A NEW APPROACH TO ASSESSING THE REVERSE TRANSCRIPTASE INHIBITOR SUSCEPTIBILITY OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 USING ULTRASENSITIVE REVERSE TRANSCRIPTASE ASSAYS AND REAL TIME PCR

DUMRONG SAKULPONGYUENYONG

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ABSTRACT

The potential for drug resistance among HIV-1 viruses has led to the development of drug susceptibility assays. Currently, an ultrasensitive assay has been developed to aid in determining drug susceptibility. This involves obtaining HIV positive supernatant and utilizing an ultrasensitive reverse transcriptase (RT) assay to measure the RT activity. The RT product is then amplified using PCR and post PCR manipulation, such as an Enzyme-linked-immunosorbent Assay (ELISA). A new approach has been introduced as an alternative assay. The new approach is similar to the current ultrasensitive assay, however, real time PCR is substituted over conventional PCR and post PCR manipulation. In this project, avian myeloblastosis virus reverse transcriptase (AMV-RT) and the wild type HIV-1 virus (HXB2) will be used to simulate and observe benefits from the new approach. Initial observation of this new alternative assay showed increased sensitivity by at least three log fold as compared to the conventional PCR. The alternative approach consumed at least half the amount of time (4 hours) compared to the conventional method. Quantification of the samples was more precise when sample runs were repeated, expenditures were less costly, and the new approach offered a safer procedure with less toxic chemicals used such as Ethidium Bromide. And preliminary results indicate that clinical applications on HIV patients are possible. Overall the new approach offers an alternative method which is comparable if not advantageous to conventional means.

KEY WORDS: HIV-1 / DRUG SUSCEPTIBILITY / REAL TIME PCR

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วิธีการใหม่ในการตรวจสอบการดื้อยาต้านไวรัสกลุ่ม reverse transcriptase inhibitor ต่อเชื้อ HIV-1 โดยใช้ Ultrasensitive reverse transcriptase assay ร่วมกับ Real-time PCR (A NEW APPROACH TO ASSESSING THE REVERSE TRANSCRIPTASE INHIBITOR SUSCEPTIBILITY OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 USING ULTRASENSITIVE REVERSE TRANSCRIPTASE ASSAY AND REAL TIME PCR)

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บทคัดย่อ

อัตราการเสียชีวิตเนื่องจากการติดเชื้อ HIV-1 ลดลงอย่างเห็นได้ชัดเมื่อเริ่มใช้ยาต้านไวรัส แต่เนื่องจากความสามารถในการดื้อยาของเชื้อไวรัส ดังนั้นการพัฒนาวิธีการตรวจวัดความไวต่อยาของเชื้อไวรัสจึงเกิดขึ้น ในการวิจัยนี้ได้มีการพัฒนาวิธีตรวจวัดความไวของเชื้อไวรัสต่อยาที่มีความไวสูง (Ultrasensitive assay) โดยการนำของเหลวที่มีเชื้อ HIV มาตรวจวัดโดยวิธี Ultrasensitive reverse transcriptase assay โดยเริ่มต้นจะใช้เอนไซม์ Reverse transcriptase จากนั้นผลิตภัณฑ์ที่ได้จะถูกนำไปเพิ่มจำนวนโดยวิธี PCR และขบวนการหลังจากทำ PCR เช่น วิธี ELISA สำหรับการวิจัยครั้งนี้ได้เสนอทางเลือกใหม่ที่คล้ายกับวิธีที่ใช้อยู่ในปัจจุบัน แต่ใช้ Real time PCR แทนที่วิธี PCR ปกติและขบวนการหลังจากทำ PCR การวิจัยนี้ใช้เอนไซม์ Reverse transcriptase จาก avian myeloblastosis virus ในการทดลองและสังเกตผล การทดสอบเบื้องต้นพบว่าวิธีใหม่นี้สามารถเพิ่มความไวในการตรวจสอบขึ้นอย่างต่ำ 3 เท่าในมาตรา log และใช้เวลาการตรวจสอบลดลงจากเดิมครึ่งหนึ่งโดยเปรียบเทียบกับวิธี PCR ปกติ และการทดลองซ้ำพบว่าความแม่นยำของการตรวจสอบด้วยวิธีนี้จะเพิ่มขึ้น วิธีการตรวจสอบใหม่นี้จะลดค่าใช้จ่ายในการตรวจสอบต่อหนึ่งตัวอย่างลงอย่างมากทั้งเป็นวิธีที่ปลอดภัยจากสารเคมีอันตราย เช่น Ethidium Bromide โดยสรุปแล้ววิธีใหม่นี้เสนอทางเลือกใหม่ในการตรวจสอบการดื้อยาของเชื้อไวรัสซึ่งมีผลไม่ด้อยไปกว่าวิธีเดิมเลย

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
/ or :	per
AMV-RT	Avian Myeloblastosis Virus
bp	base pair
cDNA	Complementary deoxyribonucleic acid
Cm	Centimeter
Cm^3	Cubic centimeter
CT	Threshold Cycle
dCTP	deoxyribocytosinetriphosphate
ddATP	2',3'-Dideoxy-adenosine-5' triphosphate
dGTP	deoxyriboguaninetriphosphate
dNTP	deoxyribonucleotidetriphosphate
dT	deoxyribose-thymine
dTTP	deoxyribothyminetriphosphate
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis Virus
et al.	Et. All (Latin), and others
etc.	Et Cetera
FIG.	Figure
H, h	Hour
HAART	Highly Active Anti Retroviral Therapy
HIV-1	Human Immunodeficiency Virus Type-1
i.e.	id est (Latin), that is
IC_{50}	Inhibitory Concentration at 50%
IU	International Unit
l	Liter
LTR	Long terminal repeat

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LIST OF ABBREVIATIONS (CONTINUE)

mg Milligram
min Minute
mRNA messenger RNA
ng Nanogram
nm Nanometer
PBMCs Peripheral blood mononuclear cells
PCR Polymerase chain reaction
PP Plateau Phase
RT Reverse transcription
SDS Sodium deocyl sulphate
TP Transition Phase
U Unit
U Units
µg microgram
µl Micro liter
µl microliter
µM Micro molar

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CHAPTER 1
INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) has been responsible for a significant number of morbidity and mortality among the human population. Up to date, 21 million deaths have been report with at least 40 million infected worldwide(3, 4). With increasing knowledge of the viral life cycle, mortality rate has been on the decline. Human Immunodeficiency Virus Type 1 (HIV-1) is a retrovirus that relies on a specific enzyme necessary for replication(3, 5). The first enzyme is the reverse transcriptase enzyme. This enzyme serves to convert HIV-1 viral RNA into DNA, which is then integrated into the host cell(6). At an appropriate time, the viral DNA is simultaneously replicated along with the host genome, utilizing the cellular mechanism contributed by the host cell. The second enzyme is the HIV-1 protease enzyme. This enzyme serves to cleave non-functional proteins into functional proteins. It is those functional proteins that are involved in the formation of infectious viral particle of the HIV-1 (FIG 1). Since the introduction of antiretroviral drugs, morbidity and mortality caused by HIV-1 has declined significantly. However, like other forms of viruses and bacteria, evolution selects for fit strains. And because the HIV virus has a tendency to mutate very frequently, the virus eventually obtains the capability of escaping or even impeding the effects of human immunity and drug inhibition against the virus. Resistance develops. In the past, HIV-1 therapy relied on a hit or miss method in which medications were administered and until viral load increases, one continues to administer those drugs without consideration to efficacy and resistance. However, in current times, the expenditure of these HIV-1 therapies is very costly. Since Each HIV-1 patient varies in their susceptibility to certain drugs., it is beneficial that treatment of HIV therapy is one that relies on individua highly active antiretroviral therapy (HAART) for each patient)(7). It would be too costly, time consuming, and health hazard not to consider using the most optimal drug possible against the virus.

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Assays have been developed to determine drug susceptibility of HIV-1. Once drug susceptibility is determined, the appropriate HAART therapy may be used on each individual patient(8). There is a specific type of approach, called enzymatic based assay, which aids in detecting drug susceptibility in HIV-1 patients(9, 10). Enzymatic based assay allows for an ultrasensitive reverse transcriptase assay. In an enzymatic based assay, a heterologous RNA template (EMCV RNA template), serves as a template for reverse transcription (RT) activity. Product cDNA is obtained from the reverse transcription activity and then amplified using a conventional polymerase chain reaction (PCR). An ELISA hybridization utilizing a specific internal probe will be used to further detect cDNA. Through measurement of the RT activity and its production of cDNA, the ability of viral replication may be determined quantitatively(11, 12). In turn, phenotypic resistance to RT inhibitors may be determined quantitatively by simply comparing the patients drug susceptibility results with those of the wild type HIV-1 (i.e., HXB2). RT product will serve as the scale of measurement in determining RT inhibition under increasing or decreasing amount of reverse transcriptase inhibitor drugs. A new approach will be designed to eliminate PCR variations and post PCR (ex. ELISA) manipulation seen in an enzymatic assay. This involves utilization of a real-time PCR as a method for detection. Real-time PCR has become more widely accepted as a new approach to the conventional PCR due to increase in speed, sensitivity, reproduction, and reduction of possibility for contamination. The basic principle of real-time PCR is similar to that of conventional PCR; however, in real-time PCR one is able to see amplification in progress. This involves usage of primers or probes that are labeled with flourogenic molecules. This new approach will serve an alternative method in utilizing ultrasensitive reverse transcriptase assay and real-time PCR over conventional means.

In order for new methods to be considered useful, it must at least be comparable to conventional means and provide an alternative choice for researchers. Even better, those new methods should have certain advantages that benefit researcher which might not be evident in the traditional methods. In this study, a new approach to drug susceptibility testing will be compared with those of conventional means. Real-time PCR will be the key device which differs from conventional methods. Initially, optimization of the new assay will be the focus of the project. The assay will be

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optimized so that it will be comparable to those of conventional assays. Next, the new assay will be observed for advantages over traditional methods. Considerations for such advantages are sensitivity, reproducibility, cost effectiveness, time, and safety. These advantages will be observed under simulation of RT-PCR reactions utilizing Avian Myeloblastosis Virus reverse transcriptase (AMV-RT) and wild type HIV-1 (HXB2). Lastly, the study will be applied to a clinical setting in which HIV-1 patients are used for drug susceptibility testing. Clinical applications for drug susceptibility testing will be an ongoing project since determination for scale of resistance in this study will determine significantly the sample size of the project. At this point, approximately 100 patients were analyzed, of which only 20 samples were used for drug susceptibility. However, this is still not enough to justify any correlations or conclusion. This study will only suggest that there are possibilities for application on a clinical level. This will allow researchers to further their applications to clinical studies in which they will eventually obtain a correlation and trend in terms of drug susceptibility testing. In the end, this new approach will offers an alternative choice over traditional methods.

OBJECTIVES

1. To introduce a new approach to assessing the reverse transcriptase inhibitor susceptibility of human immunodeficiency virus type 1 using ultrasensitive reverse transcriptase assay and real time PCR.
2. To observe advantages and disadvantages in using the new approach over conventional means. AMV-RT and HXB2 will be used to similar the assay. Results from the new approach will also be compared with genotypic analysis.
3. To further apply this new approach on a clinical level to determine if it’s possible to observe drug susceptibility in patients.

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CHAPTER 2
LITERATURE REVIEW

1. Human immunodeficiency virus

1.1 General characteristic

Human immunodeficiency virus type 1 (HIV-1) has been responsible for a significant number of morbidity and mortality among the human population. There are presently 4 million infections of HIV-1 per year with approximately 40 million already infected worldwide. Over the past two decades, the virus has become one the biggest cause of human mortality (3, 4). HIV-1 is a retrovirus that relies on two major types of enzyme for replication within the host cell. The first enzyme is the reverse transcriptase enzyme. This enzyme serves to convert HIV-1 viral RNA into DNA, which is then integrated into the host cell. At an appropriate time, the viral DNA is simultaneously replicated along with the host genome, utilizing the cellular mechanism contributed by the host cell. The second enzyme is the HIV-1 protease enzyme. This enzyme serves to cleave non-functional proteins into functional proteins. It is those functional proteins that are involved in the formation of infectious viral particle of the HIV-1 (FIG 1). Since the introduction of antiretroviral drugs, morbidity and mortality caused by HIV-1 has declined significantly.

2. HIV-drugs

2.1 Classification of antiretroviral drugs

Currently, there are 3 major types of antiretroviral drugs (Table 1): Nucleoside reverse transcriptase inhibitors (NRTI), Non-nucleoside reverse transcriptase inhibitors (NNRTI), and Protease inhibitors (PI)(13). NRTIs serve as a competitive inhibitor of reverse transcriptase in which the drug targets the active site of the enzyme. This enzyme requires intracellular activation in which the drug is phosphorylated into the 5'-triphosphate form (14-17). It is the 5'-triphosphate form

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that competes with existing deoxynucleosides for the active site of the reverse transcriptase.

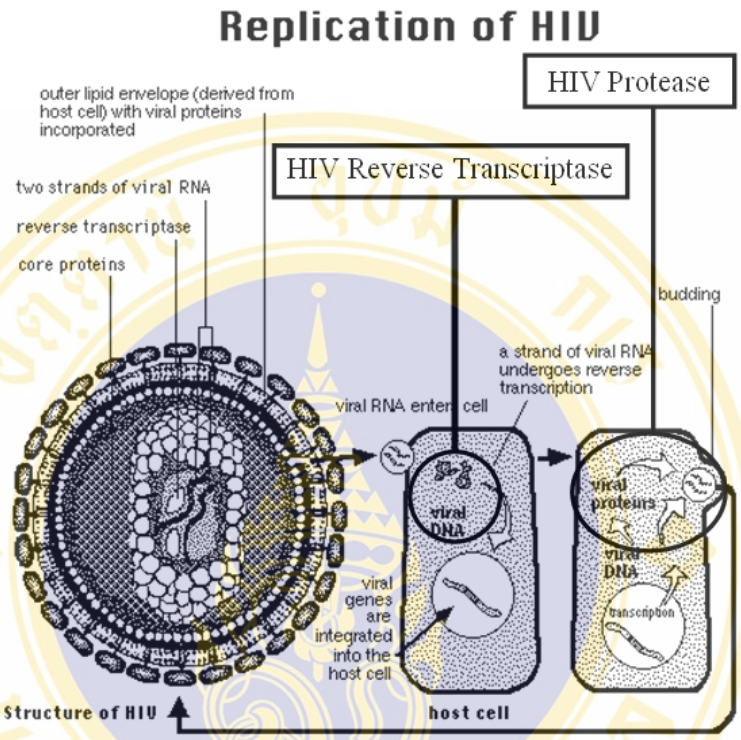

Figure 1. The replication cycle of HIV-1. Names located in the box indicate major enzymes which contribute to the replication of HIV. It is these two major enzymes which are the current target of HIV-1 drugs.

NRTIs serve as a non-competitive inhibitor of reverse transcriptase. Unlike the NRTIs, the NNRTI does not require any cellular activation or phosphorylation. It non-competitively binds to conserved residues of the p66 subunit of the reverse transcriptase (18). This in effect will manipulate the structure of the reverse transcriptase enzyme in such a way that the active site on the enzyme is changed(15, 17).

Protease inhibitor competitively binds to the active site of the protease enzyme. In this respect, it prevents the cleavage of non-functional proteins into functional ones. Infectious viral particles are not produced(19, 20).

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Table 1. Some examples of three groups of HIV-1 Drug commonly used.(21)

Generic name (Abbreviation)	**Trade Name**
Nucleoside RT inhibitors	
Abacavir (1592U89)	Ziagen
Didanosine (ddI)	Videx
Lamivudine (3TC)	Epivir
Stavudine (d4T)	Zerit
Zalcitabine (ddC)	Hivid
Zidovudine (ZDV)	Retrovir
Non-nucleoside RT inhibitors	
Delavirdine (U-90152)	Rescriptor
Efavirenz (DMP 266)	Stocrin
Nevirapine (BI-RG-587)	Viramune
Protease inhibitors	
Amprenavir (141W94)	Agenerase
Indinavir (MK 639)	Crixivan
Nelfinavir (AG 1343)	Viracept
Ritonavir (ABT 538)	Norvir
Saquinavir (RO 31-8959)	Invirase

2.2 Drug Resistance

The combination of reverse transcriptase and protease inhibitors has proven effective in suppressing viral replication(15). However, with long term usage of such drugs come resistances. Drug resistance is defined as the ability of the virus to replicate even under increasing drug concentration when compared to the wild type virus (7, 22). The basis for such resistance lies on the ability of the HIV-1 to mutate rapidly. Such mutation lies in the genes that express proteins that are targets for the antiretroviral drugs. When those genes mutate, the proteins that are expressed will change in such a way that antiretroviral drugs will have no impeding effect on it(9, 23). On a clinical level, resistance can be manifested by other factors such as insufficient drug adherence, insufficient drug potency, and insufficient knowledge of drug interaction(22). There are different degrees of resistance to current antiretroviral drugs. And some resistance to a particular antiretroviral drug may lead to a domino effect in which cross resistance may occurs with other types of drug(17).

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For NRTIs, a single mutation in the HIV-1 sequence may lead up to 100-1,000-fold increase in resistance(2). For PIs, clinical studies have confirmed the laboratory findings and document high treatment failure rate (75-80%) among urban HIV patients who have failed an initial protease inhibitor regimen(2). NNRT resistance has increased among individuals from 0% in 1996-1997 to 13.2% in 2000-2001. Presently, the prevalence of HIV-1 drug resistance is 70% for NRTI, 31% for NNRTI, and 42% for PI(2). In addition, previous studies have found that if patients were infected with drug-resistant viruses, initial antiretroviral treatment would most likely fail(2). Therefore, it is important that the choice for an appropriate antiretroviral drug should be one that is highly active, and not particularly choosing new drugs(2, 22). When prescribing HIV-1 drugs, a combination (drug cocktail) of NRTI, NNRTI, and Protease inhibitors are generally used. It is important that every drug within the combination must be effective against the drug target. This is referred to as highly active antiretroviral drug therapy (HAART), in which all drug combinations are functionally active against the virus. Figure 2 explains the importance of HAART in reducing mortality rate over a period of years. Therefore, on a clinical level, drug susceptibility testing is an important part in determining optimal patient therapy against HIV-1. The goal of drug susceptibility testing for HIV-1 is to determine which drug combination is optimal for a specific patient and which drug combination is substandard, or resistant(24, 25).

3. Resistance Testing

3.1 Type of Resistance Testing

Currently, there are two main types of resistance testing for HIV-1: Genotypic and phenotypic assay(26, 27). Genotypic assay involves the observation of mutation in the viral genome on drug resistance. Phenotypic assay involves measuring the level of viral replication relative to drug resistance(1, 28).

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3. Resistance Testing

3.1 Type of Resistance Testing

Currently, there are two main types of resistance testing for HIV-1: Genotypicand phenotypic assay. Genotypic assay involves the observation of mutation in the viral genome on drug resistance. Phenotypic assay involves measuring the level of viral replication relative to drug resistance.

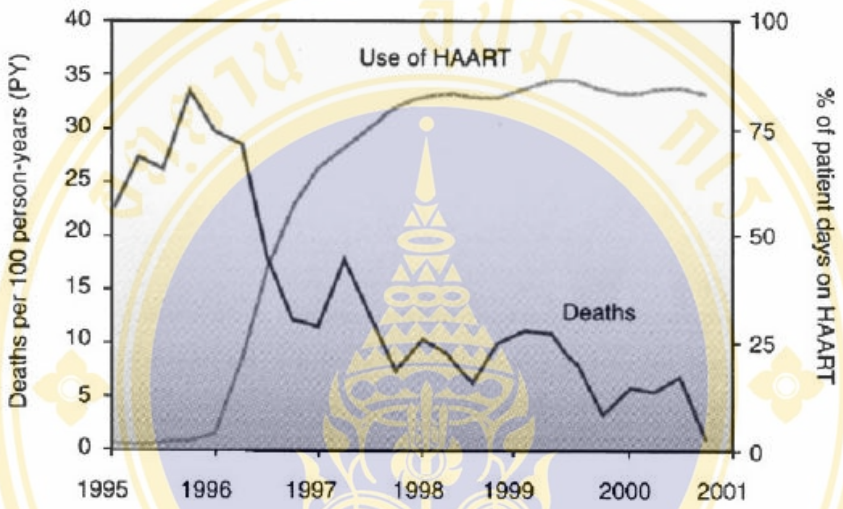

Figure 2. Quarterly mortality rates and HAART usage among patients (7, 8)

3.2 Genotypic assay

Genotypic assay essentially involves amplification and sequencing of the reverse transcriptase or protease genes(29). Dye labeled nucleotides or primers are used to pinpoint specific regions of the enzyme gene. These nucleotides and primers are dideoxynucleotides that initiate termination of DNA elongation. Through analysis of different DNA fragment length, the specific location of those nucleotides or primers on the enzyme gene could be detected. When compared to nonresistant strain, such as HXB2, any deviations or variability in DNA fragment length could be a sign of possible mutation that might be associated with drug resistance. Interpretation of the results involves paralleling patient derived DNA sequence with a reference strain. Sequence for the reference strain and location sequence of genes associated with mutation for drug resistance may be found from the Gene Bank or from a web-based site(1, 23). Currently in Thailand, a genotypic assay cost approximately 2,500 baht per patient and requires approximately one week for processing which is fairly cheap

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when compared to some phenotypic assays(6). The analysis is not as technically demanding as some phenotypic assay(6). However, there are certain disadvantages to a genotypic assay. First, a great deal of genotypic knowledge is required for analysis of the results. Second, synergistic or antagonistic results are not seen in the genotypic assay. Certain resistance to one type of drug may cause a domino effect in which resistance to other drugs occur. Or conversely, resistance to one drug may heighten the susceptibility of another. This is not seen in a genotypic assay since the focus of the assay is not a collective analysis, rather a mutation to mutation analysis affecting resistance to a single drug rather than a collective group.

3.3 Phenotypic Assay

There are two types of phenotypic assays: Replicative assay and enzymatic assay. In a replicative assay, peripheral blood mononuclear cells (PBMCs) are innoculated with the HIV-1 virus and allowed 2-8 weeks for cultivation (FIG. 1)(8). As a standard of measurement, p24 antigen is read. Because two weeks is required for cultivation, there is a tendency for manifestation of new mutations due to drug resistance selection. More current phenotypic assay involves transformation of the patient derived fragment and the proviral deletion mutants into a human T cell line and observed for replication. Usage of indicators such as alkaline phosphatase and beta-galactosidase are incorporated into the cell line or into the viral backbone. This allows for improved detection. This assay provides a more direct measure of drug susceptibility where complex resistance pattern are capable of being detected (1, 8, 19). However, there are certain disadvantages of using this assay. First, replicative assay is very expensive, costing as much as 40,000 baht per patient. Second, replication assay consumes a great of time ranging from 2-4 weeks. And third, in countries such as Thailand, replicative assay are not available and must be done abroad.

Enzymatic-based assay (Table 2) serves as a way to observe the function of specific HIV-1 enzymes, particularly those of the reverse transcriptase enzyme(30, 31). The enzymatic-based assay allows for an ultrasensitive reverse transcriptase assay. AMP-RT is one example of an enzymatic-based (32, 33). Initially, a heterologous RNA template (ie, the EMCV RNA template), serves as a template for RT activity. EMCV cDNA is obtained from the reverse transcription activity and then

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amplified using the PCR(34). An ELISA hybridization utilizing a specific internal probe will be used to further detect EMCV cDNA (12, 32, 33, 35). Through measurement of the RT activity and its production of cDNA, phenotypic resistance to RT inhibitors may be determined. RT product will serve as the scale of measurement in determining RT inhibition under increasing or decreasing amount of RT inhibitor drugs. IC_{50} and IC_{90} for drugs are used to test for susceptibility of HIV-1 RT. Drug resistance is determined by comparing IC_{50} results of viral strains within the patient relative to those of the wild type (2). A scale of fold resistance (IC_{50} drug of Patients / IC_{50} drug of HIV-1 Wild Type) will determine whether the non wild-type virus has become resistant(16). When compared to the replication-based assay, several wild type and resistant isolates have shown correlations between replication-based assay and enzymatic-based assay (1). However, in contrast to the replication-based assay, enzymatic assay does not require virus isolation and cultivation. This shortens the assay time to 1-2 days, as opposed to approximately two weeks for a replication based assay. Sensitivity is reasonable good when compared to the replication based assay. The disadvantages of enzymatic assay is that it is used mainly to detect susceptibility to reverse transcriptase inhibitors and, therefore, does not yet provide Table 2 explains the advantages and disadvantage of phenotypic and genotypic assay (1).

4. Drug Susceptibility Analysis Using Real-Time PCR.

4.1 New Approach to Drug Susceptibility

While enzymatic based assay have an advantage over other reverse transcriptase assay in terms of time consumption, a new approach will be used to assess the reverse transcriptase inhibitor susceptibility of Human Immunodeficiency Virus Type 1 using ultrasensitive reverse transcriptase assays and real time PCR(10, 36). This approach is similar to an enzymatic phenotypic assay. However, instead of utilizing conventional PCR and post PCR manipulation (ex. ELISA) seen in an enzymatic assay, a new approach would eliminate those processes and in turn use real-time PCR as a method for detection (FIG 3).

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Table 2A Disadvantages of genotypic and phenotypic assay (1).

Assay	Disadvantages
Phenotypic Assay A) Replication based assay	• Very expensive: Approximately 40,000 Baht in Thailand. • Long time for results (2-4 weeks) • No clinical relevant cut off values that are clearly defined.
B) Enzymatic Assays	• Not available in Thailand. • Rapid protease detection not available. • Not able to detect AZT resistance. Useful for subtype B and non-subtype B HIV-1. • Possible contamination.
Genotype assay	• More indirect measure of susceptibility. • Requires more expertise knowledge to interpret. • Not a complete defined resistance pattern.

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Table 2B Advantages of genotypic and phenotypic assay (1).

Assay	Advantages
Phenotypic Assay B) Replication based assay	• Directly measures drug susceptibility • Detect complex resistance patterns • More understanding among clinicians
B) Enzymatic Assays	• Rapid (1-2 days), not complex, and cheap • Good sensitivity
Genotype assay	• Cheaper than replication based assay. However, in Thailand, in-house assay cost approximately 2500 (Chulalongkorn University). • Not as technically demanding than replication based assay

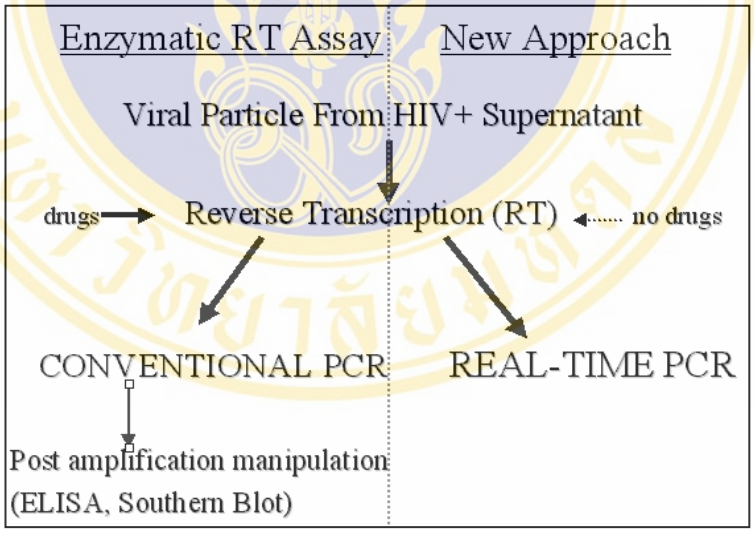

Figure 3. Comparing present enzymatic RT assay with a new approach assay.

Initially, the introduction of conventional PCR has revolutionized the field of genetics in such a way that DNA analysis could be amplified and observed with the naked eye(37). The process of amplification also enables post PCR manipulation, such as ELISA, to be sensitive enough to detect and genetically quantitate values from PCR products. In terms of drug susceptibility assays for HIV-1, PCR is an important part in detecting RT activity of the virus. However, a new approach may serve as an alternative to the conventional PCR. This new approach involves the usage of real-

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time PCR. Although conventional PCR has been an important tool for drug susceptibility testing in HIV-1, there are certain disadvantages.

4.2 Obstacles in Using Conventional PCR

In a conventional PCR, genetic amplification undergoes three phases: lag phase, exponential phase, and linear phase. Lag phase is the initial phase of amplification where a lag or delay in amplification occurs. Exponential phase is a phase where exact doubling of DNA occurs. Linear phase is a phase where the reaction starts to slow down. The linear phase occurs either because reactions are thoroughly consumed or there are product degradations. The first disadvantage lies in the linear phase. When PCR cycle is complete and genetic amplification has been achieved, the PCR will have undergone all three phases, including the linear phase. Previous documents have shown that at the linear phase, replicated runs do not produce identical results (FIG4) (38).

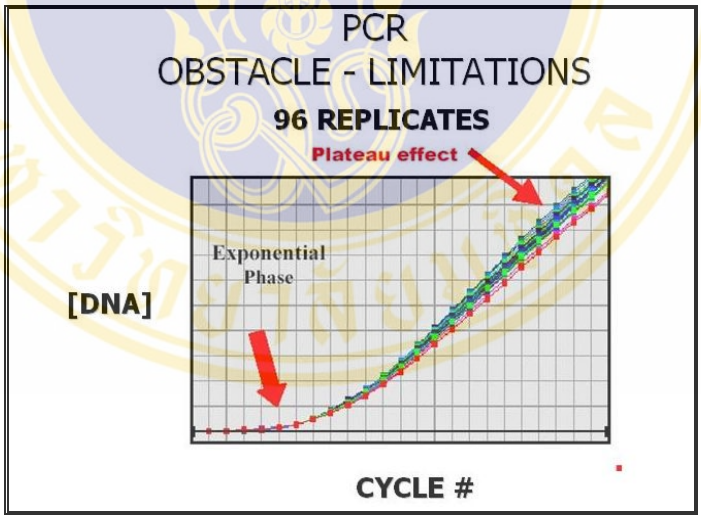

Figure 4. Obstacles in replicated runs for conventional PCR

Another disadvantage involves the ability of the conventional PCR to provide reliable quantitative results among different initial template concentrations. If a series of fold dilutions were done on a template DNA and then amplified in the conventional PCR, all dilutions would have undergone the linear phase. Previous research have shown that at serial dilutions done on a set of template DNA, the linear phase is a not a reliable phase to be quantitating initial template concentration. It is the exponential

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phase of the PCR which should be the region where quantitation occurs (FIG 5)(38). However, in the conventional PCR, most template will have undergone the linear phase. Therefore, quantitating initial template concentration may not be reliable when using the conventional PCR.

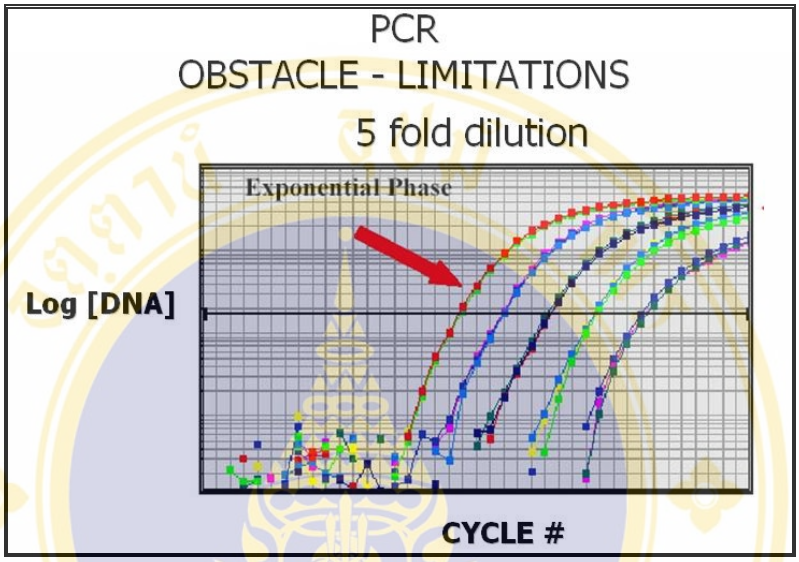

Figure 5. Obstacles for conventional PCR when doing fold dilutions.

In order to genetically quantitate values with increase sensitivity, a post PCR manipulation is used. Examples of post PCR manipulation is the Enzyme Linked Immunosorbent Assay (ELISA) and Southern Hybridization(39). With increasing steps comes the possibility for contamination. Another disadvantage of the conventional PCR is that in order to increase its sensitivity, post PCR manipulation is used which can lead to possible contaminations.

Safety issue is another reason for using alternatives to conventional PCR. Ethidium Bromide is a chemical used as fluorescence for DNA fragments after amplification. This chemical is highly toxic and carcinogenic.

4.3 Real time PCR

Real-time PCR has become more widely accepted as a new approach to the conventional PCR due to increase in speed, sensitivity, reproduction, and reduction of possibility for contamination (40). The basic principle of real-time PCR is similar to that of conventional PCR; however, in real-time PCR one is able to see amplification in progress. This process of amplification is achieved through usage of primers or probes that are labeled with flourogenic molecules. Typical of all conventional PCR,

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real-time PCR also has a PCR cycle: The first step in the PCR cycle involves denaturing of the double stranded DNA (at >90°C), the second step involves primer annealing (at 50-75°C), and the third step involves extension or elongation of the DNA strand by a DNA polymerase (at 72-78°C). The more cycles completed the more amplification of the target DNA strand (FIG. 6)(40).

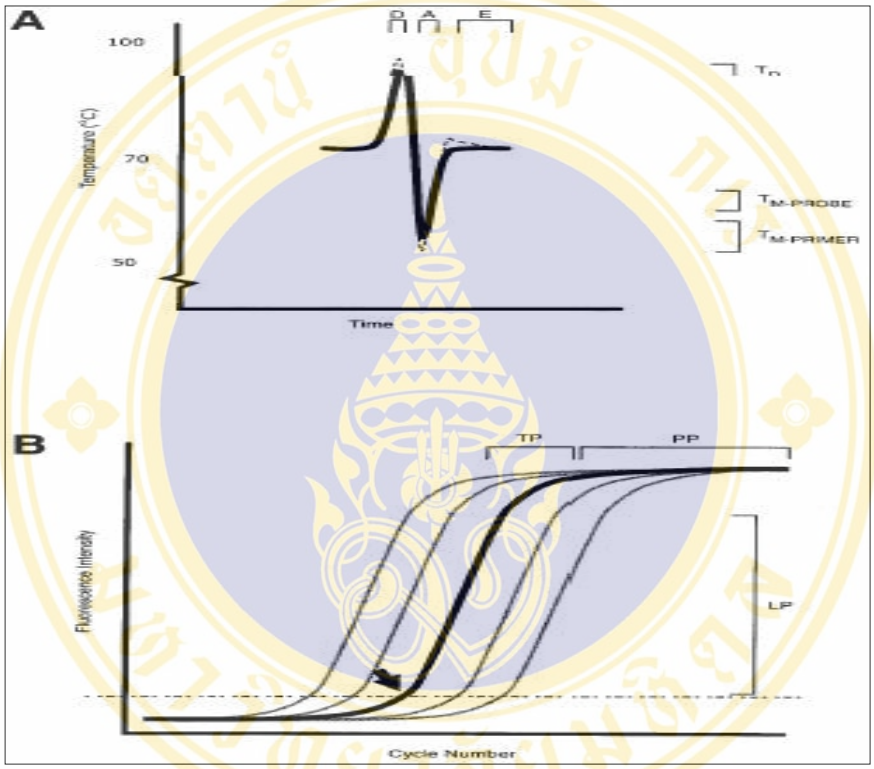

Figure 6. The PCR cycle.

Figure 6A illustrates a typical PCR cycle found in both conventional PCR and real-time PCR: (D) Signify the annealing temperature, (A) Signify a primer annealing temperature, and (E) Signify a DNA extension temperature.

Figure 6B illustrates an ideal amplification curve for a real-time PCR. In a normal real-time PCR cycle, the rate of amplification is approximately one $\log_{10}$ for every three cycles. When the sample reaches the transition phase (TP), the reaction starts to slow and reach saturation, either because the primer or DNA or polymerase has been used up. At plateau phase (PP), the reaction reaches saturation point and very little or no yield is observed. At early stages of the PCR cycle, the flourescence of the reading moves from a stage of insignificant level to a more detectable level. The stage where florescences transitions from insignificant to clearly detectable level, called the

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threshold cycle, is the point where the template quantity is calculated in the real-time PCR (Noted by arrow). In situations where serial titration is used to provide a standard curve, the log phase (LP) is the location where standard curves are determined.

4.3.1 Types of Real Time PCR

There are two major classes of real-time PCR. First is the Light Cycler real-time PCR (Roche Light Cycler) which utilizies glass capillary tubes to quickly change the temperature of the PCR content during the PCR cycle. The ramp-rate, or temperature fluctuation, may occur at 2°C per second. This is approximately ten times faster than with a conventional PCR. The second type of real-time PCR is called a Taqman Cycler real-time PCR. The speed is relatively similar to that of the conventional PCR. However, Taqman Cycler utilizes specific oligoprobes which fluoresces proportional to the amount of DNA amplification(40-42).

4.3.2 Chemistry of Real Time PCR

Two main chemistry are used for detecting amplicon in a real time PCR. First is the usage of specific primers or probe that has been labeled with flourogenic molecules. Second is the usage of a nonspecific DNA-binding flourophores (ex. SYBR green), which associates with ds-DNA(43). As amplification continues, the amount of association of the flourophores with ds-DNA increases. Flourescence will increase relative to amplification(40).

There are approximately 4 major types of specific primers or probe that are utilized in real-time PCR(44). First is the adjacent oligoprobes which are used mainly in Light Cycler real-time PCR. Adjacent oligoprobes are two separate probes, both with different fluorescence that are undetectable by the real-time PCR when unattached to the DNA fragment but flouresces a detectable wavelength when both become attached near one another on a DNA strand. Second type of specific probe is the 5’ nuclease oligoprobe. This is more commonly used in Taqman Cycler real-time PCR. The 5’ nuclease oligoprobe contains a fluorescent area and a quencher area. Normally, the fluorescent region is attached to the quencher region which suppresses the fluorescence of the probe. When the probe attaches to the DNA strand, a polymerase will cut off the fluorescent region which will no longer be quenched and in turn allow the probe to flourese. The third type of specific probe is the hairpin

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oligoprobe. This probe is shaped similar to a hairpin in which the tip of the hairpin contains the fluorescent region and the quencher region. Similar to the 5'nuclease, the fluorescent region is quenched by the quencher region. Upon attachment of the hairpin oligoprobe to the DNA template, the fluorescence and quencher region is separated as the hairpin oligoprobe is stretched into a linear strand. The fourth type of specific probe is the sunrise or scorpio primer. This primer is shaped like a hairpin oligoprobe and functions in similarly. However, it acts as a primer in which it creates a new strand where the sunrise or scorpio primer contributes to the formation of a second amplified DNA strand. The advantage of using specific primer or probes is that the results would more specific. In terms of quantification, the usage of specific primers and probes would serve to be a more precise method. The disadvantage, however, is in the complexity of designing probes. The addition of a probe may create new problems which must be taken into consideration, such as primer-probe interactions. Also, probes are costly and will add to the expense of the overall assay.

The advantage of using DNA-binding flourophores, such as SYBR green, is that little knowledge is required in designing probes, the cost is less expensive than other methods, and sensitivity is higher (40). The disadvantage of DNA-binding flourophores is that there is an increase probability for developing primer-dimers. In the new approach, the usage of Qiagen Syber Green, which contains HotStar Taq polymerase, has proven effective in eliminating primer-dimers. HotStar Taq polymerase has a active temperature that is substantially higher than the annealing temperature of the primer. For this reason, the chance for developing primer-dimer will minimize as DNA extension occurs. If there is a chance that primer-dimer does develop, there are two methods that may aid in detection of such interference. First is simply to run a polyacrylamide gel electrophoresis to observe if multiple bands exist. Second is by observing the melting temperature (Tm) of the sample. In a real-time PCR, multiple peaks in a Tm curve may signify either contaminations or existence of primer-dimers. Analysis of the Tm curve will specifically target the correct amplicon sequence through differentiating of the Tm among different sequences (40).

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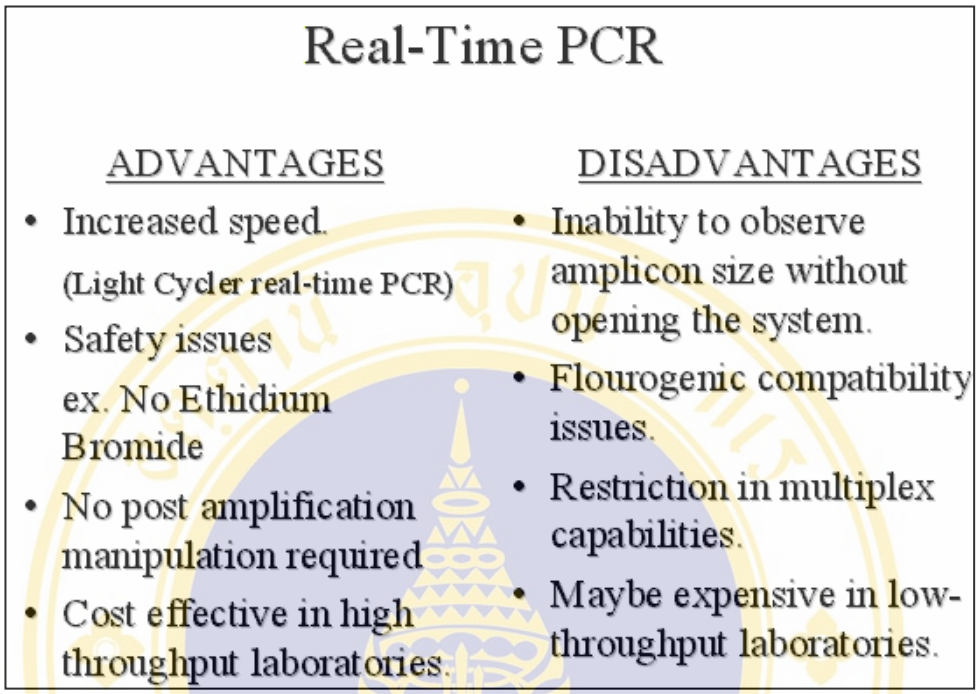
Table 3. Advantages and Disadvantages of Real Time PCR.



Through current knowledge of advantages in using real-time PCR (Table 3), this would suggest that the new approach would offer an assay that is faster, safer, cost effective (1,000 baht per sample), and reliable. There are certain disadvantages in using real time PCR. It is possible that a future multiplex system maybe used in which multiple amplicons are in one system using different flourogenic molecules to simultaneously amplify different amplicons(35). However, there are still restrictions in using multiplex system. Real time PCR still has limitations on the amount of flouregenic molecules used to detect amplicons. There may be interference or the system may not be able to discriminate the difference between the fluorescence. Although disadavantages do exist in using such a device, current knowledge and research suggests future benefits which override the negative aspec (40).

4.4 Similating drug susceptibility assay using the new approach.

4.4.1 AMV-RT

Avian myeloblastosis virus reverse transcriptase (AMV-RT) is initially used as a source of reverse transcriptase to simulate drug susceptibility assay using the new approach. There are several reasons why AMV-RT is used. First, AMV-RT is well documented and its activity and units is well understood. Second, AMV-RT is an

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active enzyme that does not require additional chemistry, such as those that are used with HIV-1 viral particles, to activate. For other source of RT-enzymes obtained from viral particles, a more complex chemistry (i.e., NP-40) is required to break down the viral particle so that active RT enzymes are exposed. Third, the AMV-RT enzyme is an active reverse transcriptase enzyme and contains no assembly enzyme for replication of infectious viruses. Therefore, the AMV-RT enzyme poses very little safety threat to laboratory staff members. Fourth, the AMV-RT enzyme is an enzyme easily obtainable from a chemical company (i.e. Promega) with exact concentration units. In a situation where AMV-RT enzyme is diluted in a fold-series, the amount of RT activity should be proportionate to the concentration of the AMV-RT(45). This is useful for quantitating a standard curve where RT activity may be quantitated based on the concentration of the AMV-RT units. If a standard curve is developed using AMV-RT, all other RT enzymes may be compared relative to the AMV-RT standard curve in which other RT enzyme activity may be based on AMV-RT concentration units(46).

4.4.2 HXB2

HXB2 is a clone of the wild type HIV-1 virus(26, 47). It is the next step used in similating drug susceptibility assay using the new approach. There are several reasons why HXB2 would be the choice to use. First, HXB2 is a viral particle that is similar to the wild type HIV-1 virus and may have similar RT activity comparable to those of the wild type. This is a useful tool which initially proves that the new approach is capable of analyzing viral particles seen in clinical applications such as those in patient serum. Second, the viral load of HXB2 may easily be obtained, and a standard curve may also be used utilizing the units of viral copy number relative to the HXB2 virus. It may also be used to observe limitations within the new approach so that one is aware of obstacles within the assay. Third, since the HXB2 is basically a wild type clone, it may be used as a positive control which is susceptible to all antiretroviral drugs(48, 49).

4.4.3 Patient Serum

Similar to HXB2, patient serum has been applied to conventional ultrasensitive drug susceptibility assay and shown satisfactory outcome. Given the advantages of real-time PCR over conventional methods, the new approach would suggest that

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applications using patient serum would benefit from those offered using the real-time PCR(32, 50).

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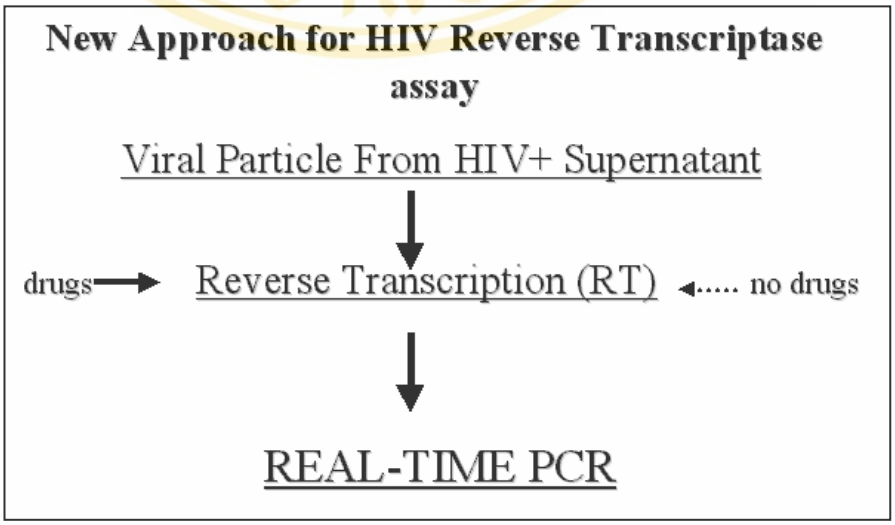
CHAPTER 3
MATERIALS AND METHODS

1. SUMMARY OF NEW APPROACH

Table 4 summarizes an HIV reverse transcriptase (RT) assay using the new approach. HIV+ supernatant is obtained either from a company stock, culured supernatant, or human serum. The HIV+ supernatant is then used for reverse transcription. At the point of reverse transcription is where drugs are utilized. The RT product is then amplified on a real-time PCR (Rotor gene real time PCR, Roche Light cycler real time PCR) and then analyzed to observe the RT activity of each sample.

Table 4. Viral particle is obtained from patient serum that has been centrifuged to eliminate cellular components. Those viral particles will serve as source of reverse transcriptase for RT activity. It is during the reverse transcription where anti-retroviral drugs are used to assess drug susceptibility. Chromosomal DNA obtained from reverse transcription will be amplifiedutilizing Roche LightCycler real-time PCR.



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2. SOURCE OF ENZYME FOR REVERSE TRANSCRIPTION

Initial step of the project involved utilizing reverse transcriptase from the Avian Myeloblastosis Virus (AMV-RT). AMV-RT is not a viral particle, rather, it is a purified reverse transcriptase extracted from the virus. This enzyme will serve as a standard curve on which all other samples are compared with. Second step of the project involves usage of a modified non-infectious viral particle (MC99) that contains the reverse transcriptase enzyme. This viral particle allows for observation of reverse transcriptase activity without safety threats and utilizing of the laboratory infectious level 3. A third step of the project involved utilizing a drug susceptible viral particle from HXB2 (CI/Wildtype), which will be used as an alternative standard which indicates the effectiveness of the RT assay. Fourth step of the project involves usage of a drug susceptible molecular clone, HXB2 (SUM9), and drug resistance molecular clone, HXB2 (SUM13). This will serve as a positive control during the RT activity. Finally, HIV viral particle will be extracted from the supernatant of patient serum, obtained from the virology laboratory, Ramathibodi Hospital. The samples will be obtained in the evening and immediately analyzed for viral load and drug susceptibility. These patient samples will not undergo any freeze and thaw until drug susceptibility assay has been complete.

3. THE ULTRASENSITIVE ASSAY

3.1 Reverse Transcription

Intially, the protocol, AMP-RT assay (10) was utilized during the initial stage of the project. Utilizing the AMP-RT assay protocol (10), a reverse transcription master mix should contain 9 reagents: 5X RT buffer C (250 m*M* Tris-HCl, pH 8.3, 250 m*M* KCl, 50 m*M* $MgCl_2$), dNTP (10 m*M*), EGTA (10m*M*), NP-40 (2%), DTT (100 m*M*), Rnasin (40,000 U/ml), EMCV RNA (20 ng/μL), Antisense Emc R2 primer (5'-GTTCATGACAGGCCGATACAGAGG-3') (200 ng/μL), DEP-C water. The total volume of the master mix is 41 μL per reaction. Nine microliters of the viral particle is added to make a final volume of 50 μL. Reverse transcription occurs when the sample is incubated at 37°C water bath for 2 hours. After reverse transcription, the

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left over reverse transcriptase must be denatured to inactive the enzyme. This requires heating the tube at 95°C for 5 minutes, and then chill on ice or store at -70°C.

Because of complications with the AMP-RT protocol, a modified form of the AMP-RT assay protocol (32) was developed. Within this modified form of AMP-RT , a reverse transcription master mix should contain 8 reagents: 5X AMV-RT Buffer (Qiagen), 0.1 M DTT, 10 mM dNTP, Antisense Emc R2 primer (5'-GTTCATGACAGGCCGATACAGAGG-3') (50 ng/µL), NP-40 (2%), Rnasin (10,000 Units), EMCV RNA (20 ng/µL), DEP-C water. A template (RT Enzyme) of one microliter is added to the master mix to make a total volume of 20 microliter (Table 5). To test susceptibility of the drug, antiretroviral drugs are added at the reverse transcription phase of the experiment (Table 6). DEP-C is adjusted accordingly to the amount of drugs used. Two types of HIV-1 drugs are used: ddATP and Nevaripine. For all samples and controls, there are five concentrations of ddATP used (25 µM, 6.25 µM, 3.12 µM, 1.56 µM , and 0.39 µM). For all samples and controls, there are five concentrations of Nevaripine used (100 µM, 10 µM, 1 µM, 0.1 µM, 0.01 µM) Reverse transcription occurs when the sample is incubated at 37°C water bath for 2 hours. After reverse transcription, the left over reverse transcriptase must be denatured to inactive the enzyme. This requires heating the tube at 95°C for 5 minutes, and then chill on ice or store at -70°C.

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Table 5. Modified form of AMP-RT Assay. Table of reagent contained within the master mix for one reaction for reverse transcription. This master mix is for samples which do not have HIV-1 drugs.

REAGENT	AMOUNT (µL)
5X BUFFER (AMV-RT BUF)	4
0.1 M DTT	1
10 mM dNTP	1
EMCR2 Primer (4 uM0	1
RNAsin (10,000 U)	0.2
EMCV-RNA (20ng/ul)	0.5
DW (DEP C)	5.3
NP-40 (2%)	6
Template (RT enzyme)	1
TOTAL VOLUME	20

Table 6. Modified form of AMP-RT Assay . Table of reagent contacted within the master mix for one reaction for reverse transcription. This master mix is for samples containing HIV-1 drugs.

REAGENT	AMOUNT (UL)
5X BUFFER (AMV-RT BUF)	4
0.1 M DTT	1
10 mM dNTP	1
EMCR2 Primer (4 uM0	1
RNAsin (10,000 U)	0.2
EMCV-RNA (20ng/ul)	0.5
DW (DEP C)	4.5
NP-40 (2%)	6
HIV-1 Drug	0.8
Template (RT enzyme)	1
TOTAL VOLUME	20

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3.2 Conventional PCR

With the initial use of the AMP-RT protocol in reverse , a PCR master mix was made specifically for that protocol. The PCR master mix should contain 4 reagents: 10x PCR buffer, Sense EmcF1 primer (5'-CTCCCATCAGGTTGTGCAGCGACC-3') (200 ng/μL), *Taq* DNA polymerase (5000 U/ml), and water. However, from current observation in the new approach, there was modification done to the master mix in order to optimize the amplification. An additional 3 reagent was added to the original master mix in order to optimize the amplification: $MgCl_2$ (25 m*M*), 5X Q solution, and an antisense Emc R2 primer (5'-GTTCATGACAGGCCGATACAGAGG-3') (200 ng/μL). Total volume of the PCR master mix per sample is 50 μL. Added with the initial reverse transcription product, the total volume is 100 μL. The condition for the PCR cycle was set at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The expected sequence length of the PCR product should be 318 base pairs. This is verified on SDS-PAGE at 2%.

3.3 Real-time PCR

3.3.1 Roche Light Cycler Real Time PCR

Initially, QuantiTech SYBR Green PCR kit (by Qiagen) is used as the source of reagent for real time PCR. The 2x QuantiTect SYBER Green PCR Master Mix is provided with the kit containing HotStar Taq DNA polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix (including dUTP), SYBR Green I, ROX (Passive reference dye), and 5 mM $MgCl_2$. The reaction component of the 2x QuantiTect SYBER Green PCR kit suggests a total volume of 20 μL per reaction. Of the 20μl total volume, 10 ul is the 2x QuantiTect SYBR Green PCR Master Mix, which decreases the final concentration of the master mix to 1x. Two specific internal primers are used: Antisense Emc-NewR1 (5'-GCGTCGAATTAAGAGGAAAGGTAG-3')(0.5 μ*M*), and Sense Emc-NewF1 (5'-GATGTGAAGGTGTTGTCGTACGG-3')(0.5 μ*M*). These specific internal primers will select and amplify approximately 145 bp of PCR product. Chromosomal DNA ($\leq$ 1ug/reaction) will serve as the template for the real-time PCR reaction. RNAse-free water is used to adjust the volume to 20 μl. The master mix is first prepared at 20 μl per reaction, mixed throughly , and dispensed into a LightCycler PCR capillary.

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Negative control is included which contains no cDNA. DNA template is then added to the individual PCR capillaries, capped, and spun on a centrifuge. The LightCycler capillaries is then inserted into the well of the Roche LightCycler real-time PCR and programmed for 55 cycles. After the real-time PCR run, the PCR product is analyzed using both PCR curve and melting curve provided by the build in software. Table 7 lists the LightCycler real-time PCR condition. The sample was run at 55 PCR cycles.

Table 7. PCR Cycle for Roche Light Cycle Real Time PCR

Step	Time	Temperature	Ramp
PCR Initial activation step	15 min	95 °C	20 °C/s
PCR Cycle: Denaturation Step	15 sec	94 °C	20 °C/s
PCR Cycle: Annealing Step	20 sec	55 °C	20 °C/s
PCR Cycle: Extension Step	5-25 sec	72 °C	20 °C/s

3.3.2 Corbette Research Rotor Gene Real-Time PCR

The project then utilized a Corbette Research Rotor Gene Real-Time PCR. The master mix of this real-time PCR was adjusted because probe was used instead. The SYBR green had a tendency to have more background and caused deviations in quantitations. Therefore, a new master mix was developed which utilized a flourogenic probe. The master mix of the real time PCR reagent with probe contained the following substance: 10X buffer (Qiagen), 5 mM $MgCl_2$, 100 µM Probe (6-FAM-5'-TCCTgTCTTTgCgAggCTTGCTC-3'-BSQ), 10 mM dNTP, Antisense Emc-NewR1 (5'-GCGTCGAATTAAGAGGAAAGGTAG-3')(0.5 µ*M*), and Sense Emc-NewF1 (5'-GATGTGAAGGTGTTGTCGTACGG-3')(0.5 µ*M*), Dep C, and Hot Star Taq (Qiagen). Table 8A explains the content of the master mix containing a probe.

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Table 8A. Master mix with probe for Corbett Research Rotor Gene real time PCR. This is for total volume of 20 microliter per sample.

REAGENT	AMOUNT (UL)
10X BUFFER	2
MgCl2	1.2
Probe (100 uM)	0.25
dNTP (10 mM)	0.4
NF1 Primer	0.5
NR1 Primer	0.5
DW (DEP C)	14.05
Taq	0.1
Template	1
TOTAL VOLUME	20

Table 8B describes the PCR condition for the Corbett Research Rotor Gene real time PCR. The sample was run at 40 PCR cycles.

Table 8B. PCR condition for the Corbett Research Rotor Gene real time PCR.

Step	Time	Temperature
PCR Initial activation step	15 min	95 °C
PCR Cycle: Denaturation Step	20 sec	95 °C
PCR Cycle: Annealing Step	55 sec	55 °C
PCR Cycle: Extension Step	30 sec	72 °C

4. PROCESSING RESULTS

The Inhibitory concentration of 50% (IC50) was calculated for all samples which were exposed. This was done by plotting the percent inhibition of the drug on the

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sample versus the Log concentration of the drug. Linear regression was utilized to calculate the slope of the graph and the IC50 was obtained from the linear equation of the slope. The patient IC50 was obtained and calculated for fold resistance. Fold resistance is defined as the IC50 of the sample divided by the IC50 of the wild type. It is this fold resistance where the scale of resistance is categorized. In certain literature, the fold resistance of 10 indicates resistance to drugs. In other literature, fold resistance of 100 indicates resistance. In order to determine the scale of resistance for this project, one has to compare the results against the genotypic results to see where limitations occur and the region of fold resistance that may actually be labeled as drug resistance.

Fold Resistance = IC50 Patient / IC 50 Wild type (51)

Resistance is then categorized based a scale established by the laboratory.

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CHAPTER 4
RESULTS

1. ANALYSIS USING ROCHE LIGHT CYCLER REAL TIME PCR

1.1 AMV-RT AS A STANDARD CURVE.

AMV-RT has initially been used to simulate the new approach. Reasons for such usage have been described in the literature review. To compare results with conventional techniques, a traditional ultrasensitive method called AMP-RT protocol was used(10). Encephalomyocarditis Virus (EMCV) RNA was purified and confirmed on gel run. EMCV RNA is the choice of template for the experiment mainly due to two reasons: The EMCV rarely exists along with HIV infected patients; therefore, there is little chance for any form of interference between the two viral components. The EMCV genome is well known and sequenced; therefore, primers or probes may easily be developed to target a specific sequence in the viral genome. Initially, reverse transcriptase (AMV-RT), done in a ten fold serial dilution, was used for reverse transcriptase and PCR run (RT-PCR) on conventional PCR, utilizing external primers (FIG 7).

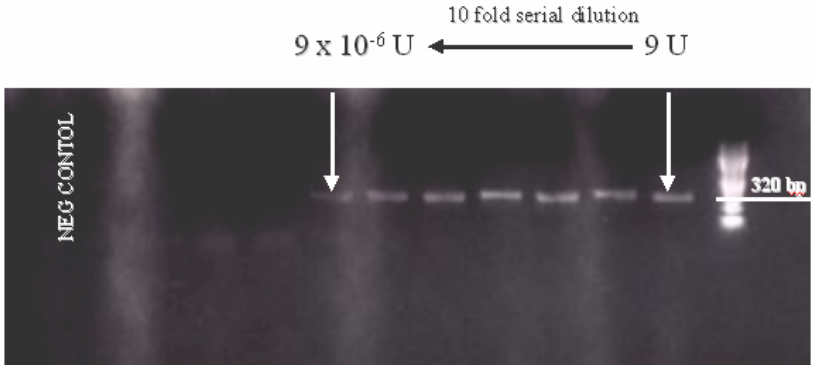

Figure 7. The gel illustrates that sensitivity of the AMV-RT on conventional PCR is detected down to a level of $9x10^{-6}$ U. The base pair length of the PCR product

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confirms the expected length of 320 base pair. In addition, the negative control confirms that the reagent is not contaminated and the EMCV RNA template does not contain any DNA residue.

To compare those results obtained from traditional techniques with the new approach, new primers (Emc-NewR1 and Emc-NewF1) were then designed and used for real-time PCR. Reverse transcriptase (AMV-RT), done in serial dilution, was used for RT-PCR on a Roche Light Cycler real-time PCR (FIG 8). Figure 8 illustrates that sensitivity of the AMV-RT on real-time PCR is detected down to a level of $9x10^{-9}$ U. The base pair length of the PCR product confirms the expected length of 145 base pair. In addition, the negative control confirms that the reagent is not contaminated and the EMCV RNA template does not contain any DNA residue. Compared to the RT-PCR done on a conventional PCR, the sensitivity of the real-time PCR has shown an increase of three $\log_{10}$. However, since the serial dilution done on the experiment was done up to $9x10^{-9}$ U, it is possible that detection levels on the real-time PCR can exceed those diluted concentrations. Figure 8, illustrates two types of curve seen on a Roche Light cycler real-time PCR utilizing SYBR Green chemistry: The PCR curve (FIG 9A) and the melting curve (FIG 9B). The fundamental idea of the real-time PCR is that amplification may be seen in progress. The intensity of the flourescence is proportional to the amount of cDNA amplified (FIG 9A). With serial dilution done on AMV-RT, there would be expectation for different degrees of intensities of flourescence due to different cDNA production. Figure 9A shows that as concentration of AMV-RT decreases, the amount of cycles necessary to amplify the sample will increase. More time is required to amplify products of more diluted AMV-RT. Figure 9 also shows that the sensitivity of the Roche Light Cycler PCR is at least three logarithm more than conventional PCR. Peaks that are found in the melting curve may suggest whether the PCR product is specific to the target cDNA of choice (FIG 9B). If multiple peaks are found in the melting curve, this might suggest that either contamination of primer-dimers exist. The specific melting temperature is specific to the type of cDNA. If two curves have completely two different melting temperatures, most likely there are two different types of cDNA. Figure 9B illustrates that there seems to be a consistency in terms of the melting curve peak (~80 degrees

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Celsius). That suggests that all the samples are likely to have a single type of cDNA, in this case the EMCV cDNA.

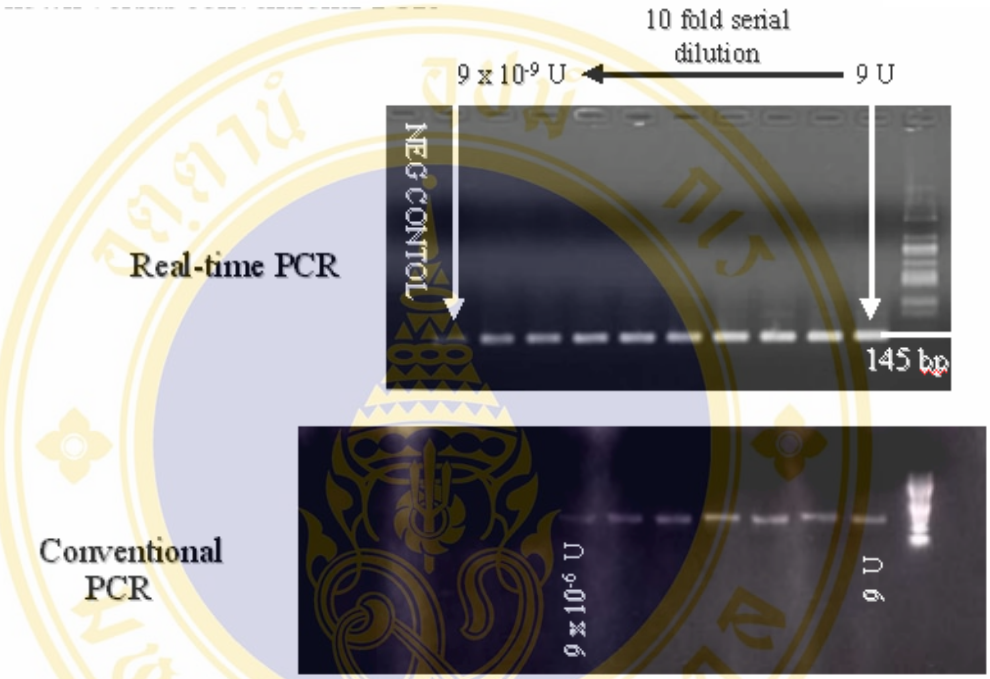

Figure 8. Comparing sensitivity of conventional PCR versus Roche Light Cycler PCR on gel electrophoresis.

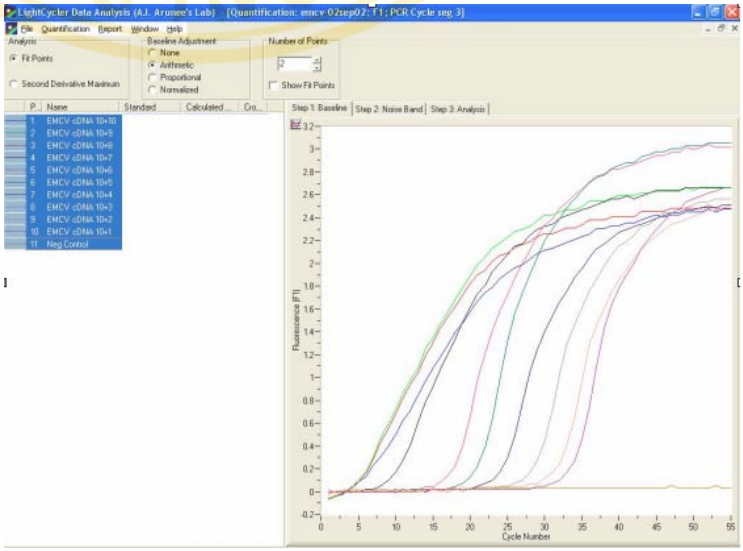

Figure 9A. PCR profile of Roche Light Cycler. The results show that as the amount of concentration of AMV-RT used was serially diluted, the RT activity

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signified by the curve was proportional to the decline in AMV-RT concentration. Farthest right indicate highest RT activity proportional to highestAMV-RT concentration. Farthest left indicate lowest RT activity proportional to lowest AMV-RT concentration.

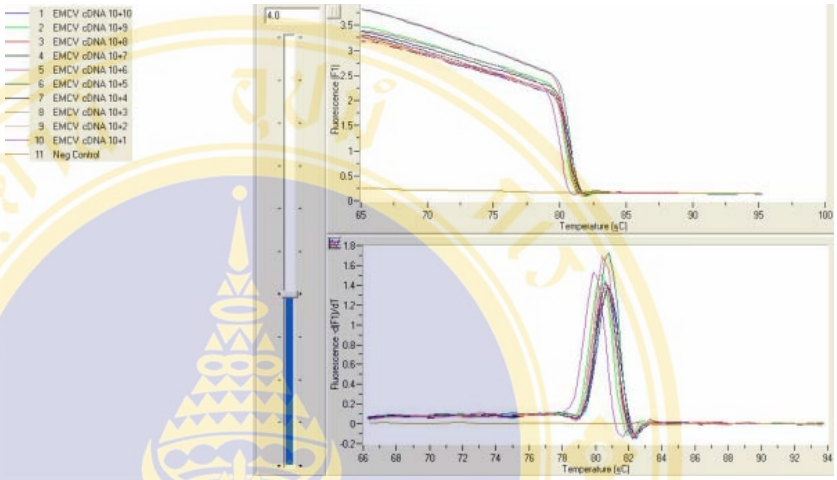

Figure 9B. Melting Curve profile of Roche Light Cycler. The results show that similar amplicon exhibited similar melting curve. In this situation the amplicon of the AMV-RT had a melting curve of approximately 80 degrees Celsius.

1.2 HXB2 ANALYSIS.

Although AMV-RT was successfully simulated in the new approach, the results alone do not justify clinical applications. In order to determine if the new approach is applicable in a clinical level, it must be used on viral particles that respond similarly as if in an in vivo situation. Wild type HIV-1 clone serves to simulate new approach by providing the necessary viral particles needed as source of RT Enzyme. Viral particle, HXB2 (CI/Wildtype), was serially diluted and observed for sensitivity under Roche Light Cycler real time PCR (FIG 10). The result shows that serial dilution has been observed from a diluted concentration of 63,286.40 International Standard Unit (IU) per milliliter (ml) to a concentration of 0.63286 IU/ml. What this indicates is that with serial dilution of viral copy numbers, the amount of RT activity response is relative to the viral concentration. Therefore, another standard curve may be developed using viral copy number of HXB2 as the concentration unit in which all other RT enzymes

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are compared to. Aside from developing a standard curve, the utilization of HXB2 do show that detection limits for the real time PCR exist within those concentrations.

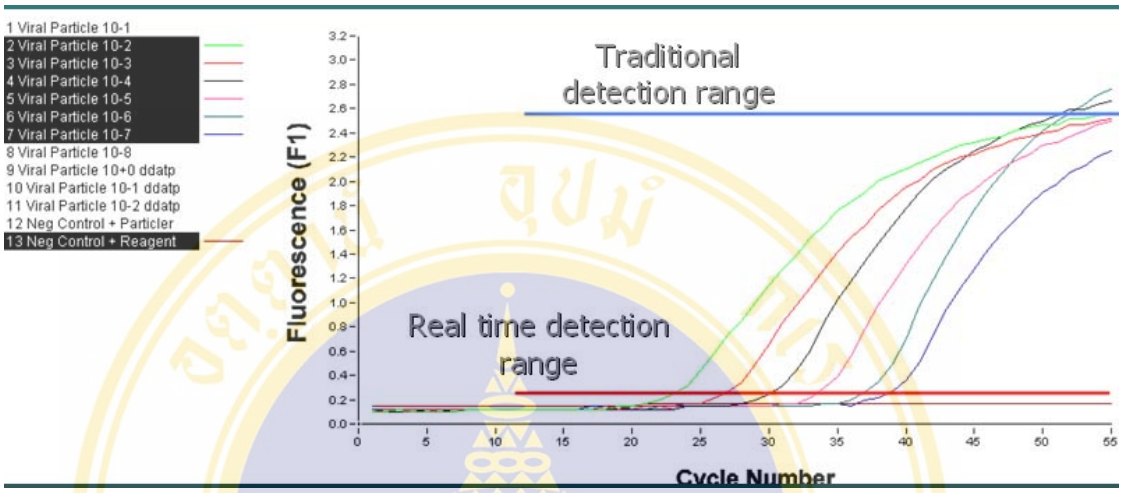

Figure 10. HXB2 serially diluted and observed under Roche Light Cycler. The farthest left is the lowest dilution of HXB2 used (63,286.40 IU/ml). The farthest right is the highest dilution used (0.63286 IU/ml). Each curve represented from left to right is in the same consecutive order as the dilution factor of HXB2.

Detection limits were obtained when analysis from the real time PCR showed that above and below the range of detection, a clustering of the curves occur (FIG 11). This indicates that beyond the limit of detection (63,286.40 IU/ml to a concentration of 0.63286 IU/ml), the results are unreliable and cluttered.

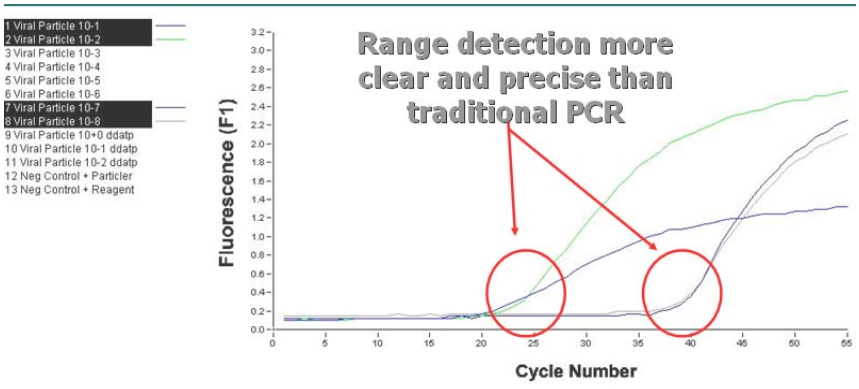

Figure 11. Clustering of results when samples are above and below the detection limit.

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As the melting curve (Tm) is specific to an amplified template cDNA, contamination by foreign strand would exhibit different melting curve if the contaminated DNA strand is of different fragment size (FIG 12). This is very beneficial if one suspects that a sample contains foreign DNA strands. The melting curve also shows that samples may have residues of background from such phenomenon as primer-dimers. In situations where background does occur, one is able to detect it and eliminate the background from other samples that might contain it. This is simply done by deducting the calculated concentration of the background from the calculated concentration of the sample. This is beneficial when doing quantitations, as background interference might impede the actual quantitative results.

1.3 HXB2 INITIAL DRUG SUSCEPTIBILITY TRIAL

In order to determine the effects of HIV-1 drugs on wild-type, ddATP at concentrations of 100 µM, suggested by the DAIDS virology manual, was used and viewed initially on the real-time PCR to observe if all reagents and devices were effective in viewing drug suppression(9). The results confirm that suppression of the wild type may be seen utilizing the new approach (FIG 13).

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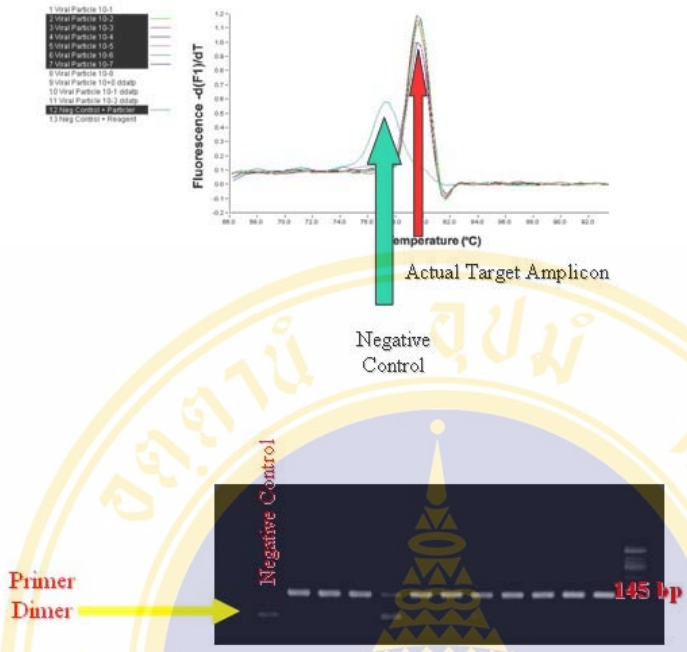

Figure 12. The melting curve analysis of Roche Light Cycler. The negative control should appear to have no melting curve. However, the appearance of a different melting curve from the actual sample is an indication of contamination or background such as primer-dimers. Gel stains confirm the existence of a primer dimer.

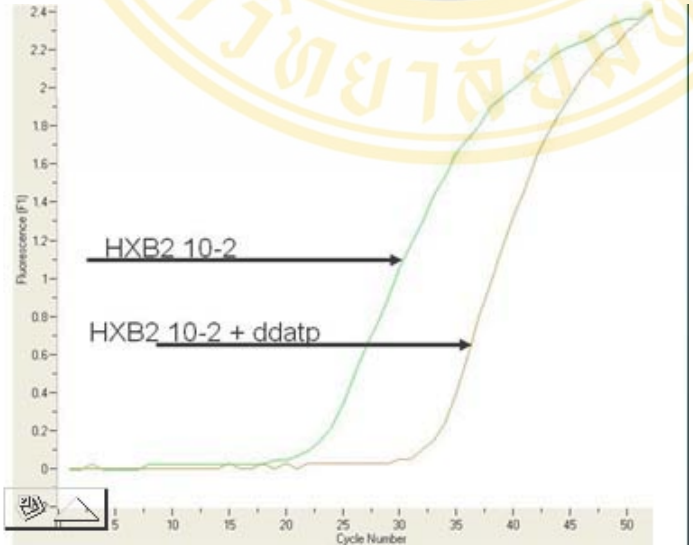

Figure 13. Observing initial effects of the suppression of ddATP on wild type HIV-1. This is a preliminary observation to observe drug suppression in the new approach.

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1.4 PATIENT INITIAL DRUG SUSCEPTIBILITY TRIAL

Preliminary observation of drug susceptibility assay using the new approach on a HIV positive patient serum initially showed that suppression of RT activity may be seen in the patient serum when using a high concentration of antiretroviral drug, in this case ddATP. In order to determine the effects of HIV-1 drugs on patients, ddATP at concentrations of 100 µM, a suggested concentration in DAIDS virology manual, was used and viewed initially on the real-time PCR to observe if all reagents and devices were effective in viewing drug suppression(9). The results confirm that suppression of the patient serum may be seen utilizing the new approach (FIG 14).

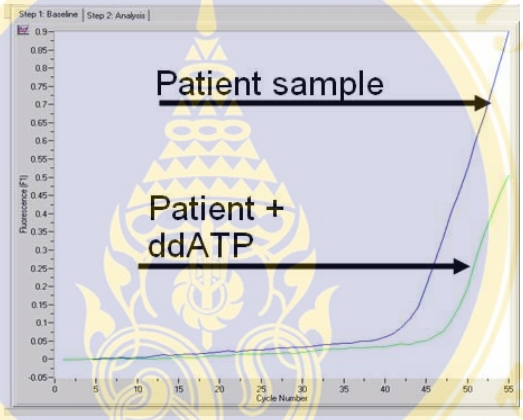

Figure 14. Observing initial effects of the suppression of ddATP on patient serum.

2. ANALYSIS USING CORBETT RESEARCH ROTOR GENE REAL TIME PCR

Midway through the research, the laboratory obtained a Corbett Research Rotor Gene real time PCR. This type of real time PCR is similar to the Taq man PCR. The chemistry was modified using a modified form of the ultrasensitive assay by Heineine. A probe was used instead of SYBR to increase specificity of the sample.

2.1 AMV-RT AS A STANDARD CURVE

A comparison was made with Roche Light cycler real time PCR by utilizing serial dilutions of AMV-RT (FIG 15). The results were similar to those analyzed on a Roche real time PCR. A standard curve was obtain using the AMV-RT. The concentration of AMV-RT was expressed in units per microliter. This standard curve will be used on future samples so that all RT activity would be expressed in concentrations of AMV-RT.

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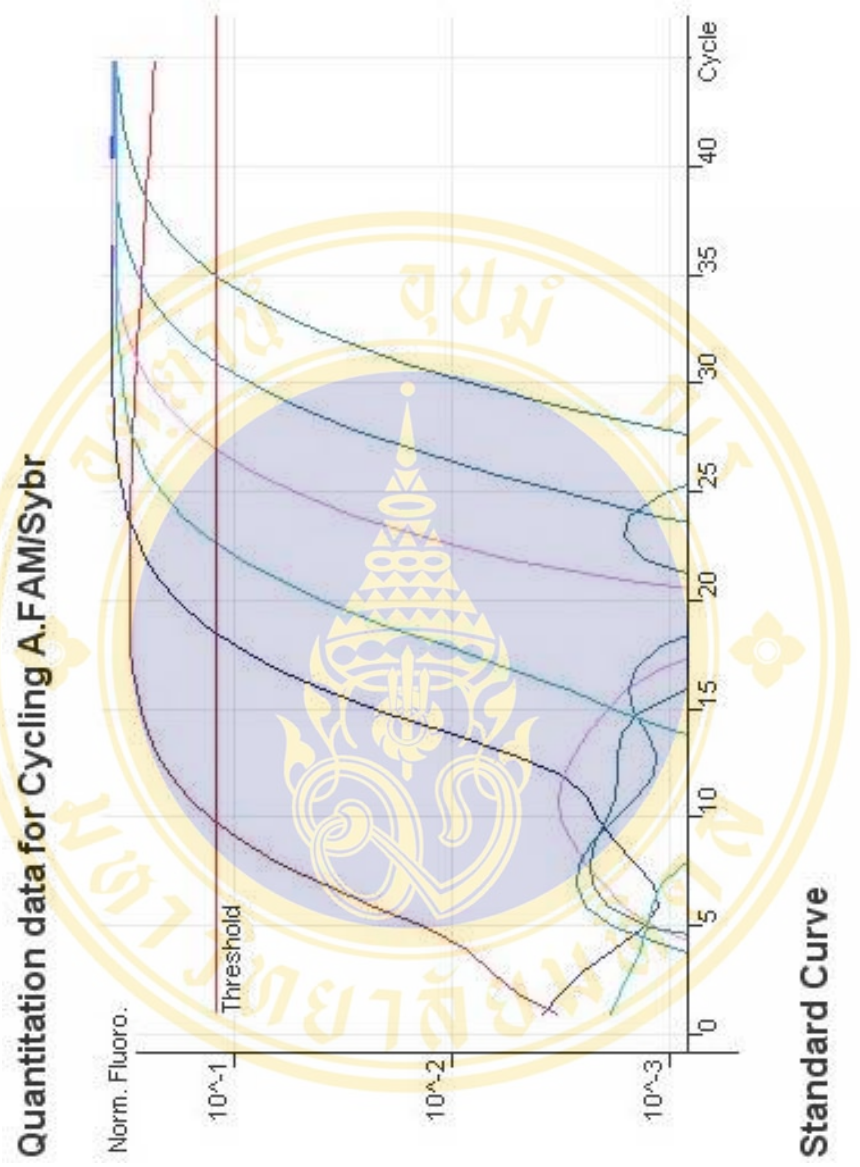

Figure 15. The real time PCR curve of serial dilution of AMV-RT (Concentration of 10 U/microliter) from ten fold serial dilution of 10^{-2} to 10^{-8} using the Rotor Gene. The order of the curve is proportional to the concentration of AMV-RT.

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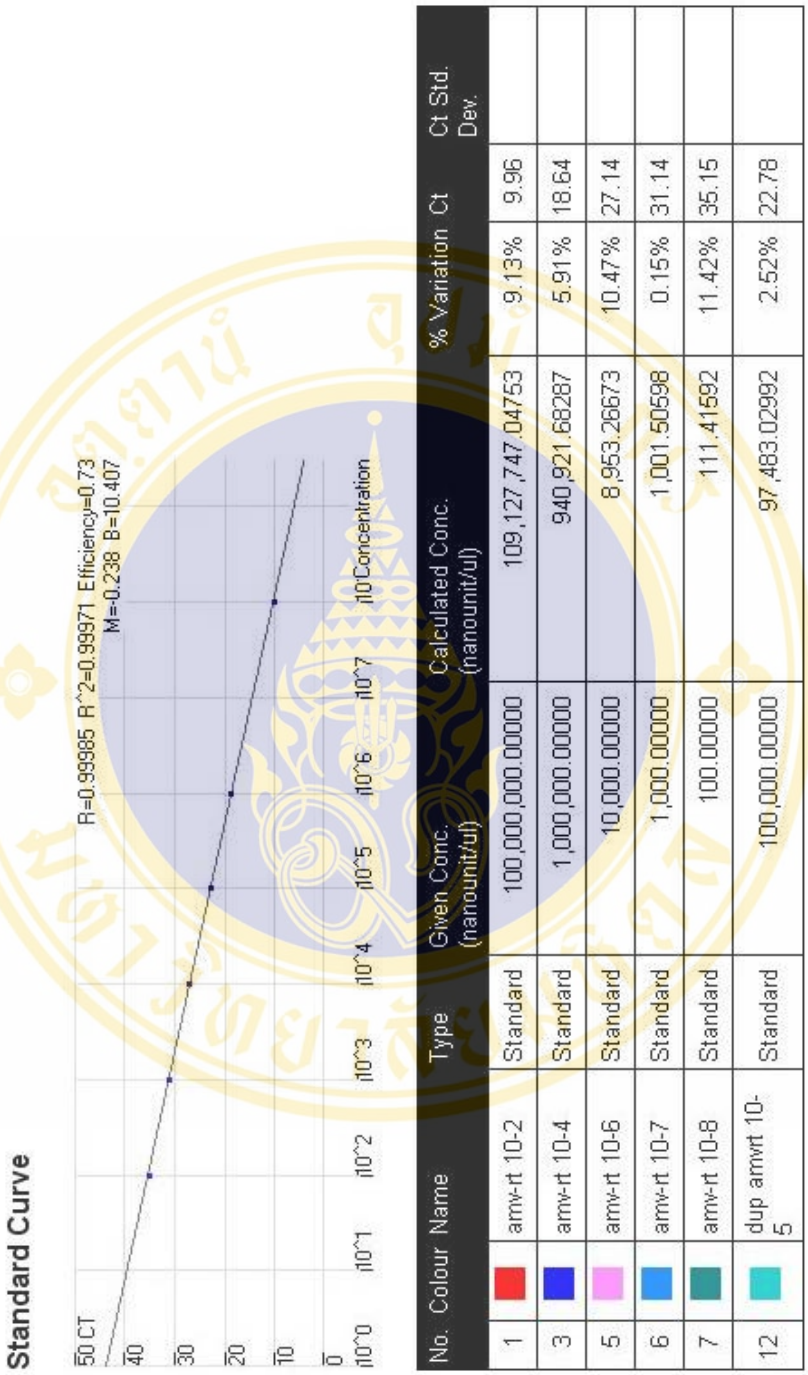

No.	Colour	Name	Type	Given Conc. (nanounit/ul)	Calculated Conc. (nanounit/ul)	% Variation	Ct	Ct Std. Dev.
1		amv-rt 10-2	Standard	100,000,000.00000	109,127,747.04753	9.13%	9.96	
3		amv-rt 10-4	Standard	1,000,000.00000	940,921.68287	5.91%	18.64	
5		amv-rt 10-6	Standard	10,000.00000	8,953.26673	10.47%	27.14	
6		amv-rt 10-7	Standard	1,000.00000	1,001.50598	0.15%	31.14	
7		amv-rt 10-8	Standard	100.00000	111.41592	11.42%	35.15	
12		dup amvrt 10-5	Standard	100,000.00000	97,483.02992	2.52%	22.78	

Figure 16. Calculated standard curve and correlation values for AMV-RT amplified on a Corbette Research Rotor Gene real time PCR. The standard was created and confirmed for correlations.

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Similar to the results of HXB2 seen in the Roche Light Cycler PCR (FIG 10), detection limit was observed. Anything above 100,000,000 nanounits/µl and below 100 nanounits/µl of AMV-RT exhibited unreliable quantitations. Clustering of the PCR curve did occur below and above the detection limit. Therefore, all future samples may only be quantitated when the curve of those samples lie within the detection limit. Any quantitation made beyond the detection limit were noted as unreliable and inconsistent.

2.2 HXB2 ANALYSIS

Similar to the results of HXB2 seen in the Roche Light cycler PCR (FIG 10), detectin limit was also observed for HXB2 on the Rotor Gene real time PCR (FIG 18). The detection limit ranges from a dilution factor of 10 to 10^7. This confirms two observation: First, the sensitivity of the detection range of HXB2 on the Rotor Gene real time PCR was similar to the Light Cycler real time PCR. Second, the new chemistry, which utilized probes instead of SYBR green, exhibited the same detection range as those of the Light Cycler real time PCR.

A standard curve was developed using HXB2 CI units. The full concentration of the HXB2 CI unit was 632,864 International units per milliliter (IU/ml) (FIG 17). The development of the standard curve utilizing HXB2 CI confirms the ability of the new approach to be able to detect and create standard curves utilizing viral particles. Therefore, comparisons of other RT enzymes, especially in a clinical sense, may be compared to the concentration units of HXB2 CI, as opposed to just pure enzyme concentration units of AMV-RT.

2.3 SUM 9 AND SUM 13 ANALYSIS

SUM 9 and SUM 13 were used as HIV -1 molecular clones that served as a control. SUM 9 is susceptible to both ddATP and Nevaripine. SUM 13 is resistant is only resistant to ddATP. Upon calculation of the IC_{50} of both SUM 9 and SUM 13, the results do show correlations between resistanct and non resistant strains. The IC50 for ddATP and Nevaripine are listed on Table 9. A fold resistance of 5.95 for the SUM 13 resistant strain confirms that resistance to ddATP is a characteristic of SUM 13 molecular clone. However, SUM 13 is not resistant to Nevaripine, and this has been observed also. This indicates that the modified ultrasensitive RT assay and real time PCR are sensitive enough to detect drug susceptibility and drug resistance.

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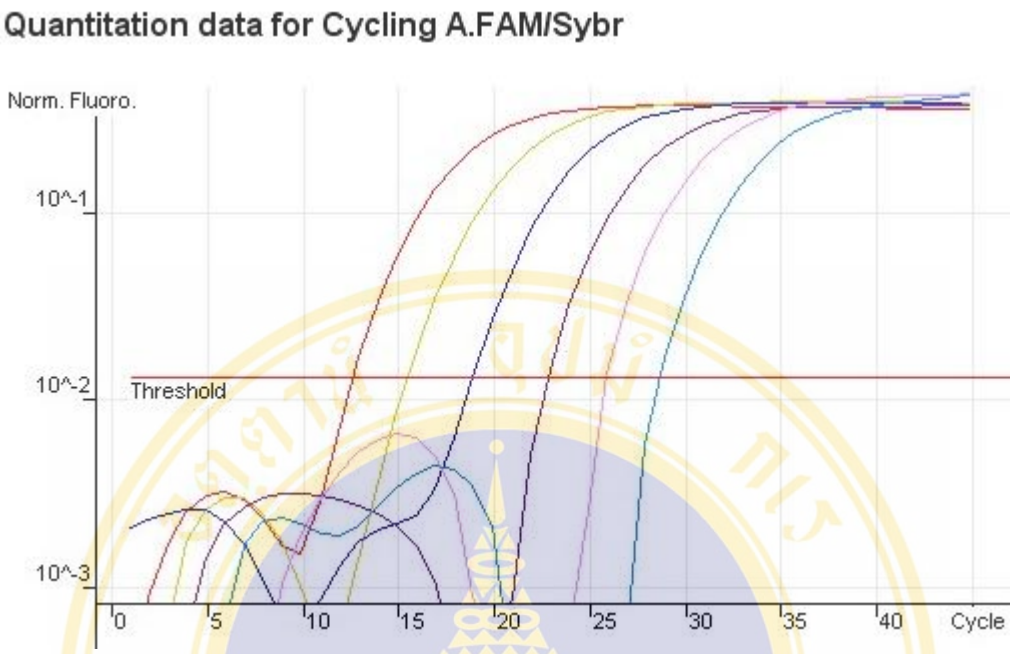

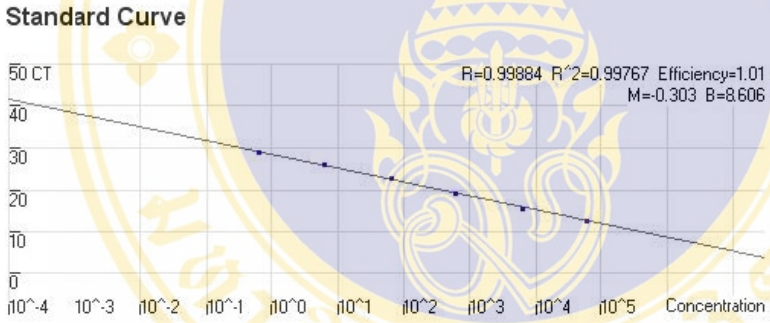

No.	Colour	Name	Type	Given Conc. (nanounit/ul)	Calculated Conc. (nanounit/ul)	% Variation	Ct	C
1		CI 10-1	Standard	63,286.40000	57,354.45304	9.37%	12.7	
2		CI 10-2	Standard	6,328.64000	7,747.50265	22.42%	15.57	
3		CI 10-3	Standard	632.86400	703.21311	11.12%	19.01	
4		CI 10-4	Standard	63.28640	46.55951	25.80%	22.89	
5		CI 10-5	Standard	6.32864	5.55599	12.21%	25.95	
6		CI 10-6	Standard	.63286	.78806	24.52%	28.75	

Figure 17. Developing a standard curve utilizing HXB2 CI. The farthest left is the lowest dilution of HXB2 used in the standard curve. The farthest right is the highest dilution used in the standard curve. Each curve represented from left to right is in the same consecutive order as the dilution factor of HXB2. Since the full concentration of the HXB2 is known (632,864 International Units per milliliter), a standard curve may be developed.

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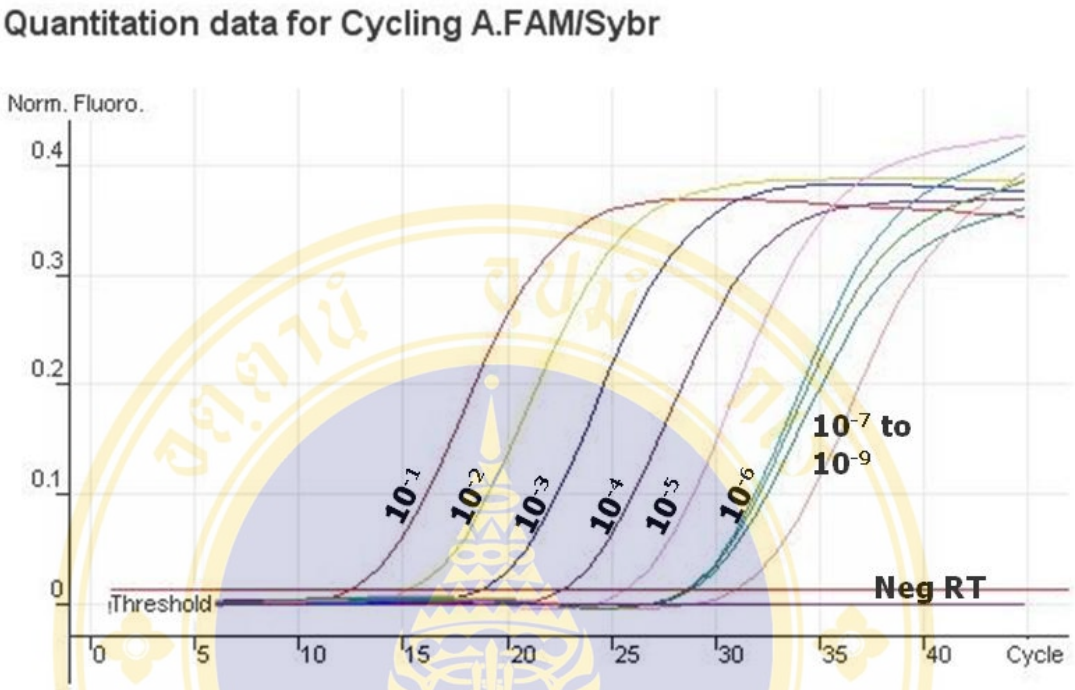

Figure 18. The detection range of HXB2 (CI/Wildtype) for the Corbett Research Rotor Gene real time PCR. Detection limit ranges from a dilution factor of 10 to 10^7. Clustering occurs from the dilution factor of 10^7 onward. The detection range, although limited in high dilutions, are more sensitive to lower dilution factor. Therefore, the range of detection is comparable to Light Cycler Real-time PCR.

Table 9. IC50 of ddATP and Nevaripine on SUM 9 and SUM 13.

Molecular Clone	IC50 of ddATP (µl)	IC 50 of Nevaripine (µl)
SUM 9	1.92	1.18
SUM 13	11.43	Similar to SUM 13

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2.2 PATIENT SERUM

HIV-1 patients were collected daily from Ramathibodi hospital and immediately undergone first RT-PCR to determine the RT activity of the patient sample. Initially, the samples undergone the first RT-PCR and then frozen. The next day the sample that had high RT activity within the detection limit was subjected to drug susceptibility. However, this new approach shows that freeze and thaw method does significantly decrease RT activity. In one patient (#2413), freeze and thaw cause a drop of approximately 88% of RT activity. Therefore, samples were retrieved and subjected to drug susceptibility without the freeze and thaw method. Table 10 summarizes current up to date results of drug susceptibility of patients collected up to date. Figure 18 and 19 summarizes the results of Table 10 in graph format to observe the relative drug resistance compared to the control. Results were compared with genotypic results to observe if correlations do exist. Samples that were non-amplifiable were due to uncertain reasons such as technical problems. Figure 19 illustrates the graph which determines the IC_{50} of the drug for each patient. It is possible that discrepancies in determination of the IC_{50} is partly due to the curve, the amount of points in the graph, and the correlations of the concentration points involved in the graph. The results of the patients do show that although some correlations do not exist between patient IC50 obtain in the new approach versus those obtained in the genotypic analysis, there are not enough sample size to justify a scale or limit of where resistance begins.

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Table 10. Summary of current drug susceptibility results for patients and molecular clone. Genotypic analysis was analyzed at Chulalongkorn Hospital and compared to the IC50 results obtained in the new approach.

Sample Description	ddATP IC50 (uM)	Nevaripine IC50 (uM)	Fold Resistance	Fold Resistance
			ddATP	Nevaripine
SUM 9	1.92	1.18	1	1
SUM 13	11.4	1.18	5.95	1
Patient 2552	21.6	1.46	11.2	1.24
Patient 2441	7.23	4.77	3.76	4.04
Patient 2448	8.81	23.8	4.58	20.2
Patient 2513	17.1	2.61	8.90	2.21
Patient 2413	6.62	6.92	3.45	5.86
Patient 2574	Not Done	3.53	Not Done	2.99
Patient 2593	0.172	0.598	0.0895	0.507
Patient 2713	1.04	1.1	0.541	0.932
Patient 2484	9.38	1.53e312	4.88	1.30e312
Patient 2502	6.83	0.83	3.55	0.703
Sample Description	**Genotypic Analysis**		**Genotypic Analysis**	
	ddATP		Nevaripine	
SUM 9	Sensitive		Sensitive	
SUM 13	Resistant		Sensitive	
Patient 2552	Sensitive		Sensitive	
Patient 2441	Sensitive		Sensitive	
Patient 2448	Sensitive		Sensitive	
Patient 2513	Sensitive		Sensitive	
Patient 2413	Sensitive		Sensitive	
Patient 2574	Sensitive		Sensitive	
Patient 2593	Sensitive		Sensitive	
Patient 2713	Sensitive		Sensitive	
Patient 2484	Possible Resist		Possible Resist	
Patient 2502	Not Done		Not Done	

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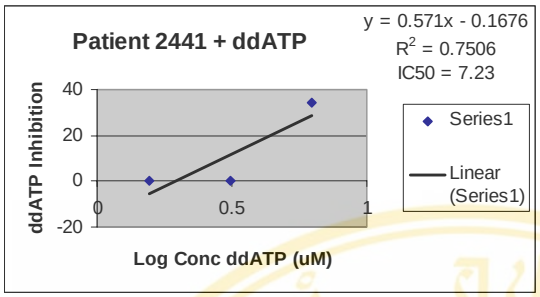
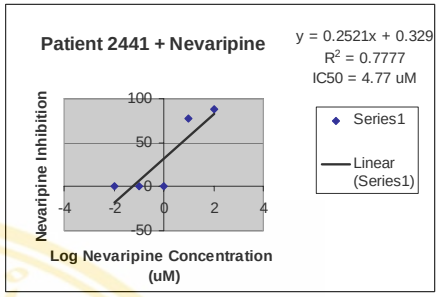
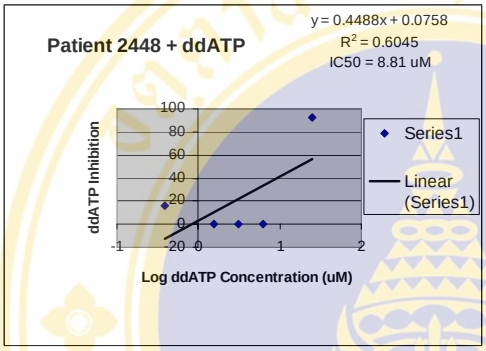
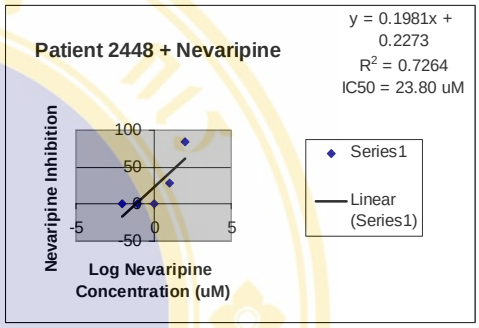
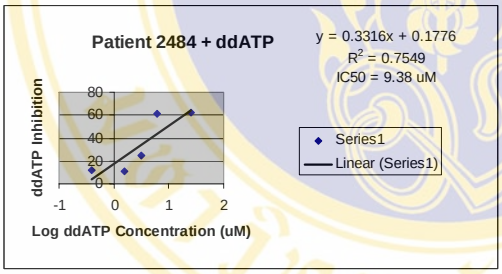
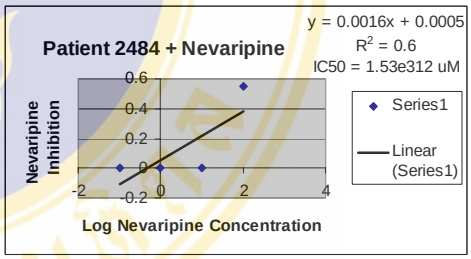
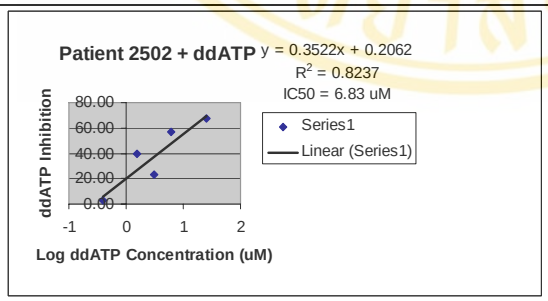
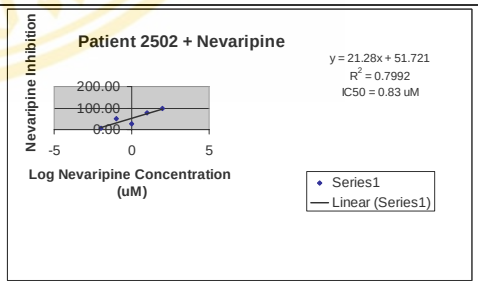
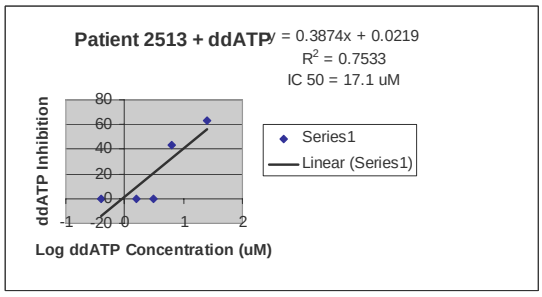
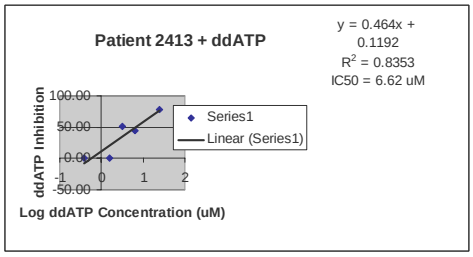
IC_{50} of ddATP	IC_{50} of ddATP
	
	
	
	
	

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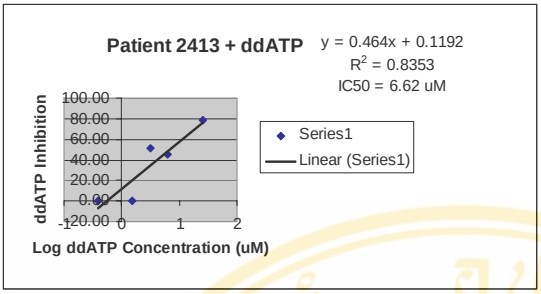
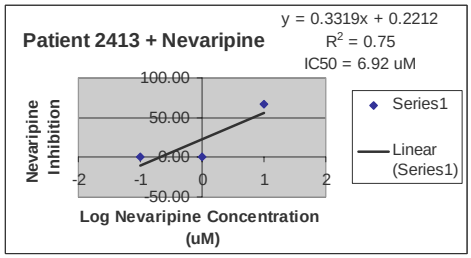
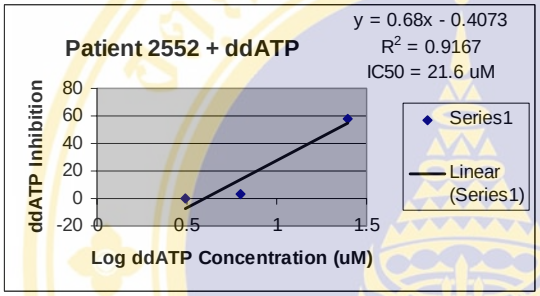
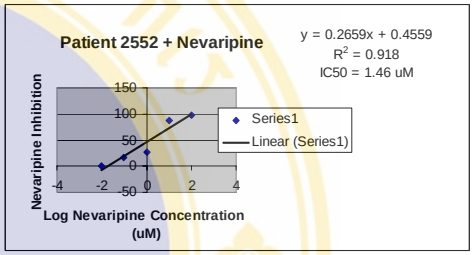
Continue Figure 19

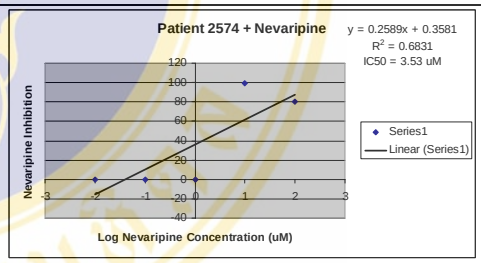


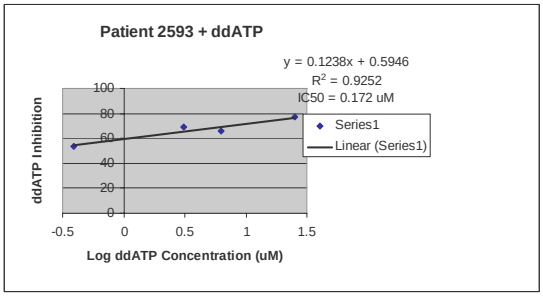


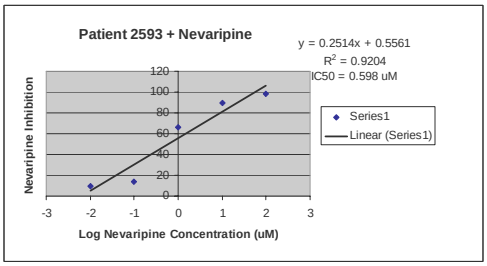




2574 FOR DDATP NOT DONE







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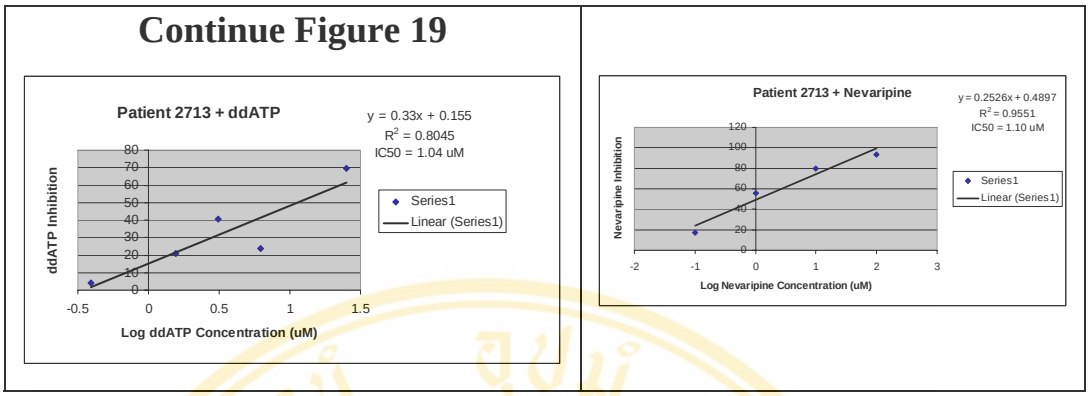

Figure 19. Graph which shows the process of determining the IC_{50} of the drug. Patient ddATP log Concentration is plotted against the percent inhibition of the drug. A slope is drawn between the plots and the IC_{50} of the drug is determined from the slope.

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CHAPTER 5
DISCUSSION

1. CHARACTERIZATION OF THE NEW APPROACH

Criterias for comparison of the new approach to drug susceptibility assay using an ultrasensitive assay and real time PCR will be based on speed, sensitivity, specificity, cost value, and safety. AMV-RT and HXB2 has been used in both types of real-time PCR to simulate RT-PCR utilizing the new approach. Avian myeloblastosis virus reverse transcriptase (AMV-RT) is initially used as a source of reverse transcriptase to simulate drug susceptibility assay using the new approach. AMV-RT is a well documented, active, and safe enzyme used as a preliminary method in observing the effectiveness of the new approach in detecting RT activity. Once AMV-RT has been successfully observed in the new approach, the next step is to utilize viral particles that exhibit similar clinical activities found HIV patients. HXB2, which a clone of the wild type HIV-1 virus, is a useful source to simulate actual clinical conditions. The reason for this is because HXB2 are viral particles that are clones of the wild-type HIV-1 virus and may have similar RT activity that is comparable to those of the wild type. In this sense, HXB2 is not resistant and be used as a positive control for drug susceptibility testing. Also, since the viral load of HXB2 is known, which is 632,864 IU/ml, a standard curve may also be developed in which all RT activity of other enzymes are expressed in viral concentration comparable to the HXB2 virus. Once results from AMV-RT and HXB2 has been achieved and has shown consistency and benefits, one is able to move on to clinical trails where actual patient serum is used to test for drug susceptibility.

1.1 Roche Light Cycler real time PCR

Real-time PCR has become more widely accepted as a new approach to the conventional PCR due to increase in speed, sensitivity, reproduction, reduction for possibility of contamination, and reduction in contamination. Therefore, the main objective of the project is to provide an insight as to the alternatives and benefits of

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utilizing combined ultrasensitive reverse transcriptase assay and real-time PCR over conventional means.

In terms of speed, using Roche's LightCycler PCR has proven very effective in amplifying DNA quickly, at about 20°C per second ramp rate (Table 7). Preliminary results have shown that the LightCycler PCR can run 55 cycles in approximately one hour and 27 minutes, which is substantially faster than a conventional PCR. . In addition, time is not consumed on post PCR manipulation, such as ELISA (FIG 3). Approximately one to two days are consumed when conducting ELISA assays. This is eliminated in the new approach, where time consumption is approximately five to six. When avoiding post PCR manipulation, the chances for contamination decline. There will be no need for a nested PCR or post PCR assays that will heighten the chance for contamination. Reduction for possibility of contamination in turn reduces the wasted time spent on rerunning the assay.

Results have shown that sensitivity of the real-time PCR is significantly greater, by at least three $\log_{10}$, than a conventional PCR (FIG 8). The sensitivity is so great that other form of contaminants may be observed in real-time PCR that would not be detected by conventional means.

In terms of specificity, as describe in Figure 6, since real time PCR utilizes the threshold cycle to quantitate the amplicon, values are more consistent and specific than those calculated from the plateau phase of the conventional PCR (FIG 4). In situations where other contaminants are found, analysis of the melting curve may discriminate the difference between target products and contaminants (FIG 9B)

In terms of value, one sample costs approximately 1,000 baht per run for approximately duplicate runs of two drugs susceptibility testing utilizing five drug concentration points. This is significantly cheaper than the replicative phenotypic assay, which cost approximately 40,000 baht. However, in comparison to genotypic assay, this new approach might not be as cost effective. Genotypic analysis can analyze a multiple of drugs by observing changes in the genetic content of the RT gene. Therefore, genotypic assay prices are usually fixed and not dependent on the amount of drugs used for analysis. In terms of the new approach, the cost of the drug susceptibility assay is proportional to the amount of drugs tested. Therefore, the more drugs tested the more expensive the new approach assay becomes. However,

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genotypic assay, as described later on, is limited in its capability for detecting synergistic activities among drugs. Therefore, the new appraoch may be important in complementing analysis with those of genotypic assays.

1.2 Corbett Research Rotor Gene real time PCR

The chemistry of the assay used in the Corbette Research real time PCR has been modified. The reason why the chemistry was modified was because the original chemistry seems to have variations that prevent one from having consistent reproducible results. It is possible that NP-40 concentration might have been too high from the original chemistry since lowering the concentration in the modified chemistry did not impede the effects of the RT activity (Table 5, Table 8A). Also, it is possible that the original chemistry has a tendency to degrade faster than the modified version. Difference in the chemistry used in Rotor Gene provided an additional benefit. SYBR green has a tendency to bind to any double stranded DNA, regardless of whether the strand is a target strand or a contaminant. If the real time PCR has not been optimized primer-dimer may occur and SYBR green may nonspecifically bind to those dimers and interfere with the results. If there are other contaminants, SYBR green will bind to the contaminants also. Utilizing a probe is more specific. Since the probe is specifically designed to bind to a specific region of the target DNA, the chance of nonspecific binding is unlikely. However, there are possibilities that sensitivity of the chemistry might decline. In Figure 17, a probe was used to determine the serial dilution of HXB2. This shows that the new approach does have the capability to detect RT activities and discriminate serial dilutions. The diluted concentration of 10^{-7} and 10^{-8} seems to cluster slightly earlier than those seen in SYBR green chemistry (FIG 18). It is possible that either usage of the probe is less sensitive than with SYBR green or the overall modified chemistry is less effective than the original unmodified chemistry. The detection range is similar, however, the rotor gene detects at high concentration than the Light Cycler, while the Light cycler detects at lower concentration than the Rotor Gene (FIG 10, FIG 17). Similar to the Light Cycler, the sensitivity of Rotor Gene is higher than those of conventional means, by at least one log (FIG 17).

This device is slower than Light Cycler real time PCR but still faster in speed to conventional PCR (Table 8B). The PCR cycle used in the Rotor gene transitions

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faster than those used in conventional PCR (Table 8B). Therefore, a 45 PCR cycle run in a Rotor Gene would take 2 ½ hours which is faster than conventional PCR, which consumes about 3 ½ hours. Also, because this device eliminates the work of having to undergo post PCR manipulation, time consumption of the overall assay was still significantly less than those used in conventional methods. Approximately 4 hours were consumed from beginning of the susceptibility testing to the end of the real time PCR amplification. This would take approximately one to two days if done by conventional means.

Similar to all advantages of a real time PCR in the new approach, the Light Cycler decrease in the possibility for contamination since post PCR manipulation has been eliminated from the assay.

Cost value of drug susceptibility testing using the Rotor Gene real time PCR was comparable to that of the Light Cycler. This is because AMV-RT buffer was used, which had to be purchased at a more costly rate than if a developed by a home made buffer.. The advantage of using the Rotor gene real time PCR is that the tubes used to hold the samples as the same as conventional PCR and relatively cheap (20 baht a tube). Light cycler PCR, on the other hand, has a glass capillary tube which is quite expensive (100 baht per tube) and easy to break. Similarly to the Light Cycler, one sample costs approximately 1,000 baht per run for approximately duplicate runs of two drugs susceptibility testing utilizing five drug concentration points. The cost of the new assay is relative to the amount of drugs used in the drug susceptibility assay. If many drugs were used in the new assay, cost will be higher than the genotypic assay, in which results are not dependent on the amount of drugs analyzed in the drug susceptibility assay. However, as described previously, genotypic assay is limited in its capability for detecting synergistic activities among drugs. Therefore, the new appraoch may be important in complementing analysis with those of genotypic assays.

2. DRUG SUSCEPTIBILITY TESTING.

Samples that were collected from the hospital were initially tested to determine the RT activity of the patient sample. Viral load and RT activity should be proportional to each other (52). Therefore, one would expect that if RT activity is high, viral load is also proportionally high. However, when compared to viral load

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results obtained from Ramathibodi hospital, viral load and RT activity did not seem to correlate unless the viral load value is extremely high (>100,000 copies/microliter). There are several reason why this might occur. First, this may be explained in the method of determining viral load. Viral load is determined by assessing the quantities of viral particles within the human serum. RT activity is a reflection of the activity of functional viral particles(52). Therefore, it is possible that although samples obtain from Ramathibodi hospital contains a relatively moderate amount of viral load, a substantial amount of the viral particles within those samples might be nonfunctional(45). Therefore, this new approach does offer a more realistic idea of the true RT activity of the sample. Second, there is a detection range in which the real time PCR is capable of analyzing reliable results. This is seen in both Light Cycler and Rotor Gene real time PCR (FIG 11, FIG 18). This might be one of the reasons for the inconsistencies between patient viral load and RT activity.

From Table 10, there seems to be slight deviations in the correlation between genotypic analysis and those obtained from the new approach. For example, Patient 2552, 2448 and 2513 had relatively higher fold resistance when compared to those of SUM 13, which is the resistant molecular clone for ddATP. Also genotypic assay concludes that those patients had sensitivity to ddATP. This may suggest certain possibilities. First, in each individual, there are inhibitory enzymes and proteins that vary(19). This may interfere with the assay, especially when the total volume of the assay is 20 microliter, which might be proned to assay variabilities.. In each reaction during the reverse transcription assay, the volume of drugs used per total volume is 0.8 µl per 20 µl total volume (Table 8A). At such a low level, deviations may occur which overall may effect the outcome of the RT activity. Second, the concentrations used for plotting the IC_{50} may be insufficient or need to be adjusted. The assay uses five concentrations of ddATP and Nevaripine to determine the IC_{50} (FIG 19). The range of those concentrations may be either too wide or too narrow. Also with such variations in viral load as compared to those of the molecular clones, more concentration points might be required to prevent shifting of the IC_{50}(9). With more points, the possibility of deviation of the IC_{50} is buffered(9). Shifting of the IC_{50} will occur more likely when concentration points are limited. Third, the patient sample size is too small to scale any detection limits for which one can claim resistance. Because the overall

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research was to introduce the new approach assay, applications of drug susceptibility testing on patients is still in its infancy. The problem is that unless the RT activity of the patient is within the detection limit of the real time PCR, the patient sample cannot undergo drug susceptibility testing since further suppression of the RT activity would only push the curve beyond the detection point. Therefore, not every patient sample is capable of undergoing drug susceptibility testing. This limits the sample size. With only eight to ten patients undergoing drug susceptibility testing, it is not possible to find a point where one can label samples as resistant. Therefore, this is left to future projects to gather up enough patient samples capable of undergoing drug susceptibility testing. When enough sample size is obtained, a correlation may be observed in which one can find a scale where samples maybe be reliably labeled as resistant. It also provides a challenge to develop further RT assay that is more optimal with the real time PCR. In this respect, the sensitivity of the new approach would detect sample size with even more lower functional viral load.

2.1 Overall comparison with genotypic analysis.

Currently, some hospitals prefer the usage of genotypic analysis for drug susceptibility. The advantage is that it is not expensive when comparing to replication based phenotypic assays. It is also not as technically demanding in interpretation, such as mutations, since results are easily obtainable from web based sites. The basic method for genotypic analysis is amplification and sequencing of the reverse transcriptase or protease genes. When compared to nonresistant strain, deviations or variability in DNA fragment lengths could be a sign of possible mutation. The results are obtainable within a week and is extremely consistent. However, the disadvantage lies in the assays incapability to detect synergistic or antagonistic results. For example, a mutation in the RT gene may cause a domino effect in which multiple drugs are resistant. In terms of utilizing genotypic analysis, the interpretation of synergistic resistance may be difficult. Genotypic analysis does not provide collective analysis.

In terms of the modified new approach done in this project, results are faster and complementary to the results of the genotypic assay. However, the difficulty seen in the clinical application for drug susceptibility assay poses some disadvantages that make the genotypic analysis a necessary tool. Cost and consistency are current

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obstacles that is seen in this project for the new approach. The new approach is only cost effective if the amount of drug used is small. If more drugs are used in the assay, the cost rises and may exceed those of the genotypic assay. As seen in the results of Table 10, there are inconsistencies in the new approach, reasons which have been explained previously. Given that that there are limitations in both analysis, it is beneficial to parallel both genotypic and phenotypic analysis to guarantee that results are consistent and that phenotypic synergism of resistance will be observed. Basically, the new approach offers an alternative method which complements certain deficits in which genotypic analysis does not provide. In third world countries, the new approach may be a useful and cost effective way to provide preliminary results for HIV therapy.

Although there are many advantages for using real-time PCR, there are some obstacles that should be taken into account when assessing susceptibility of reverse transcriptase inhibitor using ultrasensitive assay and real-time PCR:

1. Since flourescence is the key mechanism for detection in real-time PCR, there could be problems when dealing with multiplex systems. If there were multiple sets of primers used to detect multiple region of a genome, or multiple genes from different genome, in the same system, there could possible interference between difference flourescence (40).
2. There is a controversy as to the interference of endogenous retrovirus found in human cells(53). This could manipulate the results of the experiment in such a way that a false positive may occur. Possible ways to alleviate such a problem involves appropriate centrifuging to remove as much cellular component as possible, which is where endogenous retrovirus are thought to linger. It is still a controversy as to the possible interference of endogenous retrovirus on HIV assays. Some research has claimed that the amount of interference is so low that results are not significant enough to make a difference in the overall results. Either way, this will be an issue that must be noted when used in clinical trials.
3. Interpretation of the results may be a problem in terms of labeling degrees of drug resistance. This has been a problem for most currently. (Korn, 2002) Every experiment seems to have their own criteria's and categories. In analysis of worldwide variation in HIV-1phenotypic susceptibility testing, researchers have found that there has been no correlation in terms of resistance baseline where one

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can claim a point of resistance. It is important that comparisons be made with a standard curve (ex. Titrations done on AMV-RT) so that an appropriate interpretation is made. Also sample size must be big enough to conclude correlations for drug resistance. This project has gathered no more than 100 samples of which only 20 were available for resistance testing. Even with other prior research, over a 1000 patients did not provide enough data to conclude a criterion or limit necessary to draw a baseline for resistance (24)

4. The volume of drug in the master mix reagent must be relatively high so that variations will not occur. In this project, using 0.8 µl of drug per 20 µl total volume was relatively too small. Volume should be adjusted to a level where variations are less like to occur.
5. Since hospital viral load results do not seem to correlate well with results obtained from the new approach, another assay, such as a replication based assay, would be beneficial in confirming that functional viral particles are necessary for RT activity.

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CHAPTER 6
CONCLUSION

This project has introduced a new approach to assessing the reverse transcriptase inhibitor susceptibility of human immunodeficiency virus type 1 using ultrasensitive reverse transcriptase assay and real time PCR. Observation of this new alternative assay showed increased sensitivity by at least three log fold as compared to the conventional PCR. The alternative approach consumed at least half the amount of time compared to the conventional method. Quantification of the samples was more precise when sample runs were repeated. Expenditures were less costly in the new approach. The new approach offered a safer procedure with less toxic chemicals used. When samples were eventually tested for drug susceptibility, results of each sample seems to correlate with the actual high viral load and other genotypic assays. Preliminary results do indicate some correlation in drug susceptibility testing. Overall, the new approach will offer an alternative choice for drug susceptibility testing. It will also benefit future projects in the analysis of functional and nonfunctional viral particle, and the effects of freeze and thaw on HIV-1 RT activity.

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REFERENCE

1. Lerma JG, Heneine W. Resistance of human immunodeficiency virus type 1 to reverse transcriptase and protease inhibitors: genotypic and phenotypic testing. J Clin Virol 2001;21(3):197-212.
2. Sebastian J, Faruki H. Update on HIV resistance and resistance testing. Med Res Rev 2004;24(1):115-25.
3. (WHO) JUNPoHAUaWHO. AIDS epidemic update 2003. In; 2003.
4. Cann AJ, Karn J. Molecular biology of HIV: new insights into the virus life-cycle. Aids 1989;3 Suppl 1:S19-34.
5. Dar L, Vajpayee, M. Antiretroviral Drugs in HIV Infection. Indian Journal of Pharmacology 1999;31:96-103.
6. Cimarelli A, Darlix JL. Assembling the human immunodeficiency virus type 1. Cell Mol Life Sci 2002;59(7):1166-84.
7. Aboulker JP, Babiker A, Chaix ML, Compagnucci A, Darbyshire J, Debre M, et al. Highly active antiretroviral therapy started in infants under 3 months of age: 72-week follow-up for CD4 cell count, viral load and drug resistance outcome. Aids 2004;18(2):237-45.
8. Paolucci S, Baldanti F, Zavattoni M, Comolli G, Labo N, Menzo S, et al. Comparison of levels of HIV-1 resistance to protease inhibitors by recombinant versus conventional virus phenotypic assay and two genotypic interpretation procedures in treatment-naive and HAART-experienced HIV-infected patients. J Antimicrob Chemother 2003;51(1):135-9.
9. Beatty C, Bradley, M., Brambilla, D., Breaken, F., Bremer, J., Dragavon, J., Ladd, B., Livnat, D., Michels, C., Mundy, C., Price, V., Ramacciotti, T., Reichelderfer, P., Staes, B., Starkey, C., Winter, M. Drug Susceptibility Assay. In: DAIDS Virology Manual for HIV Laboratories: NIH Publishers; 1997. p. 78-92.

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10. Heneine W, Lerma, G.J. Ultrasensitive Detection of Reverse Transcriptase Activity by the Amp-RT assay. Methods in Molecular Medicine 1995:291-299.
11. Jonson T, Mahlamaki EH, Karhu R, Gorunova L, Johansson B, Hoglund M. Characterization of genomically amplified segments using PCR: optimizing relative-PCR for reliable and simple gene expression and gene copy analyses. Genes Chromosomes Cancer 2000;29(2):192-9.
12. Goldschmidt PL, Devillechabrolle A, Ait-Arkoub Z, Aubin JT. Comparison of an amplified enzyme-linked immunosorbent assay with procedures based on molecular biology for assessing human immunodeficiency virus type 1 viral load. Clin Diagn Lab Immunol 1998;5(4):513-8.
13. Sarin PS, Goldstein AL. Treatment of AIDS with drugs targeted to inhibit different stages of the HIV life cycle. Immunopharmacol Immunotoxicol 1995;17(2):217-45.
14. Arts EJ, Wainberg MA. Mechanisms of nucleoside analog antiviral activity and resistance during human immunodeficiency virus reverse transcription. Antimicrob Agents Chemother 1996;40(3):527-40.
15. Basavapathruni A, Bailey CM, Anderson KS. Defining a molecular mechanism of synergy between nucleoside and nonnucleoside AIDS drugs. J Biol Chem 2004;279(8):6221-4.
16. Qari SH, Winters M, Vandamme AM, Merigan T, Heneine W. A rapid phenotypic assay for detecting multiple nucleoside analogue reverse transcriptase inhibitor-resistant HIV-1 in plasma. Antivir Ther 2002;7(2):131-9.
17. Quan Y, Brenner BG, Marlink RG, Essex M, Kurimura T, Wainberg MA. Drug resistance profiles of recombinant reverse transcriptases from human immunodeficiency virus type 1 subtypes A/E, B, and C. AIDS Res Hum Retroviruses 2003;19(9):743-53.
18. Kinchington D, Raymond, Schinazi. Antiviral Methods and Protocols. London: Humana Press; 2000.
19. Gehringer H, Von der Helm K, Seelmeir S, Weissbrich B, Eberle J, Nitschko H. Development and evaluation of a phenotypic assay monitoring resistance

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formation to protease inhibitors in HIV-1-infected patients. J Virol Methods 2003;109(2):143-52.

20. Gulick RM, Ribaudo HJ, Shikuma CM, Lustgarten S, Squires KE, Meyer WA, 3rd, et al. Triple-nucleoside regimens versus efavirenz-containing regimens for the initial treatment of HIV-1 infection. N Engl J Med 2004;350(18):1850-61.
21. Harper DR. Molecular Virology; 1998.
22. Schmidt B, Korn K, Walter H. Technologies for measuring HIV-1 drug resistance. HIV Clin Trials 2002;3(3):227-36.
23. Kagan R, Winters M, Merigan T, Heseltine P. HIV type 1 genotypic resistance in a clinical database correlates with antiretroviral utilization. AIDS Res Hum Retroviruses 2004;20(1):1-9.
24. Clavel F, Hance AJ. HIV drug resistance. N Engl J Med 2004;350(10):1023-35.
25. Saengwonloey O, Jiraphongsa C, Foy H. Thailand report: HIV/AIDS surveillance 1998. J Acquir Immune Defic Syndr 2003;32 Suppl 1:S63-7.
26. Winslow DL, Anton ED, Horlick RA, Zagursky RJ, Tritch RJ, Scarnati H, et al. Construction of infectious molecular clones of HIV-1 containing defined mutations in the protease gene. Biochem Biophys Res Commun 1994;205(3):1651-7.
27. DAIDS Virology Manual for HIV Laboratories. In: National Institutes of Health and Collaborative Investigators; 1997.
28. Sonnerborg A, Abens J, Johansson B, Strannegard O. Detection of human immunodeficiency virus-1 by polymerase chain reaction and virus cultivation. J Med Virol 1990;31(3):234-40.
29. Montes B, Vergne L, Peeters M, Reynes J, Delaporte E, Segondy M. Comparison of Drug Resistance Mutations and Their Interpretation in Patients Infected With Non-B HIV-1 Variants and Matched Patients Infected With HIV-1 Subtype B. J Acquir Immune Defic Syndr 2004;35(4):329-336.
30. Vazquez-Rosales G, Garcia Lerma JG, Yamamoto S, Switzer WM, Havlir D, Folks TM, et al. Rapid screening of phenotypic resistance to nevirapine by

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direct analysis of HIV type 1 reverse transcriptase activity in plasma. AIDS Res Hum Retroviruses 1999;15(13):1191-200.

31. Ueno T, Mitsuya H. Comparative enzymatic study of HIV-1 reverse transcriptase resistant to 2',3'-dideoxynucleotide analogs using the single-nucleotide incorporation assay. Biochemistry 1997;36(5):1092-9.
32. Heneine W, Yamamoto S, Switzer WM, Spira TJ, Folks TM. Detection of reverse transcriptase by a highly sensitive assay in sera from persons infected with human immunodeficiency virus type 1. J Infect Dis 1995;171(5):1210-6.
33. Makuwa M, Souquiere S, Niangui MT, Rouquet P, Apetrei C, Roques P, et al. Reliability of rapid diagnostic tests for HIV variant infection. J Virol Methods 2002;103(2):183-90.
34. Boni J, Opravil M, Tomasik Z, Rothen M, Bisset L, Grob PJ, et al. Simple monitoring of antiretroviral therapy with a signal-amplification-boosted HIV-1 p24 antigen assay with heat-denatured plasma. Aids 1997;11(6):F47-52.
35. Puppe W, Weigl JA, Aron G, Grondahl B, Schmitt HJ, Niesters HG, et al. Evaluation of a multiplex reverse transcriptase PCR ELISA for the detection of nine respiratory tract pathogens. J Clin Virol 2004;30(2):165-74.
36. Pyra H, Boni J, Schupbach J. Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proc Natl Acad Sci U S A 1994;91(4):1544-8.
37. Sninsky JJ. The polymerase chain reaction (PCR): a valuable method for retroviral detection. Lymphology 1990;23(2):92-7.
38. Kainz P. The PCR plateau phase - towards an understanding of its limitations. Biochim Biophys Acta 2000;1494(1-2):23-7.
39. Morata P, Queipo-Ortuno MI, de Dios Colmenero J. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. J Clin Microbiol 1998;36(9):2443-6.
40. Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. Nucleic Acids Res 2002;30(6):1292-305.

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41. Ito Y, Grivel JC, Margolis L. Real-time PCR assay of individual human immunodeficiency virus type 1 variants in coinfected human lymphoid tissues. J Clin Microbiol 2003;41(5):2126-31.

42. Ameziane N, Lamotte M, Lamoril J, Lebret D, Deybach JC, Kaiser T, et al. Combined factor V leiden (G1691A) and prothrombin (G20210A) genotyping by multiplex real-time polymerase chain reaction using fluorescent resonance energy transfer hybridization probes on the Rotor-Gene 2000. Blood Coagul Fibrinolysis 2003;14(4):421-4.

43. Karsai A, Muller S, Platz S, Hauser MT. Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification of gene expression. Biotechniques 2002;32(4):790-2, 794-6.

44. Proudnikov D, Yuferov V, Zhou Y, LaForge KS, Ho A, Kreek MJ. Optimizing primer--probe design for fluorescent PCR. J Neurosci Methods 2003;123(1):31-45.

45. Ariyoshi K, Jaffar S, Alabi AS, Berry N, van der Loeff MS, Sabally S, et al. Plasma RNA viral load predicts the rate of CD4 T cell decline and death in HIV-2-infected patients in West Africa. Aids 2000;14(4):339-44.

46. Peters IR, Helps CR, Hall EJ, Day MJ. Real-time RT-PCR: considerations for efficient and sensitive assay design. J Immunol Methods 2004;286(1-2):203-17.

47. Gibbs JS, Regier DA, Desrosiers RC. Construction and in vitro properties of HIV-1 mutants with deletions in "nonessential" genes. AIDS Res Hum Retroviruses 1994;10(4):343-50.

48. Salminen MO, Ehrenberg PK, Mascola JR, Dayhoff DE, Merling R, Blake B, et al. Construction and biological characterization of infectious molecular clones of HIV-1 subtypes B and E (CRF01_AE) generated by the polymerase chain reaction. Virology 2000;278(1):103-10.

49. Tebit DM, Zekeng L, Kaptue L, Krausslich HG, Herchenroder O. Construction and characterisation of a full-length infectious molecular clone from a fast replicating, X4-tropic HIV-1 CRF02.AG primary isolate. Virology 2003;313(2):645-52.

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50. Saag MS. Use of virologic markers in clinical practice. J Acquir Immune Defic Syndr Hum Retrovirol 1997;16 Suppl 1:S3-13.

51. Narciso P, Lazzarin A. Genotypic resistance tests for the clinical management of patients with primary HIV infection. Scand J Infect Dis Suppl 2003;35 Suppl 106:66-70.

52. Abbott MA, Poiesz BJ, Byrne BC, Kwok S, Sninsky JJ, Ehrlich GD. Enzymatic gene amplification: qualitative and quantitative methods for detecting proviral DNA amplified in vitro. J Infect Dis 1988;158(6):1158-69.

53. Qari SH, Magre S, Garcia-Lerma JG, Hussain AI, Takeuchi Y, Patience C, et al. Susceptibility of the porcine endogenous retrovirus to reverse transcriptase and protease inhibitors. J Virol 2001;75(2):1048-53.

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APPENDIX

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APPENDIX

1. Reverse Transcriptase Master Mix (Modified) – No Drug

5x Buffer (AMV-RT Buffer) (Qiagen)	4 µl
0.1 M DTT (Sigma)	1 µl
10 mM dNTP (Qiagen)	1 µl
EMCR2 Primer (4 µM) (Proligo)	1 µl
RNAsin (10,000 Units) (Promega)	0.2 µl
EMCV-RNA (20ng/µl) (In house)	0.5 µl
DW (DEP C)	5.3 µl
NP-40 (2%) (Sigma)	6.0 µl
Template	1 µl
HIV-1 Drug	0 µl

2. Reverse Transcriptase Master Mix (Modified) – With Drug

5x Buffer (AMV-RT Buffer) (Qiagen)	4 µl
0.1 M DTT (Sigma)	1 µl
10 mM dNTP (Qiagen)	1 µl
EMCR2 Primer (4 µM) (Proligo)	1 µl
RNAsin (10,000 Units) (Promega)	0.2 µl
EMCV-RNA (20ng/µl) (In house)	0.5 µl
DW (DEP C)	4.5 µl
NP-40 (2%) (Sigma)	6.0 µl
HIV-1 Drug (ddATP, Nevaripine)	0.8 µl
Template	1 µl

3. Real-time PCR Master Mix for Rotor Gene

10X Buffer (Qiagen)	2 µl
$MgCl_2$ (Qiagen)	1.2 µl

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Probe (100 uM) (Proligo)	0.25µl
dNTP (10 mM) (Qiagen)	0.4 µl
NF1 Primer (Proligo)	0.5 µl
NR1 Primer (Proligo)	0.5 µl
DW (DEP C)	14.05µl
Hot Star Taq (Qiagen)	0.1 µl
Template	1 µl

4. Reverse Transcriptase Master Mix (AMP-RT Assay – No Drug)

5X RT Buffer C (250 mM Tris-HCl, 250 mM KCl, 50 mM $MgCl_2$	10 µl
dNTP (10 mM) (Qiagen)	2 µl
EGTA (10 mM) (Sigma)	5 µl
NP-40 (2%) (Sigma)	1.5 µl
DTT (100 mM) (Sigma)	1 µl
RNAsin (40,000 U/ml) (Qiagen)	0.25 µl
EMCV RNA (20 ng/µl) (Proligo)	0.5 µl
Antisense Emc R2 Primer (200 ng/µl) (Proligo)	0.5 µl
DEP-C	13.25 µl
Template	9 µl
Drug	0 µl

5. Reverse Transcriptase Master Mix (AMP-RT Assay – No Drug)

5X RT Buffer C (250 mM Tris-HCl, 250 mM KCl, 50 mM $MgCl_2$	10 µl
dNTP (10 mM) (Qiagen)	2 µl
EGTA (10 mM) (Sigma)	5 µl
NP-40 (2%) (Sigma)	1.5 µl
DTT (100 mM) (Sigma)	1 µl
RNAsin (40,000 U/ml) (Qiagen)	0.25 µl
EMCV RNA (20 ng/µl) (Proligo)	0.5 µl
Antisense Emc R2 Primer (200 ng/µl) (Proligo)	0.5 µl

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DEP-C	11.25 µl
HIV-1 Drug	2 µl
Template	9 µl

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