 8,500 Baht. The exceed cost is responsibility of laboratory or organization where performed this test. The in-house assay can reduce the total cost 50% or more than. This reduced cost can reduce responsibility of patient, government, and laboratory or organization where performed it. Moreover, it can be another alternative for genotypic drug resistance test.

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APPENDICES

APPENDIX A

Prevalence of NRTI-associated-mutation codon

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
1	2	Kit																			N
		Inh																			N
2	3	Kit																			
		Inh																			
3	4	Kit															V				
		Inh															V				
4	5	Kit															V				
		Inh															V				
5	7	Kit					N								I/V		V		W	Y	
		Inh		D/E			N								I/V		V		W	Y	
6	9	Kit	L	A			G			I							V		W	F	Q
		Inh	L	A			G			I							V		W	F/Y	Q
7	14	Kit															V				
		Inh															V				
8	18	Kit	L	D				Si		I					I		V		W	S/Y	
		Inh	L	D				Si		I					I		V		W	S/Y	
9	22	Kit						N	K/R												
		Inh						N													
10	23	Kit																			
		Inh																			
11	24	Kit															V				
		Inh															V				

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
12	28	Kit		A/V			N								I					Y	
		Inh		A			N								I					Y	
13	30	Kit															V				
		Inh															V				
14	31	Kit															I/M/V				
		Inh															I/M/V				
15	32	Kit															I/M/V				
		Inh															I				
16	33	Kit	L					N	R		I/M/V						V			F	Q
		Inh	L					N	K/R		M						V		L/W	F	K/Q
17	35	Kit															V				
		Inh															V				
18	36	Kit															V				
		Inh							R								V				
19	37	Kit					N		R								V			F	Q
		Inh					N		R								V			F	Q
20	38	Kit					d	G	R	I										F	Q
		Inh					d	G	R	I										F	Q
21	39	Kit			A/V		D/N									M	V				
		Inh			A/V		D/N									M	V				
22	41	Kit				K/R															
		Inh				K/R															

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
23	42	Kit															V				
		Inh															V				
24	43	Kit															V				
		Inh															V				
25	44	Kit															V				
		Inh															M/V				
26	45	Kit																			K/N
		Inh																			K/N
27	51	Kit															V				
		Inh															V				
28	52	Kit															V				
		Inh															V				
29	53	Kit					D/N										V				
		Inh					D/N										V				
30	66	Kit					D/N										I/M/V				
		Inh															V				
31	68	Kit						S	K/R								V				
		Inh						S	R								V				
32	73	Kit															V				
		Inh															V				
33	76	Kit	L	D			N								I		V		W	Y	
		Inh	L	D			N								I		V		W	Y	

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
34	78	Kit									L	L		Y		M	V				
		Inh									L	L		Y		M	V				
35	79	Kit					N		E					Y		M	V				
		Inh					N		E					Y		M	V				
36	83	Kit								I/L							M/V				
		Inh								I							V				
37	84	Kit	L														V			F	
		Inh	L																	F	
38	86	Kit					N/S	N	R											F	E
		Inh					S	N	R											F	E
39	97	Kit	L				N				I						V		W	F	
		Inh	L				N				I						V		W	F	
40	99	Kit							K/R												
		Inh																			
41	100	Kit						N								K	V				
		Inh						N								M	V				
42	108	Kit															V				
		Inh															V				
43	109	Kit																			
		Inh																			E/K
44	110	Kit																			
		Inh																			

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
45	111	Kit	L	A			N	D							I		V	H/Y	W	Y	
		Inh	L	A			N	D							I		V	H/Y	W	Y	N
46	112	Kit					N		R								V			F	E
		Inh					N		R								V			F/I/S/T	E/Q
47	113	Kit																			
		Inh																			
48	114	Kit																		Y	
		Inh																		N/S/T/Y	
49	115	Kit					G	D	R								I/V				K/Q
		Inh					G	D	R								V				
50	116	Kit					D/N		K/R												
		Inh					D/N		K/R												
51	117	Kit															M/V				
		Inh															M/V				
52	118	Kit																			
		Inh																			
53	119	Kit									A/V						V			N/S/T/Y	
		Inh									A/V						V			Y	
54	120	Kit																			
		Inh																			
55	122	Kit															V				
		Inh															V				

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
56	123	Kit	L	D			N			V					I		V		W	Y	
		Inh	L	A/D			N			V					I		V		W	Y	
57	128	Kit				R					M	L				M	V				
		Inh				R					M	L				M	V				
58	130	Kit							K/R		I/V						V				E/K
		Inh						A/T	K/R		I/V						I/M/V				E/K
59	131	Kit															V				
		Inh															V				
60	132	Kit															V				
		Inh															V				
61	134	Kit					D/N										V			F/I/S/T	
		Inh					D/N										V			F/I/S/T	
62	137	Kit						N/T													
		Inh						N/T													
63	138	Kit															V				
		Inh															V				
64	139	Kit															V				
		Inh															V				
65	140	Kit								V							V				
		Inh								V							V				
66	141	Kit						N/T									V				E/K
		Inh						N/T									V				E/K

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
67	142	Kit									I						I				
		Inh									I						I				
68	145	Kit					N		R	I							V			F	Q
		Inh					N		R	I/L							V			F/I/S/T	Q
69	147	Kit	L/M						N		L						V				R
		Inh	L						N		L						V				R
70	149	Kit									L						V				
		Inh									L						V				
71	150	Kit								I							V				
		Inh								I							V				
72	151	Kit							K/R								V				
		Inh							R								V				K/R
73	152	Kit																			
		Inh																			
74	156	Kit															V				
		Inh															V				
75	158	Kit																			
		Inh																			
76	159	Kit															I/V			I	
		Inh															V				
77	164	Kit															V				
		Inh															V				

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
78	165	Kit	L	D													V		W	Y	
		Inh	L	D													V		W	Y	
79	166	Kit					N										V				
		Inh					N										V				
80	167	Kit															V				
		Inh															V				
81	168	Kit															V				
		Inh															M/V				
82	169	Kit	L				N	S			I/M/V								W	S/T	
		Inh	L				N	Si			M								W	S	
83	170	Kit			V	R											V				
		Inh				R							F/Y				V				
84	176	Kit					N										V				K/R
		Inh					N										V				K/R
85	177	Kit	L	A			G				M									Y	
		Inh	L	A			G				M									Y	
86	185	Kit																			
		Inh																			
87	192	Kit																			
		Inh																			
88	194	Kit			V						T					M	V				E
		Inh			V						T					M	V				E

Prevalence of NRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NRTI																	
			M41	E44	A62	K65	D67	T69	K70	L74	V75	F77	Y115	F116	V118	Q151	M184	H208	L210	T215	K219
89	195	Kit	L							V					I		V		W	F	
		Inh	L							V					I/V		V		W	F	
90	198	Kit	L																L/W	Y	
		Inh	L																L/W	Y	
91	200	Kit				N							F								
		Inh				N							F								
92	201	Kit															M/V				
		Inh															M/V				
93	202	Kit				R														A	
		Inh				R														A	
94	204	Kit				R					L										
		Inh				R					L										
95	205	Kit									I/V						V				
		Inh															V				
96	206	Kit															V			F	
		Inh															V			F	
97	208	Kit								I							V			F	N
		Inh								I							V			F	N
98	209	Kit															V				
		Inh															V				
99	210	Kit																			
		Inh																			

APPENDIX B

Prevalence of NNRTI-associated-mutation codon

No.	Sample No.	Test	NNRTI																
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
1	2	Kit				Q						C/Y		A/G						
		Inh				Q						C		A						
2	3	Kit												A						
		Inh												A						
3	4	Kit		G								C								
		Inh		G								C								
4	5	Kit							I			C			Y					
		Inh							I			C			Y					
5	7	Kit		G			N													
		Inh		G			N													
6	9	Kit					N					C								
		Inh					N					C								
7	14	Kit		G								C			Y					
		Inh		G								C			Y					
8	18	Kit		G			N		I											
		Inh		G			N		I											
9	22	Kit				Q						C		A/G						
		Inh				Q						C		A/G						
10	23	Kit	I										L							
		Inh											L							
11	24	Kit				E/K								A						
		Inh				E								A						

Prevalence of NNRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NNRTI																
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
12	28	Kit				E			I			C		A/G	Y					
		Inh				E			I			C		A	Y					
13	30	Kit					N					C/Y								
		Inh					N													
14	31	Kit							I/V			C/Y			H/Y					
		Inh										C/Y								
15	32	Kit					N					C		A/G						
		Inh										C								
16	33	Kit										C								
		Inh										C								
17	35	Kit																		
		Inh																		
18	36	Kit					S			Q										
		Inh					N			Q										
19	37	Kit		A/G							H/L/D/V	I								
		Inh		A/G							D	I								
20	38	Kit										C			Y					
		Inh										C			Y					
21	39	Kit						I				C		A	Y					
		Inh						I				C		A	Y					
22	41	Kit				E						C		A						
		Inh	I/V			E						C		A						

Prevalence of NNRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NNRTI																	
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
23	42	Kit					N			Q										
		Inh					N			E/Q										
24	43	Kit		A/G			K/N/R/S			E/Q				A						
		Inh		A/G			K/N/R/S			E/Q				A						
25	44	Kit							I/V			C								
		Inh										C								
26	45	Kit										C/Y		A/G						
		Inh										C/Y								
27	51	Kit					N													
		Inh					N/S													
28	52	Kit																		
		Inh																		
29	53	Kit				P/Q								A						
		Inh				A/E/P/Q								A						
30	66	Kit										C/Y		A/G						
		Inh												A						
31	68	Kit				K/Q						C								
		Inh										C								
32	73	Kit																		
		Inh																		
33	76	Kit																		
		Inh																		

Prevalence of NNRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NNRTI																
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
34	78	Kit				E								A						
		Inh				E								A						
35	79	Kit						M						A						
		Inh						M						A						
36	83	Kit					N		I/V							H/P		L/M		
		Inh					N									H				
37	84	Kit		G		H					T	C		A						
		Inh		G		H					T	C		A						
38	86	Kit				Q						C		A						
		Inh				Q						C		A						
39	97	Kit			I		N													
		Inh			I		N													
40	99	Kit				E/K	K/R			E/G										
		Inh								E/G										
41	100	Kit					R				D	C		A						
		Inh					R				D	C		A						
42	108	Kit					R				D			A						
		Inh					R				D			A						
43	109	Kit										C		A/G						
		Inh										C/Y		A/G						
44	110	Kit					N													
		Inh					N													
45	111	Kit		G			N												T	
		Inh		G			N													
46	112	Kit				E						C		A						
		Inh				E						C/Y		A						
47	113	Kit	I				N													
		Inh	I				N		I											
48	114	Kit																		
		Inh																		

Prevalence of NNRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NNRTI																	
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
49	115	Kit				Q						V			H/Y					
		Inh				Q						V			Y					
50	116	Kit					K/N													
		Inh					K/N													
51	117	Kit					K/N			E/Q									T	
		Inh					K/N			E/Q										
52	118	Kit					K/N													
		Inh					K/N													
53	119	Kit	I			E		M				C		A						
		Inh	I			E		M				C		A						
54	120	Kit					R			G	D									
		Inh					R			G	D									
55	122	Kit										C			Y					
		Inh										C/Y		A/G	Y					
56	123	Kit				E						C		A						
		Inh				E						C		A						
57	128	Kit				Q			I			C								
		Inh				Q			I			C								
58	130	Kit							I/V					A						
		Inh							I/V					A/G						
59	131	Kit											L							
		Inh											L							
60	132	Kit																		
		Inh																		
61	134	Kit				E						C/G		A						
		Inh				E						C		A						
62	137	Kit										C								
		Inh										C								
63	138	Kit					N													F/Y
		Inh					N													

Prevalence of NNRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NNRTI																	
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
64	139	Kit					S							A						
		Inh					S							A						
65	140	Kit			I		N									H/P				
		Inh			I		N									H/P				
66	141	Kit					K/N					C/Y		A/G						
		Inh					K/N			E/Q		C/Y		A/G						
67	142	Kit										C								
		Inh										C								
68	145	Kit				E								A						
		Inh				E			I/V					A						
69	147	Kit					N		I							H				
		Inh					N		I							H				
70	149	Kit				E								A						
		Inh				E								A						
71	150	Kit					N	M												
		Inh					N	M												
72	151	Kit					N													
		Inh	I/V				N													
73	152	Kit					N					C								
		Inh					K/N					C/Y								
74	156	Kit					N					V								
		Inh					N					C/F/G/V								
75	158	Kit					N			G										
		Inh					N			G										
76	159	Kit										C								
		Inh										C								
77	164	Kit		G			S							A						
		Inh		G			S							A						
78	165	Kit																		
		Inh																		

Prevalence of NNRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NNRTI																
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
79	166	Kit										C								
		Inh										C								
80	167	Kit				K/Q	N													
		Inh					N													
81	168	Kit		G		E						C		A						
		Inh		G		E						C/Y		A/G						
82	169	Kit					N			Q									K/T	F/Y
		Inh					N			Q									T	
83	170	Kit						M						A						
		Inh						M						A			F/L			
84	176	Kit	I				R				D	C			Y					
		Inh	I				R				D	C			Y					
85	177	Kit							I			V			Y					
		Inh							I			V			Y					
86	185	Kit					N													
		Inh					N													
87	192	Kit					N	I		Q										
		Inh					N	I		Q										
88	194	Kit					N					C								
		Inh					N					C								
89	195	Kit					N	I										L		
		Inh					N	I										L		
90	198	Kit																		
		Inh																		
91	200	Kit	I								D	C								
		Inh	I								D	C								
92	201	Kit																		
		Inh																		

Prevalence of NNRTI-associated-mutation codon (Cont.)

No.	Sample No.	Test	NNRTI																
			V90	A98	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	K238	Y318
93	202	Kit				Q					D	C		S						
		Inh				Q					D	C		S						
94	204	Kit				Q				E/K				E						
		Inh				Q				E/K				E						
95	205	Kit										C								
		Inh										C								
96	206	Kit				E						C		A						
		Inh				E						C		A						
97	208	Kit		G								C								
		Inh		G								C								
98	209	Kit					N													
		Inh					N													
99	210	Kit						I				C			Y					
		Inh						I				C			Y					

APPENDIX C

Antiretroviral resistance reports of each assay

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
1	2	Kit (Kit Guideline)	S	S	S	S	S	S	S	P	P	R	-
		Inhouse (Standford)	S	S	S	S	P	S	P	R	R	R	R
		Kit (Standford)	S	S	S	S	P	S	P	R	R	R	R
2	3	Kit (Kit Guideline)	S	S	S	S	S	S	S	P	S	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	P	P	R	P
		Kit (Standford)	S	S	S	S	S	S	S	P	P	R	P
3	4	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P
4	5	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P
5	7	Kit (Kit Guideline)	P	P	R	R	P	P	P	R	S	R	-
		Inhouse (Standford)	P	P	R	R	P	P	R	R	S	R	S
		Kit (Standford)	P	P	R	R	P	P	P	R	S	R	S
6	9	Kit (Kit Guideline)	P	R	R	R	P	R	P	R	P	R	-
		Inhouse (Standford)	R	R	R	R	R	P	R	R	P	R	P
		Kit (Standford)	R	R	R	R	R	P	R	R	P	R	P
7	14	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
8	18	Kit (Kit Guideline)	R	R	R	R	R	R	R	R	P	R	-
		Inhouse (Standford)	R	R	R	R	R	R	R	R	S	R	S
		Kit (Standford)	R	R	R	R	R	R	R	R	S	R	S
9	22	Kit (Kit Guideline)	S	S	S	S	S	S	S	P	P	R	-
		Inhouse (Standford)	S	P	S	S	P	S	S	R	R	R	R
		Kit (Standford)	S	P	S	S	P	S	P	R	R	R	R
10	23	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	S	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	P	R	P
		Kit (Standford)	S	S	S	S	S	S	S	R	P	R	P
11	24	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P
12	28	Kit (Kit Guideline)	S	S	S	S	R	S	R	R	P	R	-
		Inhouse (Standford)	P	P	P	P	P	P	P	R	R	R	R
		Kit (Standford)	P	P	P	P	P	P	P	R	R	R	R
13	30	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	S	R	S
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
14	31	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
15	32	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
16	33	Kit (Kit Guideline)	P	R	R	R	R	R	P	P	P	R	-
		Inhouse (Standford)	R	R	R	R	R	P	R	P	P	R	P
		Kit (Standford)	P	R	R	R	R	P	R	P	P	R	P
17	35	Kit (Kit Guideline)	S	S	R	R	S	S	S	S	S	S	-
		Inhouse (Standford)	P	S	R	R	S	S	S	S	S	S	S
		Kit (Standford)	P	S	R	R	S	S	S	S	S	S	S
18	36	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	P	R	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
19	37	Kit (Kit Guideline)	P	P	R	R	P	P	P	P	P	R	-
		Inhouse (Standford)	P	P	R	R	P	P	R	P	R	R	R
		Kit (Standford)	P	P	R	R	P	P	R	P	R	R	R
20	38	Kit (Kit Guideline)	S	S	S	S	R	S	R	P	P	R	-
		Inhouse (Standford)	R	R	P	P	R	P	R	P	P	R	P
		Kit (Standford)	R	R	P	P	R	P	R	P	P	R	P
21	39	Kit (Kit Guideline)	R	R	R	R	R	S	R	P	P	R	-
		Inhouse (Standford)	R	R	R	R	P	P	P	R	R	R	R
		Kit (Standford)	R	R	R	R	P	P	P	R	R	R	R

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
22	41	Kit (Kit Guideline)	R	R	P	P	P	R	S	R	P	R	-
		Inhouse (Standford)	P	P	P	P	P	P	S	R	R	R	R
		Kit (Standford)	P	P	P	P	P	P	S	R	R	R	R
23	42	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
24	43	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
25	44	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P
26	45	Kit (Kit Guideline)	S	S	S	S	S	S	S	P	P	R	-
		Inhouse (Standford)	S	S	S	S	P	S	P	P	P	R	P
		Kit (Standford)	S	S	S	S	P	S	P	R	P	R	P
27	51	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	S	R	S
		Kit (Standford)	P	S	R	R	S	S	S	R	S	R	S
28	52	Kit (Kit Guideline)	S	S	R	R	S	S	S	S	S	S	-
		Inhouse (Standford)	P	S	R	R	S	S	S	S	S	S	S
		Kit (Standford)	P	S	R	R	S	S	S	S	S	S	S

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
29	53	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	P	R	-
		Inhouse (Standford)	P	P	R	R	S	S	S	R	P	R	P
		Kit (Standford)	P	P	R	R	S	S	S	R	P	R	P
30	66	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	P	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	P	R	R	P	S	P	R	P	R	P
31	68	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	P	R	-
		Inhouse (Standford)	P	P	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	P	R	R	S	S	S	P	P	R	P
32	73	Kit (Kit Guideline)	S	S	R	R	S	S	S	S	S	S	-
		Inhouse (Standford)	P	S	R	R	S	S	S	S	S	S	S
		Kit (Standford)	P	S	R	R	S	S	S	S	S	S	S
33	76	Kit (Kit Guideline)	R	R	R	R	P	R	P	S	S	S	-
		Inhouse (Standford)	R	R	R	R	R	P	R	S	S	S	S
		Kit (Standford)	R	R	R	R	R	P	R	S	S	S	S
34	78	Kit (Kit Guideline)	R	R	R	R	R	S	R	P	S	R	-
		Inhouse (Standford)	R	R	R	R	R	P	R	P	P	R	P
		Kit (Standford)	R	R	R	R	R	P	R	P	P	R	P
35	79	Kit (Kit Guideline)	R	R	R	R	R	P	R	R	S	R	-
		Inhouse (Standford)	R	R	R	R	R	P	R	R	P	R	P
		Kit (Standford)	R	R	R	R	R	P	R	R	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
36	83	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	P	R	-
		Inhouse (Standford)	P	P	R	R	S	S	S	R	S	R	S
		Kit (Standford)	P	P	R	R	S	S	S	R	P	R	P
37	84	Kit (Kit Guideline)	P	P	R	R	P	P	P	P	R	R	-
		Inhouse (Standford)	P	P	S	S	P	P	P	R	R	R	R
		Kit (Standford)	P	P	R	R	P	P	P	R	R	R	R
38	86	Kit (Kit Guideline)	S	R	S	S	P	R	R	P	P	R	-
		Inhouse (Standford)	P	P	S	S	R	P	R	R	R	R	R
		Kit (Standford)	P	P	S	S	R	P	R	R	R	R	R
39	97	Kit (Kit Guideline)	R	R	R	R	P	R	P	R	P	R	-
		Inhouse (Standford)	R	R	R	R	R	P	R	R	P	R	P
		Kit (Standford)	R	R	R	R	R	P	R	R	P	R	P
40	99	Kit (Kit Guideline)	S	S	S	S	S	S	S	S	P	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	S	P	S	P
		Kit (Standford)	S	S	S	S	P	S	P	P	P	P	P
41	100	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	P	R	-
		Inhouse (Standford)	P	R	R	R	P	S	P	R	R	R	R
		Kit (Standford)	P	P	R	R	S	S	S	R	R	R	R
42	108	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
43	109	Kit (Kit Guideline)	S	S	S	S	S	S	S	P	P	R	-
		Inhouse (Standford)	S	S	S	S	P	S	P	R	P	R	P
		Kit (Standford)	S	S	S	S	S	S	S	R	P	R	P
44	110	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	S	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	S	R	S
		Kit (Standford)	S	S	S	S	S	S	S	R	S	R	S
45	111	Kit (Kit Guideline)	R	R	R	R	P	R	P	R	S	R	-
		Inhouse (Standford)	R	R	R	R	R	P	R	R	S	R	S
		Kit (Standford)	R	R	R	R	R	P	R	R	P	R	S
46	112	Kit (Kit Guideline)	P	P	R	R	P	P	P	R	P	R	-
		Inhouse (Standford)	P	P	R	R	P	P	R	R	R	R	R
		Kit (Standford)	P	P	R	R	P	P	R	R	R	R	R
47	113	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	S	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	S	R	S
		Kit (Standford)	S	S	S	S	S	S	S	R	S	R	S
48	114	Kit (Kit Guideline)	S	S	S	S	R	S	R	S	S	S	-
		Inhouse (Standford)	P	P	S	S	P	P	P	S	S	S	S
		Kit (Standford)	P	P	S	S	P	P	P	S	S	S	S
49	115	Kit (Kit Guideline)	S	P	R	R	P	S	P	S	P	S	-
		Inhouse (Standford)	P	P	R	R	P	P	P	P	R	R	R
		Kit (Standford)	P	P	R	R	P	P	P	P	R	R	R

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
50	116	Kit (Kit Guideline)	S	S	S	S	P	S	P	R	S	R	-
		Inhouse (Standford)	S	S	S	S	P	P	P	R	S	R	S
		Kit (Standford)	S	S	S	S	P	P	P	R	S	R	S
51	117	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
52	118	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	S	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	S	R	S
		Kit (Standford)	S	S	S	S	S	S	S	R	S	R	S
53	119	Kit (Kit Guideline)	P	S	R	R	P	S	P	R	P	R	-
		Inhouse (Standford)	P	P	R	R	P	P	P	R	R	R	R
		Kit (Standford)	P	P	R	R	P	P	P	R	R	R	R
54	120	Kit (Kit Guideline)	S	S	S	S	S	S	S	S	P	S	-
		Inhouse (Standford)	S	S	S	S	S	S	S	P	P	P	P
		Kit (Standford)	S	S	S	S	S	S	S	P	P	P	P
55	122	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	R	R	R
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P
56	123	Kit (Kit Guideline)	R	R	R	R	R	R	R	R	-	R	-
		Inhouse (Standford)	R	R	R	R	R	P	R	R	R	R	R
		Kit (Standford)	R	R	R	R	R	P	R	R	R	R	R

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
57	128	Kit (Kit Guideline)	R	R	R	R	R	R	R	R	-	R	-
		Inhouse (Standford)	R	R	R	R	R	R	P	P	P	R	P
		Kit (Standford)	R	R	R	R	R	R	P	P	P	R	P
58	130	Kit (Kit Guideline)	S	S	R	R	S	S	P	P	-	R	-
		Inhouse (Standford)	P	P	R	R	P	P	P	P	P	R	P
		Kit (Standford)	P	P	R	R	P	S	P	P	P	R	P
59	131	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
60	132	Kit (Kit Guideline)	S	S	R	R	S	S	S	S	-	S	-
		Inhouse (Standford)	P	S	R	R	S	S	S	S	S	S	S
		Kit (Standford)	P	S	R	R	S	S	S	S	S	S	S
61	134	Kit (Kit Guideline)	P	P	R	R	P	S	P	R	-	R	-
		Inhouse (Standford)	P	P	R	R	P	S	P	R	R	R	R
		Kit (Standford)	P	P	R	R	P	S	P	R	R	R	R
62	137	Kit (Kit Guideline)	S	S	S	S	S	S	S	P	P	R	-
		Inhouse (Standford)	S	P	S	S	P	S	S	P	P	R	P
		Kit (Standford)	S	P	S	S	P	S	S	P	P	R	P
63	138	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	S	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	S	R	S
		Kit (Standford)	P	S	R	R	S	S	S	R	S	R	S

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
64	139	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P
65	140	Kit (Kit Guideline)	P	R	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	R	R	R	R	S	S	S	R	P	R	P
		Kit (Standford)	R	R	R	R	S	S	S	R	P	R	P
66	141	Kit (Kit Guideline)	S	P	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	P	R	R	P	S	P	R	R	R	R
		Kit (Standford)	P	P	R	R	P	S	P	R	P	R	P
67	142	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	-	R	-
		Inhouse (Standford)	P	P	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	P	R	R	S	S	S	P	P	R	P
68	145	Kit (Kit Guideline)	R	R	R	R	R	P	R	P	-	R	-
		Inhouse (Standford)	R	R	R	R	P	P	R	R	P	R	P
		Kit (Standford)	R	R	R	R	P	P	R	P	P	R	P
69	147	Kit (Kit Guideline)	P	P	R	R	P	S	P	R	-	R	-
		Inhouse (Standford)	P	P	R	R	P	S	P	R	S	R	S
		Kit (Standford)	P	P	R	R	P	S	P	R	S	R	S
70	149	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	P	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
71	150	Kit (Kit Guideline)	P	P	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	P	R	R	S	S	S	R	S	R	S
		Kit (Standford)	P	P	R	R	S	S	S	R	S	R	S
72	151	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	P	S	P	R	S	R	S
		Kit (Standford)	P	S	R	R	S	S	P	R	S	R	S
73	152	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	-	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	P	R	P
		Kit (Standford)	S	S	S	S	S	S	S	R	P	R	P
74	156	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	R	R	R
		Kit (Standford)	P	S	R	R	S	S	S	R	R	R	R
75	158	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	-	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	P	R	P
		Kit (Standford)	S	S	S	S	S	S	S	R	P	R	P
76	159	Kit (Kit Guideline)	S	S	R	R	P	S	P	P	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	P	R	R	P	S	P	P	P	R	P
77	164	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	P	R	P
		Kit (Standford)	P	S	R	R	S	S	S	R	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
78	165	Kit (Kit Guideline)	R	P	R	R	R	P	R	S	-	S	-
		Inhouse (Standford)	P	P	R	R	P	P	P	S	S	S	S
		Kit (Standford)	P	P	R	R	P	P	P	S	S	S	S
79	166	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	-	R	-
		Inhouse (Standford)	P	P	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	P	R	R	S	S	S	P	P	R	P
80	167	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	S	R	S
		Kit (Standford)	P	S	R	R	S	S	S	R	S	R	S
81	168	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	R	R	R
		Kit (Standford)	P	S	R	R	S	S	S	R	R	R	R
82	169	Kit (Kit Guideline)	P	R	S	S	R	P	R	R	-	R	-
		Inhouse (Standford)	P	R	P	P	R	P	R	R	P	R	P
		Kit (Standford)	P	R	P	P	R	P	R	R	P	R	P
83	170	Kit (Kit Guideline)	P	P	R	R	S	R	S	R	-	R	-
		Inhouse (Standford)	R	R	R	R	P	R	S	R	P	R	P
		Kit (Standford)	R	R	R	R	P	P	S	R	P	R	P
84	176	Kit (Kit Guideline)	S	S	R	R	P	S	P	P	-	R	-
		Inhouse (Standford)	P	P	R	R	P	S	P	P	P	R	P
		Kit (Standford)	P	P	R	R	P	S	P	P	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
85	177	Kit (Kit Guideline)	P	P	S	S	R	P	R	P	-	R	-
		Inhouse (Standford)	P	R	P	P	R	P	R	P	R	R	R
		Kit (Standford)	P	R	P	P	R	P	R	P	R	R	R
86	185	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	-	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	S	R	S
		Kit (Standford)	S	S	S	S	S	S	S	R	S	R	S
87	192	Kit (Kit Guideline)	S	S	S	S	S	S	S	R	P	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	R	P	R	P
		Kit (Standford)	S	S	S	S	S	S	S	R	P	R	P
88	194	Kit (Kit Guideline)	R	R	R	R	R	S	R	R	-	R	-
		Inhouse (Standford)	P	R	R	R	R	P	R	R	P	R	P
		Kit (Standford)	P	R	R	R	R	P	R	R	P	R	P
89	195	Kit (Kit Guideline)	R	R	R	R	R	P	R	R	-	R	-
		Inhouse (Standford)	R	R	R	R	P	P	P	R	P	R	P
		Kit (Standford)	R	R	R	R	P	P	P	R	P	R	P
90	198	Kit (Kit Guideline)	P	P	S	S	R	P	R	S	-	S	-
		Inhouse (Standford)	P	P	P	P	R	P	R	S	S	S	S
		Kit (Standford)	P	P	P	P	R	P	R	S	S	S	S
91	200	Kit (Kit Guideline)	P	S	S	S	S	S	S	P	-	R	-
		Inhouse (Standford)	P	P	P	P	S	P	S	P	P	R	P
		Kit (Standford)	P	P	P	P	S	P	S	P	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
92	201	Kit (Kit Guideline)	S	S	R	R	S	S	S	S	-	S	-
		Inhouse (Standford)	P	S	R	R	S	S	S	S	S	S	S
		Kit (Standford)	P	S	R	R	S	S	S	S	S	S	S
93	202	Kit (Kit Guideline)	P	P	P	P	P	R	P	R	-	R	-
		Inhouse (Standford)	P	P	P	P	P	P	S	R	R	R	R
		Kit (Standford)	P	P	P	P	P	P	S	R	R	R	R
94	204	Kit (Kit Guideline)	P	P	P	P	S	R	S	R	-	R	-
		Inhouse (Standford)	P	P	P	P	P	P	S	R	P	R	P
		Kit (Standford)	P	P	P	P	P	P	S	R	P	R	P
95	205	Kit (Kit Guideline)	S	S	R	R	S	S	S	P	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	P	P	R	P
		Kit (Standford)	P	P	R	R	S	S	S	P	P	R	P
96	206	Kit (Kit Guideline)	P	S	R	R	P	S	P	R	-	R	-
		Inhouse (Standford)	P	P	R	R	P	S	P	R	R	R	R
		Kit (Standford)	P	P	R	R	P	S	P	R	R	R	R
97	208	Kit (Kit Guideline)	R	R	R	R	P	S	P	P	-	R	-
		Inhouse (Standford)	R	R	R	R	P	S	P	P	P	R	P
		Kit (Standford)	R	R	R	R	P	S	P	P	P	R	P
98	209	Kit (Kit Guideline)	S	S	R	R	S	S	S	R	-	R	-
		Inhouse (Standford)	P	S	R	R	S	S	S	R	S	R	S
		Kit (Standford)	P	S	R	R	S	S	S	R	S	R	S

R=Resistance, P=Possible Resistance, S=Susceptible

Antiretroviral resistance reports of each assay (Cont.)

No.	Sample No.	Assay (Interpretation)	NRTI							NNRTI			
			ABC	DDI	3TC	FTC	D4T	TDF	AZT	EFV	ETR	NVP	RPV
99	210	Kit (Kit Guideline)	S	S	S	S	S	S	S	P	-	R	-
		Inhouse (Standford)	S	S	S	S	S	S	S	P	P	R	P
		Kit (Standford)	S	S	S	S	S	S	S	P	P	R	P

R=Resistance, P=Possible Resistance, S=Susceptible

BIOGRAPHY

Miss Sayompoo Sanguansittianant was born on 12 June, 1987 in Bangkok. In 2008, he obtained the Bachelor of Science Program with Major in Medical Technology and Minor in Nutrition and Dietetics from faculty of Allied Health Sciences, Chulalongkorn University. Since 2008-2012, he had done postgraduate education in Clinical Biochemistry and Molecular Medicine at Department of Clinical Chemistry, faculty of Allied Health Sciences, Chulalongkorn University. He received a scholarship from Graduate School, Chulalongkorn University and teacher assistant scholarship from faculty of Allied Health Sciences, Chulalongkorn University.