การประเมินประสิทธิภาพของกระดาษกรองซับเลือดสำหรับการตรวจหาปริมาณเอชไอวี1 และการตรวจหาเชื้อเอชไอวีดื้อต่อยาต้านไวรัส

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Evaluation efficacy of dried blood spot filter paper for HIV-
1 viral load and Drug resistance genotyping detection

Miss Boontida Sermsakskul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Molecular Science of Medical
Microbiology and Immunology
Department of Transfusion Medicine and Clincal Microbiology
Faculty of Allied Health Sciences
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Thesis Title	Evaluation efficacy of dried blood spot filter paper for HIV-1 viral load and Drug resistance genotyping detection
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for HIV-1 viral load and Drug resistance genotyping detection) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร.
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ปัจจุบันจำนวนผู้ติดเชื้อไวรัสเอชไอวีมีจำนวนเพิ่มขึ้นอย่างต่อเนื่อง ในขณะที่จำนวนของผู้เสียชีวิตจากโรค
ดังกล่าวมีจำนวนลดลงเมื่อเทียบจากจำนวนผู้เสียชีวิตในอดีต เนื่องมาจากมีการตรวจติดตามปริมาณไวรัสในกระแสเลือด
และการตรวจหาเชื้อดื้อต่อยาต้านไวรัส ซึ่งเป็นสิ่งสำคัญในการตรวจติดตามเพื่อประเมินประสิทธิภาพของการใช้ยาต้าน
ไวรัส ปัจจุบันมีการใช้กระดาษกรองเป็นตัวเก็บสิ่งตรวจ เนื่องจากกระดาษกรองสามารถจัดเก็บได้ง่ายและสะดวกต่อการ
ขนส่งในระยะทางไกล

กระดาษกรอง Whatman 903 เป็นกระดาษกรองที่ยอมรับในการตรวจปริมาณไวรัสในกระแสเลือดและการ
ตรวจหาเชื้อดื้อต่อยาต้านไวรัส แต่อย่างไรก็ตามราคาของกระดาษกรองชนิดนี้มีราคาสูงและการใช้ยังไม่เป็นที่แพร่หลาย
วัตถุประสงค์ของวิทยานิพนธ์ฉบับนี้เพื่อเปรียบเทียบประสิทธิภาพของกระดาษกรองสองชนิด ได้แก่ nitrocellulose
membrane และ cellulose fiber sample pad ซึ่งมีราคาถูกกว่า Whatman 903 ในการเก็บสิ่งตรวจเพื่อทำการตรวจปริมาณ
ไวรัสในกระแสเลือดและการตรวจหาเชื้อดื้อต่อยาต้านไวรัส สิ่งส่งตรวจสำหรับการวิจัยครั้งนี้มาจากเลือดครบส่วนจำนวน
70 รายโดยทำการตรวจปริมาณไวรัสในกระแสเลือดด้วย NucliSENS EasyQ HIV-1 v2.0 และตรวจหาเชื้อดื้อต่อยาต้าน
ไวรัสด้วย ViroSeq HIV-1 Genotyping system นอกจากนี้ในวิทยานิพนธ์ฉบับนี้ได้มีการตรวจสอบประสิทธิภาพของ
gold nanoparticles และ anion polymer-coated magnetic beads ในการเพิ่มความไวในการตรวจปริมาณไวรัสในกระแส
เลือดด้วยกระดาษกรอง

จากผลการทดลองพบว่า nitrocellulose membance ไม่มีความสามารถในการดูดซับเลือดจึงทำการ
เปรียบเทียบกระดาษ Whatman 903 และ cellulose fiber sample pad เท่านั้น ในส่วนของ anion polymer-coated
magnetic beads ไม่สามารถแปลผลการตรวจปริมาณไวรัสในกระแสเลือดได้เนื่องจากมีการรบกวนการเกิดปฏิกิริยา ใน
ส่วนของ gold nanoparticle พบว่าเมื่อตรวจปริมาณไวรัสในกระแสเลือดด้วยกระดาษ Whatman 903 และ cellulose fiber
sample pad ด้วยการใช้และไม่ใช้ gold nanoparticle พบว่ามีค่าเฉลี่ยไม่แตกต่างกันซึ่งหมายความว่า gold nanoparticle ไม่
สามารถเพิ่มความไวในการตรวจวัด แต่จากผลนี้แสดงว่ากระดาษ cellulose fiber sample pad มีประสิทธิภาพใกล้เคียงกับ
กระดาษ Whatman 903 จึงนำกระดาษสองชนิดนี้มาตรวจหาเชื้อดื้อต่อยาต้านไวรัสพบว่าสามารถตรวจพบลำดับกรดอะมิ
โนที่มีการเปลี่ยนแปลงได้เช่นเดียวกัน ดังนั้นกระดาษ cellulose fiber sample pad นี้มีประสิทธิภาพเช่นเดียวกับ Whatman
903 ควรทำการศึกษาเพิ่มเติมต่อไป

ภาควิชา	เวชศาสตร์การธนาคารเลือดและจุลชีววิทยาคลินิก	ลายมือชื่อนิสิต ....
สาขาวิชา	วิทยาศาสตร์ระดับโมเลกุลทางจุลชีววิทยาทางการแพทย์และวิทยาภูมิคุ้มกัน	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
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5 6 7 6 6 5 4 0 3 7 : MAJOR MOLECULAR SCIENCE OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

KEYWORDS: DRIED BLOOD SPOT / WHATMAN 9 0 3 / CELLULOSE FIBER SAMPLE PAD / HIV-1 VIRAL LOAD / HIV-1 DRUG RESISTANCE

BOONTIDA SERMSAKSKUL: Evaluation efficacy of dried blood spot filter paper for HIV-1 viral load and Drug resistance genotyping detection. ADVISOR: ASST. PROF.PALANEE AMMARANOND, Ph.D., CO-ADVISOR: ASST. PROF.NAVIN HORTHONGKHAM, Ph.D., 81 pp.

Currently, although a number of HIV-1 infected people increases continuously, the number of death people caused by HIV-1 disease reduces as compared to the death people in the past. Because patient is monitored continually by detecting the HIV-1 viral load and HIV-1 drug resistance, which is used to guide a HIV-1 therapy. In addition, a dried blood spot (DBS), which is used for collecting sample, is used for detecting the viral load and drug resistance genotyping because it is convenience to be collected and transported to both urban and rural area.

Whatman 903 (W-903) filter paper has been used to detect HIV-1 viral load and HIV-1 drug resistance. However, the cost of Whatman 903 filter paper is still expensive. The objective of this research was to evaluate the performance of two new dried blood spot filter papers, nitrocellulose membrane and cellulose fiber sample pad, which cost is cheaper than Whatman 903 filter for collecting the dried blood spot in order to be used to detect HIV-1 load and HIV-1 drug resistance. The dried blood spot specimens were collected from 70 blood samples which HIV-1 viral load and HIV-1 drug resistance were measured by NucliSENS EasyQ HIV-1 v2.0 and ViroSeq HIV-1 Genotyping system, respectively. Moreover, we also evaluated an ability of gold nanoparticles and anion polymer-coated magnetic beads in order to increase the sensitivity of HIV-1 viral load detection of filter papers.

The experimental results showed that nitrocellulose membrane was not capable of absorbing the dried blood spot therefore only Whatman 903 and cellulose fiber sample pad performance were evaluated. On the other hand, when used anion polymer-coated magnetic beads with filter papers, the HIV-1 viral load could not be interpreted. This is because the anion polymer-coated magnetic beads interfere the reaction in HIV-1 viral load detection. Although gold nanoparticles did not interfere the HIV-1 viral load detection reaction, the detected HIV-1 viral load from Whatman 903 with/without gold nanoparticles and cellulose fiber sample pad with/without gold nanoparticles were related. This means that gold nanoparticles did not affect an increasing in an ability of HIV-1 viral load detection. Since cellulose fiber sample pad could be used to detect the HIV-1 viral load similarly to Whatman 903, we therefore evaluated the performance of Whatman 903 and cellulose fiber sample pad to detect HIV-1 drug resistance from dried blood spot. The experimental results showed that these filters detected the amino sequencing which was the cause of virus mutation.

Therefore, the performance of cellulose fiber sample pad should be investigated comprehensively in order to be used in practical.

Department:	Transfusion Medicine and Clincal	Student's Signature
	Microbiology	Advisor's Signature
Field of Study:	Molecular Science of Medical	Co-Advisor's Signature
	Microbiology and Immunology	
Academic Year:	2015	

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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LIST OF ABBREVIATIONS

HIV	Human immunodeficiency virus
SIV	Simian Immunodeficiency Virus
UNAIDS	The joint United Nations programme on HIV and AIDS
BoE	The Bureau of Epidemiology, Department of Disease Control Ministry of Public Health
NHSO	The National Health Security Office
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
FDA	Food and Drug Administration
DBS	Dried blood spot
DPS	Dried plasma spot
W-903	Whatman 903
CFSP	Cellulose fiber sample pad
AuNPs	Gold nanoparticles
RT	Reverse transcriptase enzyme
PR	Protease enzyme
IN	Integrase enzyme
NRTI	Nucleotide reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitors
PI	Protease inhibitors
3TC	lamivudine
FTC	emtricitabine
AZT	zidovudine
ddl	didanosine
d4T	stavudine

ABC	abacavir
TDF	tenofovir
DLV	delavirdine
EFV	efavirenz
NVP	nevirapine
ETR	etravirine
APV	amprenavir
FOS	fosamprenavir
IDV	indinavir
SQV	saquinavir
LPV	lopinavir+ritonavir
DRV	durunavir
NFV	nelfinavir
ATV	atazanavir
TPV	tipranavir
nm	nanometer
ul	microliter
ml	milliliter
PBMC	Peripheral blood mononuclear cell
EDTA	Ethylenediaminetetraacetic acid
NASBA	Nucleic acid sequence based amplification

CHAPTER I

INTRODUCTION

1.1 Background and Rational

Human immunodeficiency virus (HIV) is a virus that infects cells in the immune system. After, it gets into the body, virus will destroy white blood cells especially CD4+ which leads to impair the immune system. When immune systems go down, an opportunistic pathogens such as *Mycobacterium tuberculosis* will damage cells in the immune system.[1] This may cause severity to patients which leading to death.

According to the joint United Nations programme on HIV and AIDS (UNAIDS) 2014, there was approximately 36.9 million people around the world who was living with HIV infection. When compared to a number of new infected people in 2013, the number was reduced from 2.1 to 2.0 million. The number of people who died from HIV was reduced from 1.5 million (in 2013) to 1.2 million (in 2014).[2] Therefore, the Bureau of Epidemiology, Department of Disease Control Ministry of Public Health (BoE) was reported that overall in 2014, there were 388,621 of HIV infected cases and 100,617 people who died from HIV infection.[3] The maximum age range of HIV infected patients were 30-34 years old (25.83%). From previous studies, they showed that the number of newly infection and also the number of people died from HIV was reduced annually since 2001.[2, 3] This might be because of the implementation of antiretroviral drugs. Currently, the number of HIV drug resistance was increased.[4, 5] HIV drug resistance may occur when patients take low optimal antiretroviral therapy, poor drug absorption and poor adherence.[6, 7]Without continual tracking of the amount of virus in blood, the virus may mutate and resist to the antiviral drugs treatment. Therefore, antiviral treatment regimens, which is used for patients is not suite for individual appropriately. Then, the virus cannot be suppressed.

In general, the treatment of HIV is evaluated by tracking a number of CD4+ T lymphocytes in order to make a decision for starting antiretroviral therapy.[8] Then, the viral load still be detected in the presence of antiretroviral drugs, it means that virus can

resist to antiretroviral treatment. If the viral load level cannot be suppressed, it indicates the failure of antiviral drugs treatment. And also, monitoring of viral load level may help to determine the efficiency of antiretroviral drug treatment. The gold standard for detection viral drug resistance is a HIV genotyping assay. In general, the drug resistance genotyping detection is performed when the viral load is greater than 2,000 copies/ml.[9] Due to the fact, the HIV viral load and the antiviral drug resistance genotyping detection are importance to evaluate the efficacy of antiviral drugs. Moreover, they are also useful in epidemic virus tracking. The National Health Security Office (NHSO) has promoted the HIV treatment policy for the patients where the medical fee, such as antiviral drug, a CD4 tracking, HIV viral load detection and HIV antiviral drug resistance detection, is excluded.[10]

For specimen collection, HIV National guideline recommended that blood samples need to be processed within 6 hours and kept in -80°C and separated plasma must be transported in form of a cold chain. Following these requirements, cost of plasma transportation is noticeably concerned. To reduce the transportation cost, World Health Organization (WHO) proposed a sample collection technique by dropping a blood spot onto a dried blood spot filter paper and transports this paper at room temperature which does not need a centrifuge or -80°C to processed specimens.[11-13] Previous studies have reported that results of viral load detection in plasma and dried blood spot were correlated.[14-18] It showed that dried blood spot can be used for viral load testing [17-22] and drug resistance testing. [16, 23-25]Therefore, WHO supports the use of dried blood spot for HIV viral load and antiviral drug resistance.[11] However, this technique is still not widely used.

The objective of this study is to evaluate the performance of dried blood spot for HIV viral load and drug resistance genotyping detection. In our study, we evaluate the performance of three types of dried blood spot filter paper including Whatman 903, nitrocellulose membrane and cellulose fiber sample pad. And also use of gold nanoparticle, which is normally used in a lateral flow immunoassay test and anion polymer-coated magnetic beads with these filter papers in order to increase the capability of detection performance.

1.2 Objective

   1.2.1 To study an effect of gold nanoparticle and anion polymer-coated magnetic beads on the sensitivity of an HIV-1 viral load detection.

   1.2.2 To investigate the performance of different type dried blood spot filter papers, Whatman 903, nitrocellulose membrane and cellulose fiber sample pad for HIV-1 viral load and drug resistance detection.

1.3 Research Question

   1.3.1 Do gold nanoparticle and anion polymer-coated magnetic beads increase a sensitivity of HIV-1 viral load detection?

   1.3.2 Do different types of dried blood spot filter papers, Whatman 903, nitrocellulose membrane and cellulose fiber sample pad, give a different performance for HIV-1 viral load and drug resistance detection?

   1.3.3 Which type of dried blood spot filter papers, Whatman 903, nitrocellulose membrane and cellulose fiber sample pad, give the highest sensitivity of HIV-1 viral load and drug resistance detection?

1.4 Expected Benefits

   1.4.1 A new filter paper can be used to detect HIV-1 viral load as Whatman 903.

   1.4.2 To collected dried blood spot on the filter can reduce the complexity of transportation.

   1.4.3 The new filter paper can be used to collected the sample in resource limited country.

1.5 Ethical Considerations

   Blood samples in this study are obtained from Health Sciences Service Unit, the Faculty of Allied Health Sciences, Chulalongkorn University. To perform the evaluation, we perform the ethical considerations and this study was approved by the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University-COA No. 007/2558.

CHAPTER II
LITERATURE REVIEW

2.1 Discovery of HIV infection

Before 1981, there was not a specific name for AIDS. It was called by using various names such as gay compromise syndrome, Gay-related immune deficiency (GRID), acquired immunodeficiency disease (AIDS), gay cancer or community-acquired immune dysfunction.[26] In 1981, the symptoms of *Pneumocystis carinii* pneumonia (PCP) were founded in gay man in New York and California of the United State of America (USA).[27] Then, Centers for Disease Control and Prevention (CDC) specified the name for the disease to be used in the worldwide called AIDS.

In 1983, Barre-Sinoussi and colleagues at Pasteur Institute found a man who got persistent generalized lymphadenopathy (PGL) that may be associated to AIDS. Then, they investigated that the virus contains reverse transcriptase enzyme, this might be a retrovirus which property is closely to the human T-cell leukemia virus (HTLV). Moreover, they also found lymphadenopathy associated virus (LAV) which some property is different to HTLV. LAV can replicate in CD4+ T lymphocytes.

In 1984, Gall and his colleague reported that a new virus, HTLV-III, which had different properties as compared to HTLV. In the same year, Levy and his colleagues separated a virus from infected patients and high risk patients. Then, they reported a new virus known as AIDS-associated retrovirus (ARV). In 1986, the International Committee on Taxonomy of viruses (ICTV) investigated the property of three viruses including LAV, HTLV-III and ARV, they found that these viruses have the same characteristic. Then they named these viruses as Human Immunodeficiency Virus (HIV). [28]

2.2 Strains of HIV

Human immunodeficiency virus (HIV) is a kind of virus that has a genotype related to Simian Immunodeficiency Virus (SIV). The SIV, which is endemic in primates,

can be categorized into a group of Retroviridae family and genus Lentivirus. Retroviridae family has reverse transcriptase (RT) that used complementary DNA as a template for DNA replication.

In general, HIV can be categorized into two types including HIV-1 and HIV-2. HIV-1 is now pandemic over the worldwide, while HIV-2 is now endemic only in South and North Africa. To study the evolution of both HIV viruses (HIV-1 and HIV-2), molecular genetic technique is utilized by constructing a phylogenetic tree and then compared to lentivirus, where the hosts of the virus are primates. As a result, HIV-1 and HIV-2 evolve from different primates.

In general, HIV-1 strain is related to two types of SIV virus that infects chimpanzee (SIVcpz) and gorillas (SIVgor) in central and West Africa.[29, 30] On the other hand, HIV-2 is related to SIV that infects gibbon (SIVsm).[31] There are many factors that SIV virus can be infected to a human and mutate to be a new kind of disease known as HIV. The important factor that causes cross viral infection which is from a regional popularity of Africa such as animal hunting and consuming meat products.[32] The first HIV infection may caused by butchering or cooking of primates. Therefore, the first hypothesis of HIV infection was the HIV virus that cannot infect in human but it can be infected in primates. After that, there was a mutation of SIV virus in the human due to a reverse transcriptase enzyme lack of proofreading function. Since there is an error in DNA replication of retrovirus, the SIV virus is then mutated into HIV virus, where human is a specific host of HIV virus.

From a similarity study of a genome's nucleic acid sequence of HIV, HIV-1 virus can be categorized into three groups including M (major), O (outlier) and N (non-M non-O or novel). M has been subdivided further into nine major subtypes or clades: A-D, F-H, J and K, where these viruses are pandemic while O and N group infect only in a small region such as Gabon and Cameroon.[33] In some cases, different types of HIV-1 viruses infect in the same host and they can be performed a recombination and rearrangement. Then, a hybrid mosaic virus is generated. Most of hybrid mosaic virus particles cannot be productive. Some of productive hybrid mosaic virus particles are

called circulating recombinant forms (CRF), such as CRF A/B. On the other hand, HIV-2 is epidemic in Africa and a few regions of South of Europe and India.[34]

A subgroup of HIV-1, which is mostly found in south-east Asia, are CRF01_AE (84.28%). Lau Ka and his colleagues reported that the number of HIV infected in Thailand were 89% CRF01_AE and 8% group B.[35] The first HIV infected patient of Thailand was found in prostitutes in 1984. The type of HIV was CRF01_AE. In 1987, a group B of HIV was found among drug-addicted people. Afterwards, a disease of CRF01_AE group spread to injection drug use (IDU).[36]
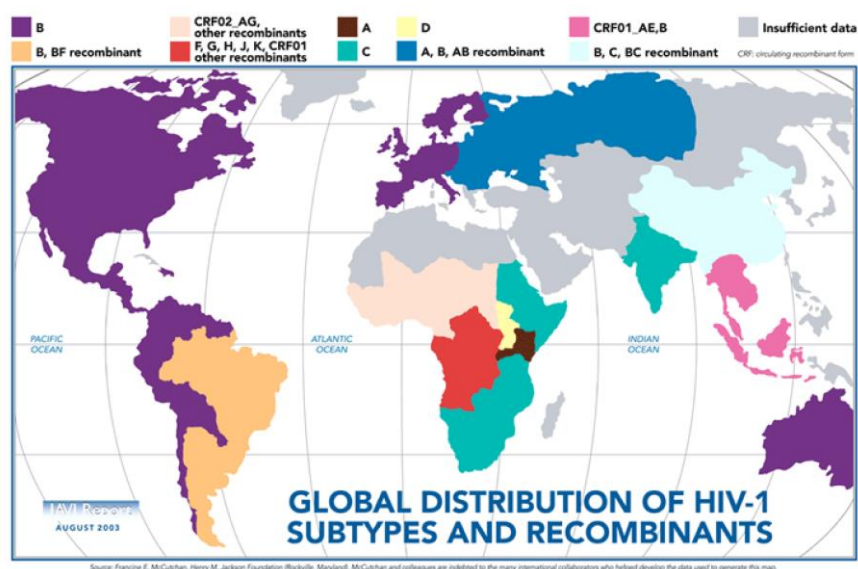
Figure 2- 1 Global distribution of HIV-1 subtype and recombinants[37]

2.3 HIV structure

The structure of HIV virus is spherical where the diameter is between 100-120 nm. It contains RNA genome, nucleocaspid (p7) and three enzymes which are reverse transcriptase enzyme (RT), protease (PR) and integrase (IN) in its core and enclosed by capsid consisted of core protein, called p24. Matrix protein (p17) lies between the envelope and the core. The exterior part is the envelope consisting of the outer glycoprotein gp120 and the transmembrane gp41 (as shown in Figure 2-2).[38]
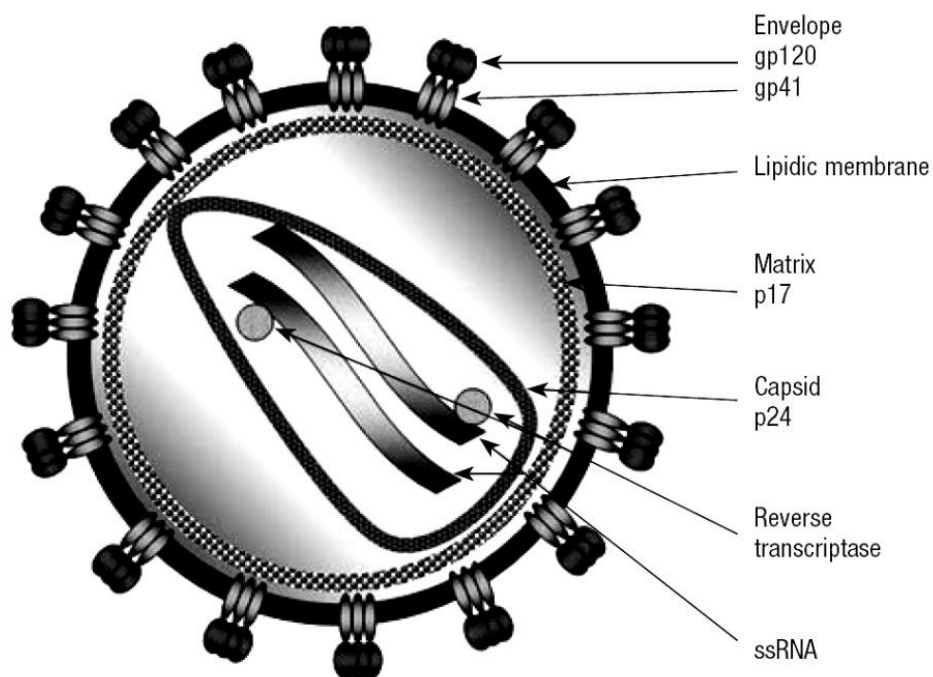
Figure 2 -2 The Structure of HIV[39]

HIV-1 genome is approximately 10 kb. It consists of structural genes, regulatory genes and long terminal repeat (LTR).
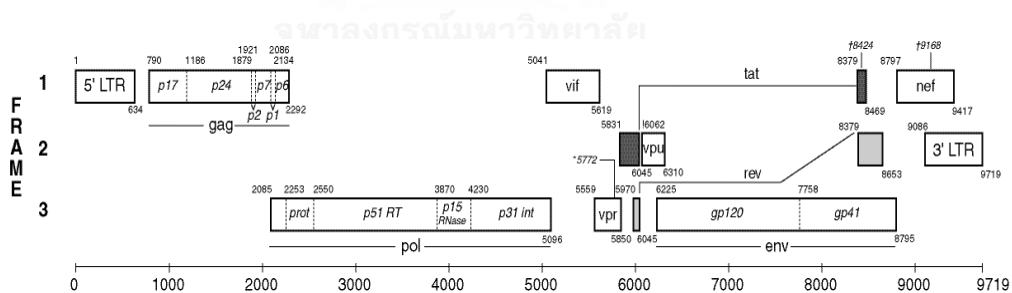
Figure 2-3 Structure of HIV-1 genome [40]

Structural gene is gene that codes for a structural protein of virus including *gag*, *pol* and *env*. These proteins are polyprotein precursor and then cleaved by protease enzyme to produce a structural protein.

- *Gag* (group-specific antigen) produces a core protein (p24), matrix protein (p17) and nucleocapsid (p7).

- *Pol* (polymerase) codes for a reverse transcriptase enzyme (RT), protease enzyme (PR) and integrase enzyme (IN).

- *Env* (envelope) is a coat of virus which some parts of the envelope are from host cell, i.e., gp160. It should be noted that gp160 is a precursor protein of gp120 and gp41.

Regulatory gene encodes a protein in order to control an increasing of virus and its ability of infecting.

Long terminal repeat (LTR), the end of HIV RNA's strand, controls a new HIV virus production where the sequence is activated by a cellular transcriptional factor such as NFkB, SP1 and TFIID. By this activating, HIV transcription process begins which resulting in a multiple copies of viral RNA production. [41]

2.4 HIV life cycle

HIV virus particles bind with a CD4 receptor and coreceptor (CCR5 or CXCR4) on CD4 T lymphocyte surface by using glycoprotein gp120. In the next step, the envelope of viral proteins fuses with host cell membranes.[42, 43] The viral RNA is released into the host cell cytoplasm and transcribes to double-stranded viral DNA by reverse transcriptase enzyme.[44] Double-stranded viral DNA transports into the cellular nucleus and the HIV integrase enzyme integrated this DNA into the host cell's chromosomes as proviral DNA.[45] Next step, proviral DNA is multiplied along with the chromosomes when the cell is replicated by host cell polymerases. The product of transcription proviral DNA is viral RNA which is exported to the cytoplasm for translation to protein or incorporation into new virions. New virions assemble and bud from the host cell's plasma membrane.[46] After buding, protease enzyme of virus cleaves HIV polyproteins into individual subunits for producing infectious mature virion.
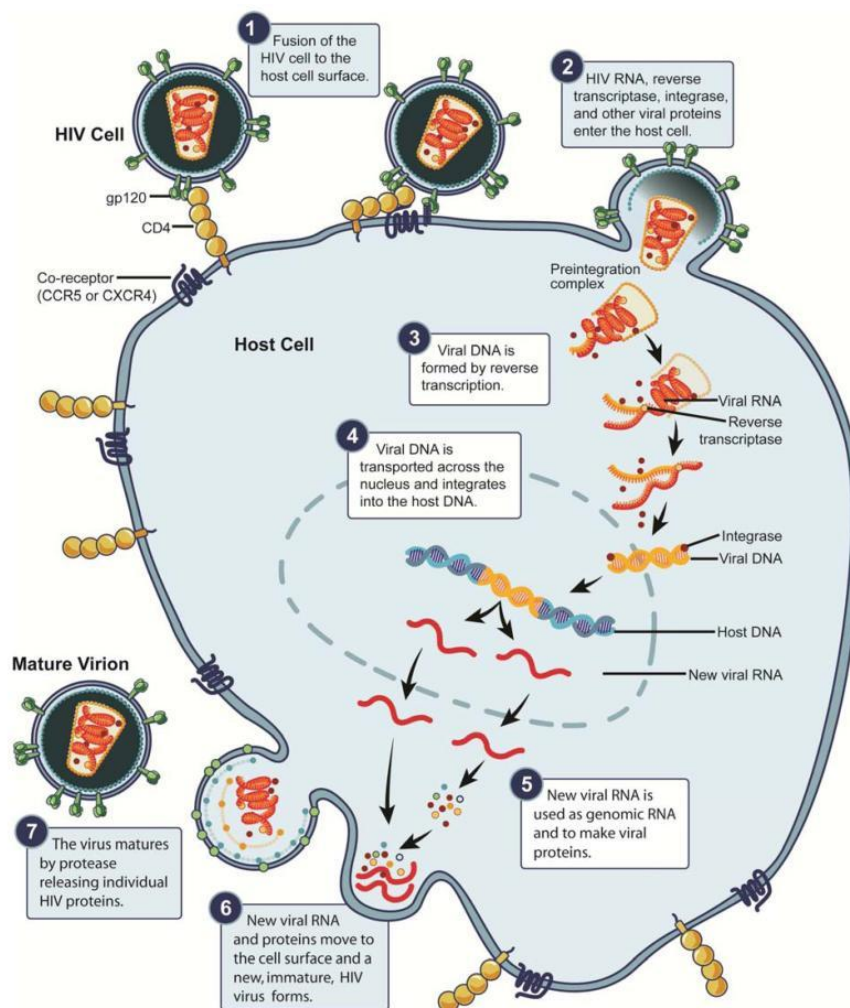
Figure 2-4 HIV life cycle [47]

2.5 HIV diagnosis

A diagnosis of HIV virus can be performed by the following procedure:

2.5.1   HIV antibodies

There are several principles to detect HIV antibodies such as ELISA, Western blot, Immunofluorescence assay, agglutination and Immunochromatography. To measure HIV antibody, at least three types of these are used. The first method, which is highly sensitive, is a screening test. If a result from the first method is positive, the detection of the second method, confirmatory test, is required. The confirmatory test is a higher specificity. In the past, Western blot is used as a confirmatory test. However, this method has a high complexity and expensive. Recently, World Health Organization (WHO) introduces a guideline that diagnoses HIV antibodies as in Figure2-5.[48]
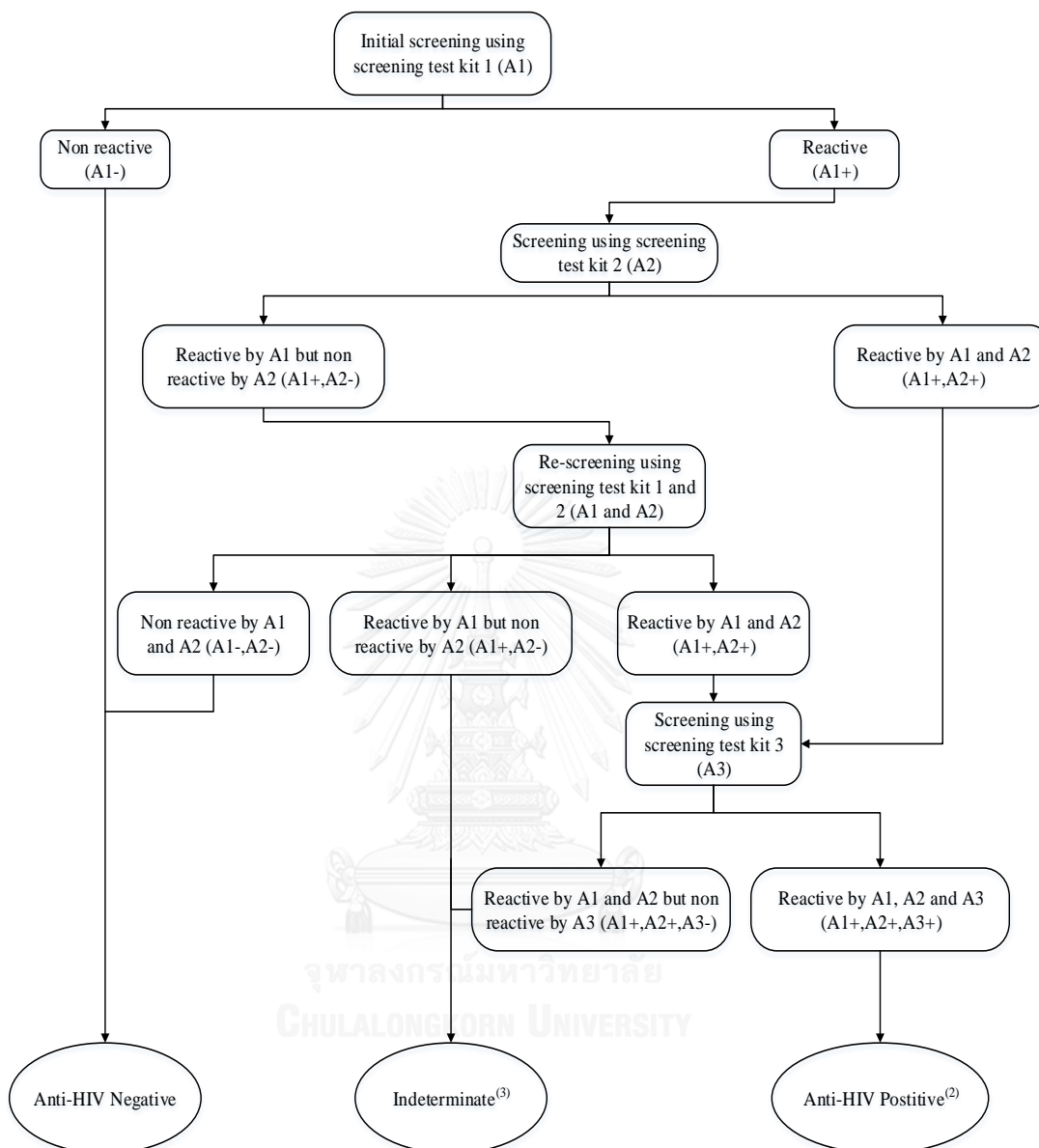
Figure 2-5 A process of HIV infection diagnosis[9]

2.5.2   Viral detection

2.5.2.1 ELISA method: A viral protein is detected from an inner protein in a part of core protein of HIV virus such as p24 Ag. This protein is detected in the first phase of an infection by using ELISA.[48]

2.5.2.2 Molecular biology method: In general, HIV viral genetic material is in a form of proviral DNA or HIV RNA. To detect a proviral DNA, peripheral blood mononuclear cell (PBMC) is extracted from EDTA blood which

contains proviral DNA of HIV virus. This method is widely used to detect an HIV viral infection of a child when the infection is occurred from his parents. To detect HIV RNA, plasma from EDTA blood was analyzed. There are several methods for detecting proviral DNA or HIV RNA such as polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA) and branched DNA (bDNA).[48]

2.6 Monitor of HIV infected patient

2.6.1 CD4+ T lymphocyte testing

CD4+ T lymphocyte testing is performed to evaluate a patient's immune system, disease progression and predict a phase of disease. The evaluation results are used to determine an effective of the treatment using antiviral drugs. The patient whose the level of CD4+ T lymphocyte is less than 350 cells/mm^3 should start taking antiviral drugs. If the level of CD4+ T lymphocyte is greater than 350 cells/mm^3, the antiviral drug is not required but the level of CD4+ T lymphocytes should be tested every 6 months.[49]

2.6.2 HIV viral load testing

HIV viral load testing is performed by testing EDTA blood which is centrifuged in order to separate the plasma. Then, HIV viral load is measured using RNA viral load. There are several HIV viral load detection principles such as reverse transcriptase polymerase chain reaction (RT-PCR), branched DNA (bDNA), nucleic acid sequence based amplification (NASBA) and real-time PCR.[9] These methods are implemented in a device which is developed by several companies such as Abbott, Roche and NucliSEN®EasyQ. Information from the viral load detection is used to determine an effective of HIV treatment, trace a patient's viral load and used to determine an appropriate antiviral drug.

Table 2-1 Specifications of FDA approved commercial HIV viral load assays [50]

Item	Abbott RealTime HIV-1 (*m2000rt*)	COBAS TaqMan 48 HIV-1 (Roche)	NucliSENS EasyQ HIV-1 v1.2 (bioMérieux)	VERSANT 440 HIV-1 RNA v3.0 (Siemens)
HIV target region	Highly conserved region within *pol* (integrase)	Highly conserved region of the *gag* gene	Highly conserved region within *gag*	Several regions of *pol*
Internal control	Yes (non-competitive pumpkin gene)	Yes	No	No
Amplification	Real-time PCR target amplification	Real-time PCR target amplification (TaqMan)	Real-time NASBA	bDNA signal amplification
Detection	Fluorescence	Fluorescence	Fluorescence – molecular beacons	Chemiluminescence
Quantitation	May be reported in multiple formats: copies/ml, log_{10} copies/ml, IU/ml or log_{10} IU/ml; conversion factor to IU/ml is 1 IU = 0.56 copies and 1 copy = 1.74 IU	Reported in copies/ml; conversion factor to IU/ml is 1 IU = 0.6 copies and 1 copy = 1.7 IU	Reported in copies/ml; 1:1 conversion to IU	Reported in copies/ml
Linear dynamic range (lower and upper limits)	40 copies/ml from 600 µl to 10 million copies/ml	40 copies/ml from 850 µl to 10 million copies/ml	25 copies/ml to 10 million copies/ml	50 copies/ml to 500 000 copies/ml
Specificity (%) (95% CI)	100 (95% CI 98.05–100)	100 (95% CI 99.3–100)	100	100 (95% CI 98–100)
HIV subtypes	Group M – subtypes A–D, CRF01_AE, CRF02_AG, subtypes F–H, group N and O	Group M – subtypes A–D, F–H; CRF01_AE	Group M – subtypes A–D, F–H, J	Group M – subtypes A–D, F–H

2.6.3 Drug resistance testing

Drug resistance testing including genotypic drug resistance testing, phenotypic drug susceptibility testing and virtual phenotype. [51]

2.6.3.1 Genotypic drug resistance testing

A genotypic drug resistance testing is based on detection of genomic sequences which control the production of reverse transcriptase and protease enzyme. Normally, the mutations which occurred at this position effects to drug treatment. Recently, Food and Drug Administration (FDA) approved two genotypic methods for drug resistance testing include, HIV-1 TRUGEN^{TM} Genotyping Assay (Siemens) and ViroSeq HIV-1 Genotyping System (Celera Diagnostics).

2.6.3.2 Phenotypic drug susceptibility testing

Phenotypic drug susceptibility testing is determined the sensitivity of HIV which isolate from patient to antiviral drug in vitro. The result shows concentration of antiretroviral drug which inhibit HIV replication 50% and 90% or it is called 50% inhibitory concentration (IC50) and 90% inhibitory concentration (IC90).

2.6.3.3 Virtual phenotype

Virtual phenotype is combined the results of phenotypic and genotypic method and analyzed by program computer to predict HIV drug resistance.

Genotypic drug resistance testing is widely used in Thailand. A testing of HIV antiviral drug resistance is performed by detecting a changing of amino acid sequence in the HIV viral genetic materials.[9] The testing results assist a physician to evaluate the effective of HIV treatment and can be used to determine an appropriate antiviral drug for the treatment. The sample that is used to detect an antiviral drug resistance genotyping should be the same type of sample that is used to detect the viral load and the viral load should be greater than 2,000 copies/ml.[10]

2.7 Capturing method for virus concentrate

2.7.1 Anion polymer-coated magnetic beads

The viro-adembeads captures the virus by using anionic polymer-coated magnetic beads which charge of Poly (methyl vinyl ether-maleic anhydride) or Poly (MVE-MA) coat on the magnetic bead is negative.[52] There were several study showed that anionic polymer-coated magnetic beads can bind the surface of some viral particle but the mechanism is unknown.[52] Sometime in clinical sample had low number of virus so the viro-adembeads is utilized in order to increase sensitivity of virus detection where the level of viruses is lower than to be detected by using a normal detection technique.

2.7.2 Gold nanoparticle

Recently, nanoparticles are widely used in medical diagnosis method. The nanoparticle is considered as a small material that is capable of interacting with a biomolecule such as proteins, lipids and nucleic acids. In the biochemical diagnosis application, various types of nanoparticles, gold (AuNPs), silver (AgNPs) and copper, are utilized. In this paper, we focus on the gold nanoparticles (AuNPs) which properties are sensitivity and cost-effectiveness. The gold nanoparticles can be utilized in many medical application such as labeling for cells and proteins and delivering therapeutic agents within cells.[53, 54]

There are three ways that the gold nanoparticles can bind with the protein. First, the gold nanoparticles bind with the surrounded protein, whose charge is positive, through ionic binding since the charge of the gold nanoparticle is negative. Second, the gold nanoparticles bind with the protein via their amino acid-containing. Third, the gold nanoparticle can be bound with the protein via gold and sulfur bonding. The factors that are corresponding to the performance of these binding methods are the size, surface charge and shape the gold nanoparticles.[54]

2.8 Specimen collection

2.8.1 EDTA blood

A type of sample that is accepted by WHO and normally used to detect HIV viral load and HIV antiviral drug resistance is plasma.[11] The plasma must be separated within 6 hours and should be kept in -80ºC. Plasma transportation must be in form of cold chain.

2.8.2   Dried blood spot filter paper

2.8.2.1 Whatman 903

Whatman 903 was firstly called Guthrie cards which is acknowledged by WHO for HIV viral load and antiviral drug resistance detection.[11] Whatman 903 was firstly developed in 1960 by Robert Guthrie for collecting dried blood spot from newborn baby's heel.[55] This dried blood spot is used to detect an abnormality of metabolism. A Whatman 903 dried blood spot filter paper is made from pure cotton linters and normally used to detect a newborn baby blood and detect HIV virus infection.

2.8.2.2 Nitrocellulose membrane

Nitrocellulose membrane is made from cellulose and purified by using nitric acid where the hydroxyl group is replaced by a nitrate group which has a high binding capability. Membranes with smaller pore sizes have higher binding capacities for macromolecules and are frequently used to bind smaller macromolecules. In 1970, a nitrocellulose membrane was widely used in DNA, RNA and proteins application such as Southern[56], Northern[57] and Western blotting[58]. After the proteins are separated by gel electrophoresis, the proteins will be transferred to a nitrocellulose membrane in order to identify the proteins by using hybridization. Due to the high blotting capability, in 1980, the nitrocellulose membrane was used in immunochemical detection such as antigens or antibodies detection using lateral-flow principle. [59]

2.8.2.3 Cellulose fiber sample pad

Cellulose fiber sample pad is made from cellulose fiber or glass fiber. An immunochromatographic strip test has been used in a point of care testing such as

pregnancy test, fertility and ovulation tests, cancer diagnostics and drug abuse testing for diagnostic diseases.[60] The immunochromatographic strip test is based on a principle of lateral flow and consists of four main parts including sample pad, conjugate pad, nitrocellulose membrane and adsorbent pad.[61] A cellulose fiber sample pad is the first part of the immunochromatographic strip where a sample, such as blood, urine and oral fluid, is applied and then the sample is transported to the other parts of the strip to complete the assay.[61] The cellulose fiber sample pad transports the sample with the capabilities of manner.

2.9 Antiretroviral Therapy

A criterion of starting HIV treatment by using antiviral drug in Thailand is shown in Table 2-2.

Table 2-2 A criterion of starting of HIV treatment using antiviral drug in Thailand[9]

Clinical Presentation	CD4+ T-cell counts (cells/mm3)	Recommendations
AIDS-defining illness	Any value	Treat
HIV-related Symptomatic	Any value	Treat
Asymptomatic	<350	Treat
Asymptomatic	≥350	Defer treatment; follow up clinical status and monitor CD4+ T-cell count every 6 months
Pregnancy	Any value	Treat, Discontinue ART after delivery if pre-treatment CD4+ T-cell count is >350 cells/mm3

Antiretroviral agent (ART) categorized by inhibited mechanism of HIV virus replication can be separated into 4 groups and are described as the following:
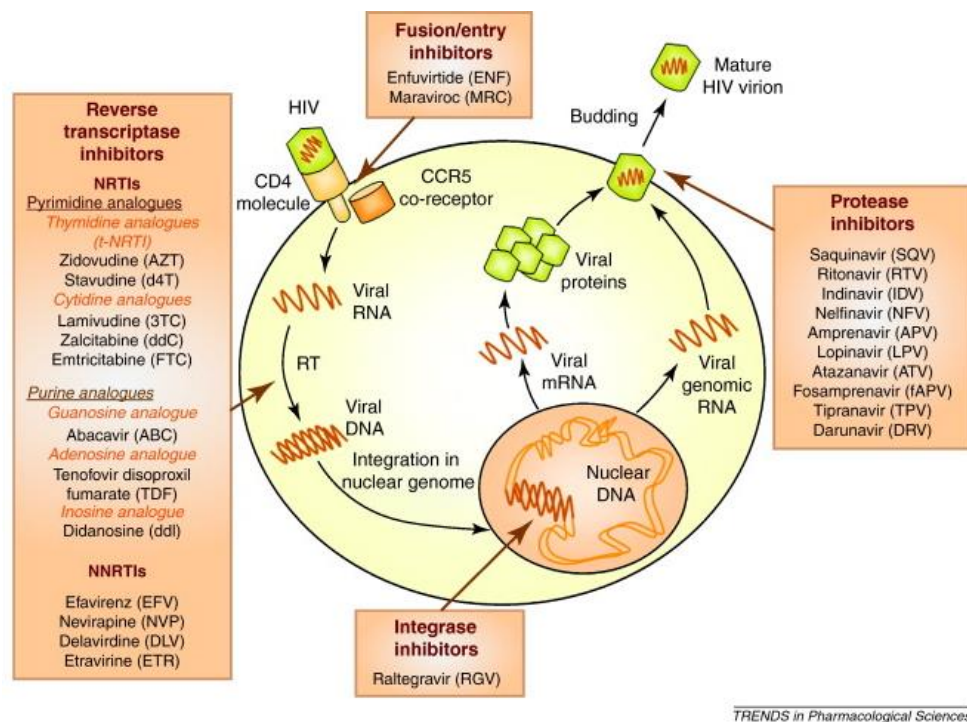
Figure 2-6 *HIV therapeutic target*[62]

2.9.1 Reverse transcriptase inhibitor (RT)

Reverse transcriptase inhibitor (RT) inhibits a generation process of reverse transcriptase (RT) enzyme in order to generate a proviral DNA of a virus.

2.9.1.1 Nucleotide reverse transcriptase inhibitors (NRTIs)

Nucleotide reverse transcriptase inhibitors (NRTIs) such as zidovudine (AZT), stavudine(d4T), lamivudine (3TC),emtricitabine (FTC), didanosine (ddI), abacavir (ABC) and tenofovir (TDF), are type of antiviral which structure are similar to an important part of DNA nucleoside. They will interrupt a viral DNA elongation process of RT enzyme. The viral RT enzyme cannot separate the difference between a natural base and a synthetic base of the medicine. Therefore, the virus cannot use the natural base to extend the DNA strand.

2.9.1.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) such as nevirapine (NVP), efavirenz (EFV) and etravirine (ETR), capture RT enzyme of Hydrophobic pocket-like binding site. This capturing affects a decreasing of a

flexibility of molecule enzyme. Then, a polymerase active site of the enzyme cannot synthesize HIV-1 cDNA.

2.9.2 Protease inhibitors (PIs)

Protease inhibitors (PIs) such as indinavir (IDV), ritonavir (RTV), nelfinavir (NFV), saquinavir (SQV), lopinavir/ritonavir (LPV/r),atazanavir (ATV) and darunavir (DRV), inhibit the operation of the protease enzyme. These inhibitors will capture a small pocket of protease enzyme using a hydrogen bond. Therefore, virus particle is not completely produced.

2.9.3 Integrase inhibitors (INIs)

Integrase inhibitors (INIs) such as raltegravir (RAL) will inhibit the action of integrase enzyme to integrate proviral DNA to the target DNA.

2.9.4 Fusion inhibitors (FIs)

Fusion inhibitors (FIs) such as enfuvirtide (ENF) it binds to gp41 transmembrane protein on HIV envelope which is the important part for HIV fusion mechanism.

A treatment of HIV infected patient is performed by using of the antiviral drug at least 3 types. Since a treatment by using only a single antiviral drug may cause an antiviral drug resistance faster than using of antiviral drug more than one type.

Table 2-3 Antiviral drug formulas.

NRTIs		NNRTIs		PIs
Preferred				**Preferred**
AZT + 3TC				LPV/r
TDF + 3TC/FTC			or	
Alternative	+	EFV	(if patient	**Alternative**
ABC + 3TC		NVP	cannot tolerate	ATV/r
d4T + 3TC			NNRTIs)	DRV/r
ddI + 3TC				SQV/r

Table 2-4 The Thailand antiretroviral therapy regimens.

	2NRTIs	NNRTIs or PIs
First-line	Zidovudine (AZT) + Lamivudine (3TC)	Nevirapine (NVP)
	Stavudine (d4T) + Lamivudine (3TC)	Efavirenz(EFV)
		Lopinavir/ritonavir (LPV/r)[a]
Second-line	Zidovudine (AZT) + Didanosine (ddI)	Atazanavir ± ritonavir (ATV±r)
	Didanosine (ddI) + Lamivudine (3TC)	
	Tenofovir (TDF) + Lamivudine (3TC)	Darunovir/ritonavir (DRV/r)
	Abacavir(ABC) + Lamivudine (3TC)	Saquinavir/ritonavir (SQV/r)

In Thailand, monotherapy using antiviral drug, Zidovudine (AZT), was firstly used in 1992 in the HIV treatment. After 1994, a performance of HIV treatment was improved by using a combination therapy where two types of NRTIs, AZT with Didanosine (ddI) or AZT with Zalcitabine (ddC), are used in the treatment. However, the performance of the treatment still needs to be improved, then a highly active antiretroviral therapy (HAART) which combines three antiviral drugs is exploited in order to suppress an HIV replication. Recommended and optional formulas of antiretroviral drugs which are used in Thailand are shown in Table 2-4

CHAPTER III

MATERIALS AND METHODS

3.1 Specimen collection and processing

Seventy samples of HIV-1 infected patients were obtained from the Health Science Service Unit, Faculty of Allied Health Sciences, Chulalongkorn University during April – September 2015. Six milliliters of ethylenediaminetetraacetic acid (EDTA) blood samples were collected to detect HIV-1 Viral Load and to prepare the dried blood spots as followed;

3.1.1 EDTA blood

One milliliter of whole blood was separated from EDTA tube and stored in 4^oC until used. The rest of EDTA blood were centrifuged for 15 minutes at 3,500 rpm. The plasma was collected to determined HIV viral load by COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, version 2.0 (Roche, USA). The step of plasma collection should be performed within 6 hours after sample collection.

3.1.2 Dried blood spot

The whole blood which had plasma viral load more than 280 copies/ml were chosen for preparing dried blood spot. Before used, whole blood was thawed at room temperature and mixed by invert. Then, two drops of 50 µl of whole blood were dropped on filter paper in different position. After that the filters were dried in a biosafety cabinet level 2 at least 3 hours. Then, the dried blood spots were kept in a zipper bag at -20^oC until used. There were three types of filter paper which used for comparing the efficiency in this study. They were Whatman 9 0 3 , nitrocellulose membrane and cellulose fiber sample pad. Dried blood spots were extracted by using NucliSENS® easyMAG® (Biomerieux, France) and detected HIV viral load by using NucliSENS EasyQ® HIV-1 v2.0 (Biomerieux, France). Nucleic acid from dried blood spots which had the level of HIV viral load more than 2,000 copies/ml were selected to perform antiretroviral drug resistance assay by ViroSeq™ HIV-1 Genotyping system (Abbott, USA).

In this study, CM244 HIV-1 subtype E which was isolated from cultured supernatant of human peripheral blood mononuclear cell (PBMC) from Department of Microbiology, Faculty of Medicine Siriraj hospital was used as a control.

3.1.3 Ethical Considerations

Blood samples in this study were taken from HIV infected patients at Health Sciences Service Unit, the Faculty of Allied Health Sciences, Chulalongkorn University. To perform the evaluation, we performed the ethical considerations. This study was approved by the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University-COA No. 007/2558.

3.2 Pre-process of dried blood spot

In this study, we applied and compared the additional process before dried blood spot collection in order to increase the sensitivity of dried blood spot. There were two methods as follow;

3.2.1 Anion polymer-coated magnetic beads (viro-adembeads)

This method was performed by incubating 100 ul of CM244 with 50 ul of viro-adembeads by thermo-mixer at 900 rpm at 20ºC for 20 minutes. Then, a magnetic field was applied in order to separate the bead fraction (BD) from supernatant fraction (SP). The supernatant were removed and resuspended bead with 100 ul PBS. Then, the suspension were dropped onto filter paper 50 ul, two drops in different position.
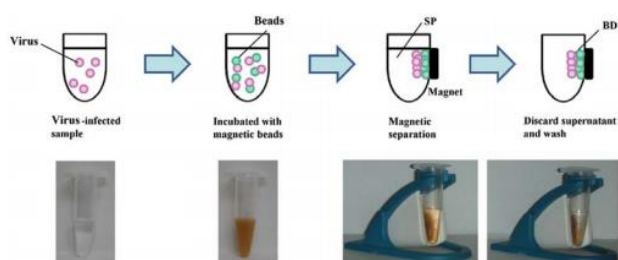
Figure 3-1 Virus capture by poly (MVE-MA)-coated magnetic beads.[52]

3.2.2 Gold nanoparticle

In this study, we used the gold nanoparticles (AuNPs) which properties are high sensitivity and cost-effectiveness. The ability of virus capturing of gold nanoparticles depends on its size and volume. Therefore, we investigated the appropriate size and volume of gold nanoparticle through Whatman 903 filter paper. We compared the viral load detection under different volume (6.25 ul, 12.5 ul, 25 ul and 50 ul) and size (10 nm, 20 nm and 40 nm) of gold nanoparticle (Sigma-Aldrich, Singapore). To investigate the level of HIV viral load, a whole blood was mixed with HIV CM244 (27,000 copies/ml) and diluted until the value of viral load was 1,125 copies/ml (from calculation). Then, two drops of different volume of gold nanoparticles were dropped onto Whatman 903 filter paper in different position and dried the filter paper over the night at a room temperature. Then, two drops of 50 ul of whole blood were dropped in the same position and dried it again. The dried blood spot was stored in a zipper bag and kept the bag in the freezer at -20 °C until used.

3.3 Extraction of dried blood spot for HIV-1 viral load and drug resistance

Genetic materials were extracted from dried blood spot by using NucliSEN easyMAG® (Biomerieux, France) which was wildly used. First, dried blood spot was cut into a circle which area surrounds the blood spot. Then, the filter paper was added into NucliSEN®Lysis buffer and incubated the tube on the roller mixer at room temperature for 2 hours to make the lysis buffer fully contacts with the dried blood spot filter paper. After that, the tube was centrifuged 1500 g for 15 seconds and the lysed samples were pipette into a NucliSEN®EasyMAG vessel. Add 50 ul of magnetic silica into a NucliSEN®EasyMAG vessel and mix them by using Biohit pipette (Multichannel pipette). The step of genetic material extraction process was started by using NucliSEN®EasyMAG machine. Finally, 25 ul of nucleic acid was collected into a 0.2 ml PCR tube. The nucleic acid was stored at -20ºC until used.

3.4 HIV-1 viral load detection

HIV-1 RNA viral load was quantified by using NucliSENEasyQ® HIV-1 v2.0 solution (Biomerieux, France). This method is based on nucleic acid sequence-based amplification (NASBA) which performs one step isothermal process to amplify RNA

and to perform real time detection using molecular beacon. The operation of NucliSENEasyQ® HIV-1 v2.0 machine should be operated with the extraction by using NucliSEN easyMAG® extraction machine. Synthetic calibrator was added into the lysed sample at the same time as the magnetic silica adding. Then, the premix was produced.

To prepare a calibration solution, 550 ul of CAL diluent was mixed with a vortex in the calibrator tube. A pre-mix was prepared by adding 550 ul of magnetic silica into the calibration solution and then mixed them using vortex. Add 100 ul of pre-mix into a NucliSEN®EasyMAG vessel. After the extraction process was completed, 15 ul of genetic material was obtained and added into a NucliSENEasyQ® 0.2 polyethylene micro tube strip. Enzyme solution was prepared by adding 45 ul of enzyme diluent into an enzyme sphere and mixed them carefully for 15 seconds. A primer solution was prepared by adding 180 ul of primer diluent into a primer spheres and mixed them with a vortex until completely smooth. Pipette 20 ul of primer solution into the NucliSENEasyQ®0.2 polyethylene micro tube.

NucliSENEasyQ®0.2 polyethylene micro tube strips was inserted into a NucliSENEasyQ®Incubator without closing the lid and incubated at 65°C for 2 seconds and at 41°C for 2 seconds. Prepare the lid of NucliSENEasyQ®0.2 polyethylene micro tube strips by pipetting 5 ul of enzyme solution onto it. After the incubation was finished, enclose the NucliSENEasyQ®0.2 polyethylene micro tube strips with the lid and spin-down the tube with a mini-strip centrifuge for 2 seconds. Mix the RNA, which was in the NucliSENEasyQ®0.2 polyethylene micro tube strips, vortex for 1 second thrice, then spin-down for 2 seconds.

NucliSENEasyQ®0.2 polyethylene micro tube strips were inserted into a NucliSENEasyQ®Analyzer in order to detect the viral load approximately for 1 hour. The result showed as a graph and copies per milliliters. Then, we converted the results in term of logarithm which expressed the viral load values as a power of ten.

3.5 A performance evaluation of new type of filter papers

In general, the volume of a whole blood that used with a Whatman 903 filter paper was approximately 50 ul per circular spot (2 spots). In this section, we evaluated the performance of two new kinds of filter paper, nitrocellulose membrane and cellulose fiber sample pad, in order to absorb the whole blood under different volume (6.25, 12.5, 25.0 and 50.0 ul).

3.6 A testing of minimum viral load

We evaluated the limit of viral load that can be measured by diluting HIV CM244 (27,000 copies/ml) with normal whole blood. The values of viral load in this evaluation were 4,500 copies/ml, 2,250 copies/ml, 1,125 copies/ml, 562 copies/ml, 281 copies/ml and 140 copies/ml. A fifty microliters of whole blood was dropped onto the filter paper, 2 spots seperately. The filter paper was dried over night at room temperature and stored in a zipper bag in -20 °C.

3.7 Effect of time of dried blood storage

Dried blood spot was determined an interval time of storage by detecting the level of viral load which kept in 1 - 5 days. We used 27,000 copies/ml of CM244 and diluted by normal whole blood to obtain 562 copies/ml. Then, sample was dropped into 5 sets of Whatman 903 filter paper. Each set was extracted and detected within difference day form 1-5 days.

3.8 Sample size calculation

Sample size was calculated by using the results of HIV viral load detection from pilot study. Fifteen samples were randomly selected from HIV infected patients at Health Sciences Service Unit, the Faculty of Allied Health Sciences, Chulalongkorn University. The samples for this study were calculated by the sample size formula:

$$n = \left[\frac{(Z_{\alpha/2} + Z_\beta)\sigma}{\Delta}\right]^2$$

where

α is a type I error which is fixed as 0.05,

β is a type II error which is fixed as 0.02,

Δ is mean difference $(\mu_2 - \mu_1)$,

σ is a standard deviation of mean difference.

It should be noted that $Z_{\alpha/2}, Z_\beta$ power were fixed as 1.96, 0.84 and 80%, respectively. The value of $Z_{\alpha/2}$ can be seen from two-tailed table.

3.9 HIV drug resistance assay (ViroSeqTM HIV-1 Genotyping system method) (Applied Biosystems, USA)

The samples which have level of HIV viral load more than 2,000 copies/ml were selected to determine HIV drug resistance assay by using ViroSeq™ HIV-1 Genotyping system method kit (Applied Biosystems, USA) followed by;

3.9.1 Reverse transcription of *pol* gene

For synthesized cDNA, a total volume of 10.4 ul of RT Mastermix was prepared by using 8 μl of HIV RT mix, 1 μl of RNase Inhibitor, 1 μl of Moloney murine leukemia virus (MuLV) reverse transcriptase and 0.4 μl of DTT (100mM). Then, 10 μl of RNA sample was added into a MicroAmp reaction tube and the tube was inserted into a thermalcycler and set a condition at 65°C for 30 seconds. Then, 10 μl of RT master mix was added into each reaction tube and incubated for 42°C for 65 minutes. Finally, the reaction of the RT Mastermix solution was terminated by heating the tube at 99°C for 5 minutes.

3.9.2 Amplification of HIV-1 *pol* gene

After terminate the reaction of RT mastermix solution, the microamp reaction tube was then performed PCR by using cDNA, as a template. Then, the double-stranded DNA that suits for a sequencing was generated. The PCR primers amplified the HIV protease and the 5´ end of the reverse transcriptase (RT) gene. A single-stranded cDNA template which was generated from reverse transcription was used to amplify the protease (PR) and reverse transcriptase (RT) region of *pol* gene (1.8 kb). The PCR master mix solution was prepared as followed; 29.5 ul of HIV PCR mix, 0.5 ul of AmpliTaq Gold polymerase and 1 ul of Amperase UNG for each reaction. The

temperature profile for the polymerase chain reaction (PCR) included 50ºC for 10 minutes and 93ºC for 12 minutes followed by first hold of 40 cycles of denaturation (93ºC, 20 seconds), annealing (64ºC, 45 seconds), and extension (66ºC, 3 minutes) with final extension of 72ºC for 10 minutes.

3.9.3 Gel electrophoresis of PCR products

The amplified PCR product of *pol* gene was detected and roughly estimated the DNA concentrate by using gel electrophoresis. 1% of agarose (ISC Bioexpress, Spain) was prepared in 0.5X TBE buffer (Wisent Inc,Canada). One kb DNA mass ladder was used as a maker to adjust the size of PCR product and DNA concentration by loading 6 ul and 3 ul into the first and second hole, respectively. Mix 5 ul of the PCR product with 5 ul of loading buffer and pour it into the next hole. Then, gel electrophoresis machine was run at 150 volts for 1 hour and stained with gel red for 10 minutes. After that, examined the gel by UV light by using gel documentation machine and recorded the gel image by using digital camera.

3.9.4 PCR products purification

A 1800 basepair PCR product of *pol* genes was purified by the PureLink^{TM} Quick PCR Purification kit (Invitrogen, Germany). To begin with, 40 μL of PCR product was mixed with 160 μL B2 (1:4) until it's homogenized. The mixture was applied to PureLink^{TM} spin column and centrifuged at $10,000 \times g$ for 1 minute. Then, discard the flow though and place the spin column into collection tube. Then, 650 μL of W1 was added into the spin column and centrifuged at $10,000 \times g$ for 1 minute. Discard the flow though and place the spin column into collection tube. Then, the spin column was dried by centrifuging at $10,000 \times g$ for 2-3 minutes in order to remove washed residual buffer in the column. Discard the collection tube and place the spin column in PureLink^{TM} elution tube. The purified PCR product was eluted by adding 50 μL elution buffer and incubating for 1 minute. Then, the spin column was centrifuged at $10,000 \times g$ for 1 minute. Finally, DNA was stored in -20°C. The DNA concentrations of the purified PCR products were measured by using the ND 1000 Spectrophotometer (NanoDrop Technologies, USA).

3.9.5 Nucleotide sequencing of HIV-1 *pol* gene

DNA sequencing was performed by using premixed BigDye terminator sequencing reagents with six different primers (A, B, C, F, G, and H). In order to perform sequencing, a MicroAmp Optical 96-well reaction plate is prepared by adding 12 ul of HIV SEQ Mix A, B, C, F, G and H into the plate on the position 1A-1G. Purified sample was loaded 8 ul into a hole 1A to hole 1G. The lid of MicroAmp Optical 96-well reaction plate was closed by MicroAmp 96-Well Full Plate Cover. The plate was inserted into a thermalcycler. Conditions for cycle sequencing (25 cycles) were 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.

3.9.6 Purification sequencing reaction

Ethanol precipitation was performed to remove dye from the sequencing reaction. Firstly, 60 μl of isopropanol and 20 μl of DNAse free water were loaded into a MicroAmp Optical 96-well reaction well and incubated plate at room temperature for 15 minutes in dark. Then, the plate was centrifuged for 45 minutes at 2,000 RCF. The supernatant were discarded and re-suspended precipitated sequencing products by adding 20 μl of Hi-Di formamide to each well and mixed. The plate was covered by reservoir septa and placed to automated ABI 3130 Genetic Analyzers (Applied Biosystems, USA) machine for sequencing and detecting the signals.

3.9.7 Sequence data analysis of *pol* gene

Sequencing data was analyzed by using Celera Diagnostics ViroSeq HIV-1 Genotyping System software (version 2.8), where sequencing data from the primers were assembled into a contiguous sequence that can be inspected to identify the mutation of drug resistance. Processed sequences include the region coding for protease amino acids 1 to 99 and reverse transcriptase (RT) amino acids 1 to 335 (full-length sequences). HXB2 was used as a reference nucleotides (2253 to 2549 for protease and 2550 to 3554 for reverse transcriptase (RT)).

3.9.8 Drug resistance interpretation

Sequence was submitted to HIVdb program in the HIV drug resistance database from Stanford University (http://hivdb.stanford.edu/), as a result, it returned the mutation patterns and the inferred levels of resistance to FDA-approved antiretroviral

drugs consist of 8 protease inhibitors (PIs) include atazanavir/r (ATV/r), darunavir/r (DRV/r), fosamprenavir/r (FPV/r), indinavir/r (IDV/r), lopinavir/r (LPV/r), nelfinavir (NFV), saquinavir/r (SQV/r) and tipranavir/r (TPV/r) ,7 nucleoside reverse transcriptase inhibitors (NRTIs) include lamivudine (3TC), abacavir(ABC), zidovudine (AZT), stavudine (D4T), didanosine (DDI), emtricitabine (FTC), tenofovir (TDF) and 4 non-nucleoside reverse transcriptase inhibitors (NNRTIs) include efavirenz (EFV), etravirine (ETR), nevirapine (NVP) and rilpivirine (RPV).

3.10 Statistical analysis

We used Person's correlation for investigation a relationship between 2 groups, Bland-Altman plot for assess agreement between two methods, One way repeated measure (ANOVA) and Wilcoxon signed-rank test for compare mean of HIV viral load.
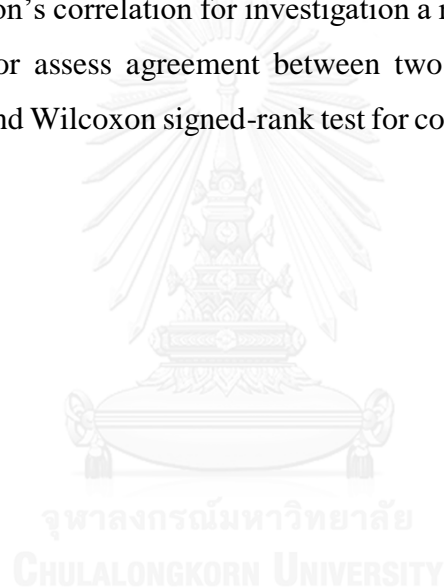
3.11 Instruments

	Instrument	Manufacturer	Country
1.	Biosafty carbonate Class II UNIFLOW UVUB 1200	Uniequip	Germany
2.	NucliSENeasyMAG®	Biomerieux	France
3.	NucliSENEasyQ®Analyze	Biomerieux	France
4.	NucliSENEasyQ®Incubater	Biomerieux	France
5.	NucliSENEasyQ®Mini-strip centrifuge	Biomerieux	France
6.	3130 Avant Genetic Analyzers	Applied Biosystems	USA
7.	Horizontal gel eletrophoresis	Toyobo	Japan
8.	Gel documentation	Alpha Innotec	Germany
9.	Thermal cycle 9700	ABI	USA
10.	IEC multi centrifuge	Thermo Electron Corporation	USA
11.	Vortex genie 2	Scientific Industries	USA
12.	Shaker VRN-360	Gemmy Indudtrial	Taiwan
13.	ThermoMixer	Eppendorf	USA
14.	Spectrafuge™ 24D Microcentrifuge	Labnet International	USA
15.	KOKUSAN Centrifuge H-11n	Euroscan	Thailand

16.	Refrigerator 4℃ Whirlpool 816xx	Whirlpool	Thailand
17.	Refrigerator 4℃ Sanden Intercool SEC-1000SBD	Sanden Intercool	Thailand
18.	Freezer -20 ℃ Sharp FC-28U	Sharp	Thailand
19.	Freezer -80 ℃ REVCO ULT-2090-7VBA	Thermo Scientific	USA

3.12 Equipment

	Equipment	Manufacturer	Country
1.	Whatman 903 paper	GE Healthcare	UK
2.	Nitrocellulose membrane	Sigma-Aldrich	Singapore
3.	Cellulose fiber Sample pads 20 mm*300mm	Merck Millipore Corporation	Germany
4.	Autopipette (10,20,200,1000 ul)	Ranin	USA
5.	Pipette tip (10,20,200,1000 ul)	Ranin	USA
6.	Serological pipette (1,5,10 ml)	Axygen Scientific Inc	USA
7.	Accu-jet pro Pipette Controller	BrandTech Scientific Inc	USA
8.	Tube 5 ml	Axygen Scientific Inc	USA
9.	Tube 1.5 ml	Quality Scientific	USA
10.	Tube 0.2 ml	Sorenson Bioscience Inc	USA
11.	Eppendorf tube	Eppendorf	USA
12.	NucliSENeasyMAG®vessel	Biomerieux	France
13.	Biohit pipette	Biomerieux	France
14.	NucliSENEasyQ®0.2 polyethylene micro tube	Biomerieux	France
15.	NucliSENEasyQ®0.2 polyethylene micro cap	Biomerieux	France

16.	MicroAmp reaction tube	Abbott	USA
17.	MicroAmp Optical 96-well reaction plate	Abbott	USA
18.	MicroAmp 96-Well Full Plate Cover	Abbott	USA
19.	Reservoir septa	Abbott	USA
20.	KIMTECH SCIENCE* KIMWIPES* Delicate Task Wipes	Kimberly-Clark	Thailand
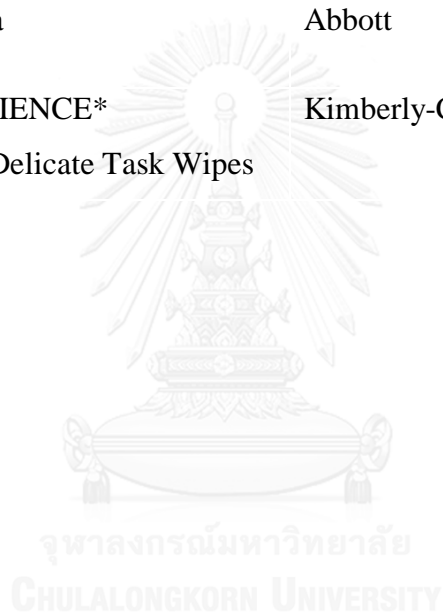
3.13 Material

	Material	Manufacturer	Country
1.	Viro-Adembead	Ademtech	France
2.	Gold nanoparticle 10 nm diameter, OD 1, stabilized suspension in citrate buffer	Sigma-Aldrich	Singapore
3.	Gold nanoparticle 20 nm diameter, OD 1, stabilized suspension in citrate buffer	Sigma-Aldrich	Singapore
4.	Gold nanoparticle 40 nm diameter, OD 1, stabilized suspension in citrate buffer	Sigma-Aldrich	Singapore
5.	NucliSENeasyMAG® Kit	Biomerieux	France
6.	NucliSENEasyQ® HIV-1 v2.0 Kit	Biomerieux	France
7.	ViroSeqTM HIV-1 Genotyping system	Applied Biosystems	USA
8.	PureLinkTM Quick PCR Purification kit	Invitrogen	Germany
9.	DNase RNase free water	Gibco	Germany
10.	Genepure LE Agarose	ISC Bioexpress	Spain
11.	TBE buffer	Wisent Inc	Canada

12.	Gel red	Biotium	USA
13.	Absolute Ethanol	RCI Labscan	Thailand
14.	Isopropanol	RCI Labscan	Thailand
15.	Fetal Bovine serum	Gibco	Germany
16.	RPMI media 1640(1X)	Gibco	Germany

CHAPTER IV
RESULTS

4.1 A performance of virus capturing of viro-adembeads

In this section, we investigated the ability of virus capturing when apply to the dried blood spot. The type of sample was CM244 (27,000 copies/ml) which was dilute to 2,000 copies/ml by using RPMI media 1640 with 2% of Fetal Bovine serum. The conditions of the testing consisted of 1. Mix the viro-adembead to the sample before drop onto the filter paper 2. Drop the viro-adembead on the filter paper before dropping the sample 3. Suck the viro-adembead out before extracting the genetic materials 4. Suck the viro-adembead out before extracting the genetic materials 5. Reduce a volume of viro-adembead 6. Wash the viro-adembead after virus capturing 7. Wash the viro-adembead during genetic materials extraction. The results of these seven conditions showed in Table 4-1. When mix the viro-adembead to the sample before drop onto the filter paper or suck the viro-adembead out before extracting the genetic materials can increased the level of viral load but duplicate sample showed invalid. Others conditions showed invalid. As a result, not only the viro-adembead did not increase the sensitivity of virus capturing, it interferedthe interaction of NucliSENeasyQ solution in order to detect the HIV viral load. Therefore, we do not recommend to use the viro-adembead to detect the viral load in this research.

Table 4-1 A performance of HIV viral load detection using Whatman 903 with viro-adembeads under various condition.

Conditions	Sample	HIV Viral load	
		copies/ml	log
1. Mix the viro-adembead to the sample before drop onto the filter paper	CM244	900	2.95
	Media with Adembead	Target not detected	Target not detected
	Media with Adembead	Invalid	Invalid
	CM244 with Adembead	1,100	3.04
	CM244 with Adembead	Invalid	Invalid
2. Drop the viro-adembead on the filter paper before dropping the sample	CM244	1,000	3.00
	Adembead -> Media	Target not detected	Target not detected
	Adembead -> Media	Invalid	Invalid
	Adembead -> CM244	Invalid	Invalid
	Adembead -> CM244	Invalid	Invalid
3. Suck the viro-adembead out before extracting the genetic materials	CM244	650	2.81
	Media with Adembead	Target not detected	Target not detected
	CM244 with Adembead	3,100	3.50
	CM244 with Adembead	Target not detected	Target not detected
4. Suck the viro-adembead out before extracting the genetic materials	CM 244	650	2.81
	Adembead -> Media	Invalid	Invalid
	Adembead -> CM244	<100	<2.00
	Adembead -> CM244	Invalid	Invalid
5. Reduce a volume of viro-adembead	CM244	860	2.93
	Media with Adembead	Invalid	Invalid
	CM244 with Adembead	Invalid	Invalid
	CM244 with Adembead	Invalid	Invalid
6. Wash the viro-adembead after virus capturing	CM244	860	2.93
	Media with Adembead	Target not detected	Target not detected
	CM244 with Adembead	Target not detected	Target not detected
	CM244 with Adembead	Target not detected	Target not detected
7. Wash the viro-adembead during genetic materials extraction	CM244 , 2 Wash	1,100	3.04
	CM244, 3 Wash	520	2.72
	CM244 with Adembead, 2 Wash	Invalid	Invalid
	CM244 with Adembead, 2 Wash	Invalid	Invalid
	CM244 with Adembead, 3 Wash	Invalid	Invalid
	CM244 with Adembead, 3 Wash	Invalid	Invalid

4.2 A testing of an appropriate size and volume of gold nanoparticles.

In this section, we investigated the appropriate size and volume of gold nanoparticles in order to be use in this research. In this investigation, we compare the viral load detection performance of Whatman 903 filter paper under different volume (6.25 ul, 12.5 ul, 25 ul and 50 ul) and size (10 nm, 20 nm and 40 nm) of gold nanoparticle. From the results, the viral load was greatly detected when the value of gold's size and volume are 20 nm and 50 ul, respectively. Then, the level viral load detected was 1,650 copies/ml (Table 4-2).

Table 4-2 A performance of HIV viral load detection using Whatman 903 with various volume and size of gold nanoparticle.

Size (nm)	Volume (ul)	HIV Viral Load		Average	
		copies/ml	log	copies/ml	log
10 nm	50 ul	960	2.98	880	2.94
	50 ul	800	2.90		
	25 ul	1,100	3.04	1,010	3.00
	25 ul	920	2.96		
	12.5 ul	390	2.59	505	2.70
	12.5 ul	620	2.79		
	6.25 ul	330	2.52	380	2.58
	6.25 ul	430	2.63		
20 nm	50 ul	1,500	3.18	1,650	3.22
	50 ul	1,800	3.26		
	25 ul	680	2.83	715	2.85
	25 ul	750	2.88		
	12.5 ul	650	2.81	580	2.76
	12.5 ul	510	2.71		
	6.25 ul	1,300	3.11	800	2.90
	6.25 ul	300	2.48		
40 nm	50 ul	340	2.53	565	2.75
	50 ul	790	2.90		
	25 ul	1,100	3.04	700	2.85
	25 ul	300	2.48		
	12.5 ul	980	2.99	950	2.98
	12.5 ul	920	2.96		
	6.25 ul	660	2.82	540	2.73
	6.25 ul	420	2.62		

4.3 A performance evaluation of new type of filter papers

In this section, we evaluated the performance of two new kind of filter paper, nitrocellulose membrane and cellulose fiber sample pad, in order to absorb the whole blood under different volume. The results showed that nitrocellulose membrane did not capable of absorbing the whole blood at any volume as showed in Table 4-3. Then, it cannot be used to store a dried blood spot. To achieve our objective, which was to find a high viral load detection performance and low cost filter paper, we compared

nitrocellulose membrane with a cellulose fiber sample pad. As a result, the cellulose fiber sample pad capable of absorbing a whole blood for all volume as showed in Table 4-3. Then, we compared the performance of viral load detection of cellulose fiber sample pad with Whatman 903 filter paper. A volume of whole blood that is used in this research was 50 ul per filter paper spot.

Table 4-3 A performance of whole blood absorbing using nitrocellulose membrane and cellulose fiber sample pad under different whole blood's volume.

Volume of whole blood	Types of filter paper	
	Nitrocellulose membrane	Cellulose fiber sample pad
50 ul	Cannot absorb	Can absorb
25 ul	Cannot absorb	Can absorb
12.5 ul	Cannot absorb	Can absorb
6.25 ul	Cannot absorb	Can absorb

4.4 A testing of minimum viral load

We evaluated the limit of viral load by diluting HIV CM244 (27,000 copies/ml) with Normal Whole blood. The values of viral load in this evaluation were load 4,500 copies/ml, 2,250 copies/ml, 1,125 copies/ml, 562 copies/ml, 281 copies/ml and 140 copies/ml. The experimental results were shown in Table 4-4. As a result, we found that the limit of viral load that can be measured was approximately 281 copies/ml and this was used as a lower of detected viral load in this research.

Table 4-4 A performance of minimal HIV viral load detection using Whatman 903.

HIV Viral load (from calculation) copies/ml	Detected HIV Viral load by Whatman 903		Average detected HIV Viral load by Whatman 903	
	copies/ml	log	copies/ml	log
4,500	2,300	3.36	2600	3.41
	2,900	3.46		
2,250	1,300	3.11	1550	3.18
	1,800	3.26		
1,125	580	2.76	460	2.65
	340	2.53		
562	220	2.34	215	2.33
	210	2.32		
281	230	2.36	165	2.18
	100	2.00		
140	Target not detected	Target not detected	Target not detected	Target not detected
	Target not detected	Target not detected		

4.5 Effect of time of dried blood storage

As shown in Table 4-5, a detected viral load at the 1-5 days were not different. A log of error between these values was ± 0.5 log that was acceptable. [63, 64]

Table 4-5 A performance of HIV viral load detection by using Whatman 903 when dried blood spots were stored during 1 to 5 days.

Days	HIV Viral Load	
	copies/ml	log
Day 1	330	2.52
Day 2	360	2.56
Day 3	420	2.62
Day 4	510	2.71
Day 5	430	2.63

4.6 A sample size based on Whatman 903 filter paper.

In this section, we calculated sample size by using pilot studies. We detected the viral load of 15 samples by using Whatman 903 and Whatman 903 with gold nanoparticles. The size and volume of gold nanoparticles were 20 nm and 50 ul, respectively.

Table 4-6 A pilot studies of HIV viral load detection from 15 samples using Whatman 903 and Whatman 903 with gold nanoparticles.

No.	HIV Viral load (copies/ml)		
	Whatman 903		
	Without AuNPs	With AuNPs	Mean Difference
1	550	610	-60
2	120	120	0
3	100	100	0
4	1,400	1,400	0
5	110	100	10
6	1,700	1,600	100
7	5,500	5,300	200
8	910	690	220
9	9,100	6,600	2,500
10	14,000	11,000	3,000
11	21,000	16,000	5,000
12	1,400,000	1,300,000	100,000
13	270,000	140,000	130,000
14	480,000	330,000	150,000
15	2,500,000	1,700,000	800,000

The results of viral load from Whatman 903 filter paper and Whatman 903 filter paper with gold nanoparticles as shown in Table 4-6. Then, we calculated the number of sample size (n) and n was given by

$$n = \left[\frac{(Z_{\alpha/2}+Z_{\beta})\sigma}{\Delta}\right]^2$$

where

α is a type I error which is fixed as 0.05,

β is a type II error which is fixed as 0.02,

Δ is mean difference $(\mu_2 - \mu_1)$,

σ is a standard deviation of mean difference.

It should be noted that $Z_{\alpha/2}$, Z_{β}, power were fixed as 1.96, 0.84 and 80%, respectively. The value of $Z_{\alpha/2}$ can be seen from two-tailed table. From the Table4-6, mean difference was 79,398 and standard deviation of mean difference was 206,134. Therefore, we found that the number of sample was 52.84 or approximately 53.

4.7 Results of HIV viral load detection

After we investigated the ability of absorption of three filter papers, we found that only Whatman 903 and cellulose fiber sample pad were capable of absorbing the dried blood spot. We used gold nanoparticle (20 nm in size, 50 ul in volume) and Whatman 903 and cellulose fiber sample pad for viral load detection. The results were shown in Table 4-7 and Table 4-8 and performed statistical analysis.

Table 4-7 HIV Viral load detection under difference types of filtter paper (copies/ml).

Viral load range (copies/ml)	sample	HIV Viral load (copies/ml)			
		Whatman 903		Cellulose fiber sample pad	
		Without AuNPs	With AuNPs	Without AuNPs	With AuNPs
Below 2,000	013	100	100	110	100
	026	100	100	100	100
	044	100	150	200	210
	053	100	120	270	480
	024	110	100	100	140
	007	120	120	320	290
	005	150	480	370	500
	057	200	160	470	1,500
	066	230	190	240	260
	069	470	580	340	300
	027	540	1,400	1,300	1,400
	023	550	610	100	100
	031	630	580	1,800	2,000
	051	740	450	1,800	710
	063	850	930	560	390
	016	870	1,600	1,600	960
	006	910	690	920	530
	061	1,200	1,300	1,500	720
	040	1,300	1,000	1,300	2,700
	025	1,400	1,400	3,500	1,600
	030	1,600	1,200	2,300	740
	001	1,700	1,600	1,700	1,400
	004	1,800	2,800	1,400	2,500
2,001 – 10,000	002	2,000	2,500	4,100	1,800
	058	2,000	1,000	1,100	1,500
	021	2,600	2,900	2,100	2,900
	037	2,600	5,600	7,400	5,200
	020	4,800	7,000	6,500	6,300
	008	5,500	5,300	6,600	5,900
	068	5,500	2,100	1,400	8,000
	032	7,900	13,000	10,000	10,000
	010	9,100	6,600	10,000	5,800
	035	9,900	5,200	4,100	3,400
	065	9,900	6,000	6,900	6,300

Table 4-7 HIV Viral load detection under difference types of filtter paper (copies/ml). (Cont.)

Viral load range (copies/ml)	sample	HIV Viral load (copies/ml)			
		Whatman 903		Cellulose fiber sample pad	
		Without AuNPs	With AuNPs	Without AuNPs	With AuNPs
10,001–100,000l	038	12,000	12,000	23,000	14,000
	012	14,000	11,000	17,000	15,000
	022	14,000	52,000	22,000	23,000
	011	15,000	18,000	14,000	20,000
	067	17,000	16,000	9,100	20,000
	070	17,000	25,000	17,000	24,000
	017	21,000	16,000	23,000	26,000
	049	24,000	25,000	23,000	30,000
	043	30,000	34,000	20,000	45,000
	056	32,000	27,000	14,000	31,000
	042	34,000	47,000	49,000	51,000
	045	35,000	46,000	42,000	53,000
	046	35,000	32,000	21,000	34,000
	039	39,000	45,000	54,000	55,000
	050	46,000	41,000	66,000	39,000
	033	52,000	86,000	54,000	74,000
	041	65,000	26,000	66,000	44,000
	047	69,000	86,000	76,000	69,000
	054	89,000	110,000	69,000	140,000
100,001–1,000,000	048	110,000	61,000	96,000	71,000
	055	140,000	200,000	200,000	120,000
	062	140,000	67,000	120,000	100,000
	009	150,000	360,000	120,000	350,000
	003	270,000	140,000	140,000	220,000
	059	280,000	260,000	380,000	220,000
	028	330,000	350,000	340,000	310,000
	060	370,000	390,000	270,000	430,000
	018	390,000	410,000	200,000	230,000
	014	480,000	330,000	350,000	370,000
	029	490,000	760,000	1,000,000	600,000
Higher than 1,000,00	034	1,100,000	1,100,000	1,500,000	1,400,000
	015	1,400,000	1,300,000	1,400,000	1,300,000
	064	1,900,000	2,200,000	1,500,000	1,600,000
	019	2,500,000	1,700,000	2,400,000	1,700,000
	036	2,700,000	2,400,000	3,000,000	2,600,000
	052	3,900,000	4,300,000	3,500,000	3,500,000

Table 4-8 HIV Viral load detection under difference types of filtter paper (log).

Viral load range (copies/ml)	Sample	HIV Viral load (log)			
		Whatman 903		Cellulose fiber sample pad	
		Without AuNPs	With AuNPs	Without AuNPs	With AuNPs
Below 2,000	013	2.00	2.00	2.04	2.00
	026	2.00	2.00	2.00	2.00
	044	2.00	2.18	2.30	2.32
	053	2.00	2.08	2.43	2.68
	024	2.04	2.00	2.00	2.15
	007	2.08	2.08	2.51	2.46
	005	2.18	2.68	2.57	2.70
	057	2.30	2.20	2.67	3.18
	066	2.36	2.28	2.38	2.41
	069	2.67	2.76	2.53	2.48
	027	2.73	3.15	3.11	3.15
	023	2.74	2.79	2.00	2.00
	031	2.80	2.76	3.26	3.30
	051	2.87	2.65	3.26	2.85
	063	2.93	2.97	2.75	2.59
	016	2.94	3.20	3.20	2.98
	006	2.96	2.84	2.96	2.72
	061	3.08	3.11	3.18	2.86
	040	3.11	3.00	3.11	3.43
	025	3.15	3.15	3.54	3.20
	030	3.20	3.08	3.36	2.87
	001	3.23	3.20	3.23	3.15
	004	3.26	3.45	3.15	3.40
2,001 – 10,000	002	3.30	3.40	3.61	3.26
	058	3.30	3.00	3.04	3.18
	021	3.41	3.46	3.32	3.46
	037	3.41	3.75	3.87	3.72
	020	3.68	3.85	3.81	3.80
	008	3.74	3.72	3.82	3.77
	068	3.74	3.32	3.15	3.90
	032	3.90	4.11	4.00	4.00
	010	3.96	3.82	4.00	3.76
	035	4.00	3.72	3.61	3.53
	065	4.00	3.78	3.84	3.80

Table 4-8 HIV Viral load detection under difference types of filtter paper (log).
(Cont.)

Viral load range (copies/ml)	Sample	HIV Viral load (log)			
		Whatman 903		Cellulose fiber sample pad	
		Without AuNPs	With AuNPs	Without AuNPs	With AuNPs
10,001–100,000l	038	4.08	4.08	4.36	4.15
	012	4.15	4.04	4.23	4.18
	022	4.15	4.72	4.34	4.36
	011	4.18	4.26	4.15	4.30
	067	4.23	4.20	3.96	4.30
	070	4.23	4.40	4.23	4.38
	017	4.32	4.20	4.36	4.41
	049	4.38	4.40	4.36	4.48
	043	4.48	4.53	4.30	4.65
	056	4.51	4.43	4.15	4.49
	042	4.53	4.67	4.69	4.71
	045	4.54	4.66	4.62	4.72
	046	4.54	4.51	4.32	4.53
	039	4.59	4.65	4.73	4.74
	050	4.66	4.61	4.82	4.59
	033	4.72	4.93	4.73	4.87
	041	4.81	4.41	4.82	4.64
	047	4.84	4.93	4.88	4.84
	054	4.95	5.04	4.84	5.15
100,001–1,000,000	048	5.04	4.79	4.98	4.85
	055	5.15	5.30	5.30	5.08
	062	5.15	4.83	5.08	5.00
	009	5.18	5.56	5.08	5.54
	003	5.43	5.15	5.15	5.34
	059	5.45	5.41	5.58	5.34
	028	5.52	5.54	5.53	5.49
	060	5.57	5.59	5.43	5.63
	018	5.59	5.61	5.30	5.36
	014	5.68	5.52	5.54	5.57
	029	5.69	5.88	6.00	5.78
Higher than 1,000,00	034	6.04	6.04	6.18	6.15
	015	6.15	6.11	6.15	6.11
	064	6.28	6.34	6.18	6.20
	019	6.40	6.23	6.38	6.23
	036	6.43	6.38	6.48	6.41
	052	6.59	6.63	6.54	6.54

4.8 Statistic Analysis of HIV Viral load

In this study, 70 EDTA dried blood spot samples were separated into 5 groups (below 2,000, 2,001-10,000, 10,001-100,00, 100,001-1,000,000 and more than 1,000,001 copies/ml) which the number of sample in each group were 23, 11, 19, 11 and 6 respectively. The results showed that Whatman 903, Whatman 903 with gold nanoparticle, cellulose fiber sample pad and cellulose fiber sample pad with gold nanoparticle had mean log ± SD 4.05 ± 1.26, 4.06 ± 1.25, 4.08 ± 1.22, 4.09 ±1.22, respectively.

4.8.1 Investigation a relationship between 2 groups

Pearson's correlation coefficient was a statistical measure of the strength of a linear relationship between paired data.
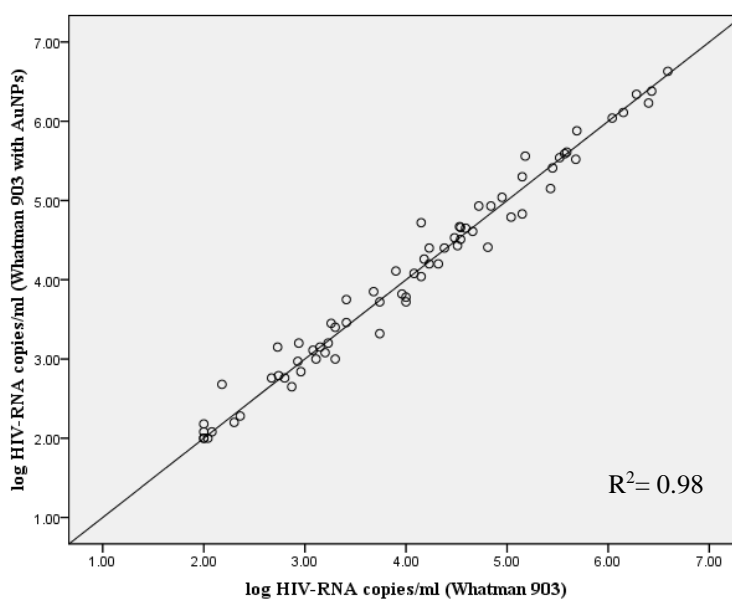
Figure 4-1 Linear regression comparing viral loads obtained with Whatman 903 and those obtained with Whatman 903 with gold nanoparticle.
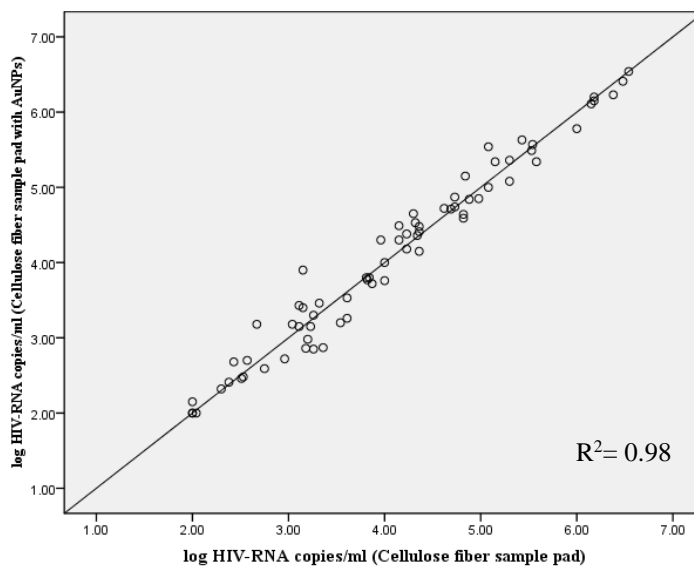
Figure 4-2 Linear regression comparing viral loads obtained with Cellulose fiber sample pad and those obtained with Cellulose fiber sample pad with gold nanoparticle.
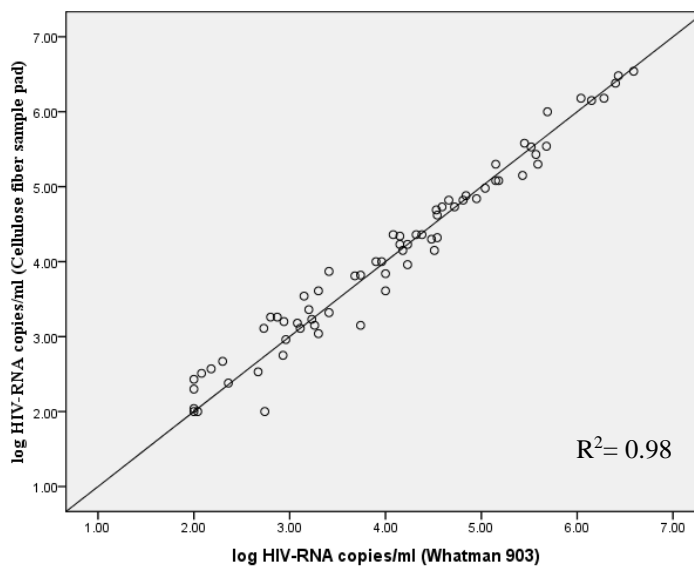
Figure 4-3 Linear regression comparing viral loads obtained with Whatman 903 and those obtained with Cellulose fiber sample pad.

The Figure 4-1 showed the correlation between the viral load determinations obtained with Whatman 903 and Whatman 903 with gold nanoparticle. The overall R^2 value was 0.99 (P<0.05).

The Figure 4-2 showed the correlation between the viral load determinations obtained with cellulose fiber sample pad and cellulose fiber sample pad with gold nanoparticle. The overall R^2 value was 0.98 (P<0.05).

The Figure 4-3 showed the correlation between the viral load determinations obtained with Whatman 903 and cellulose fiber sample pad. The overall R^2 value was 0.98 (P<0.05).

4.8.2 The Bland-Altman analysis

The Bland-Altman analysis was a process used to assess agreement between two methods of measurement.
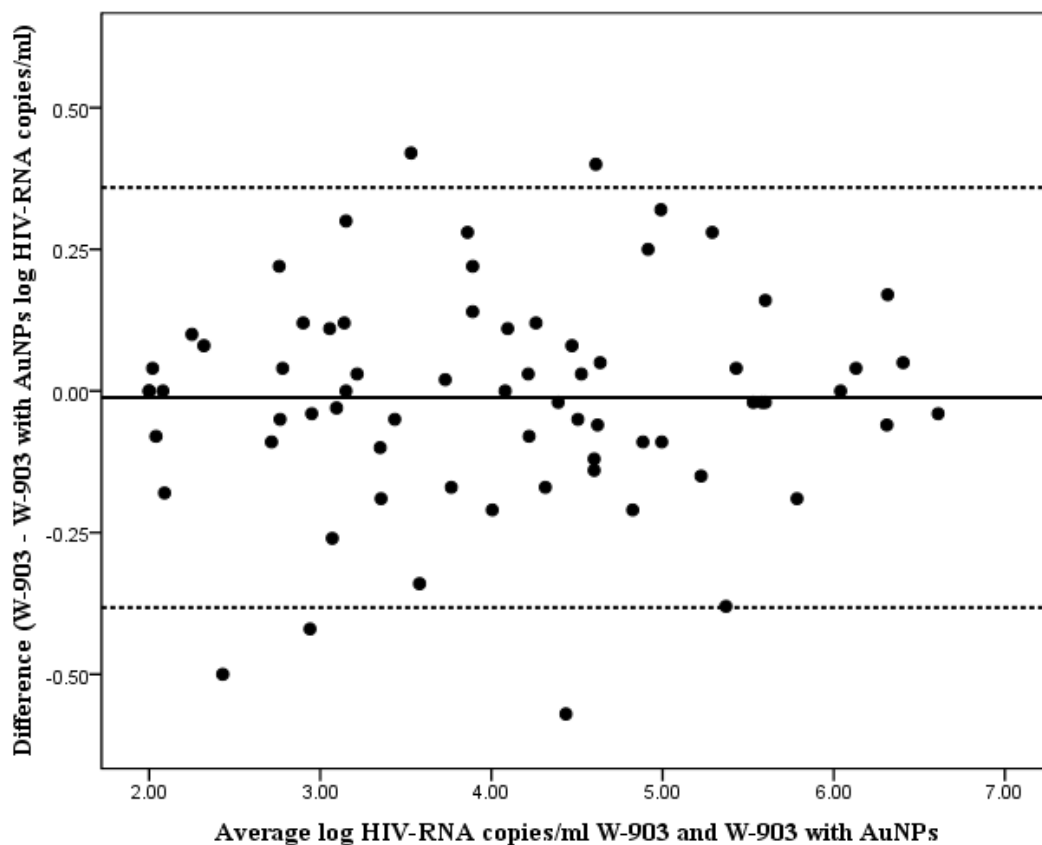
Figure 4-4 Bland-Altman plot of HIV viral load between Whatman 903 and Whatman 903 with gold nanoparticle.
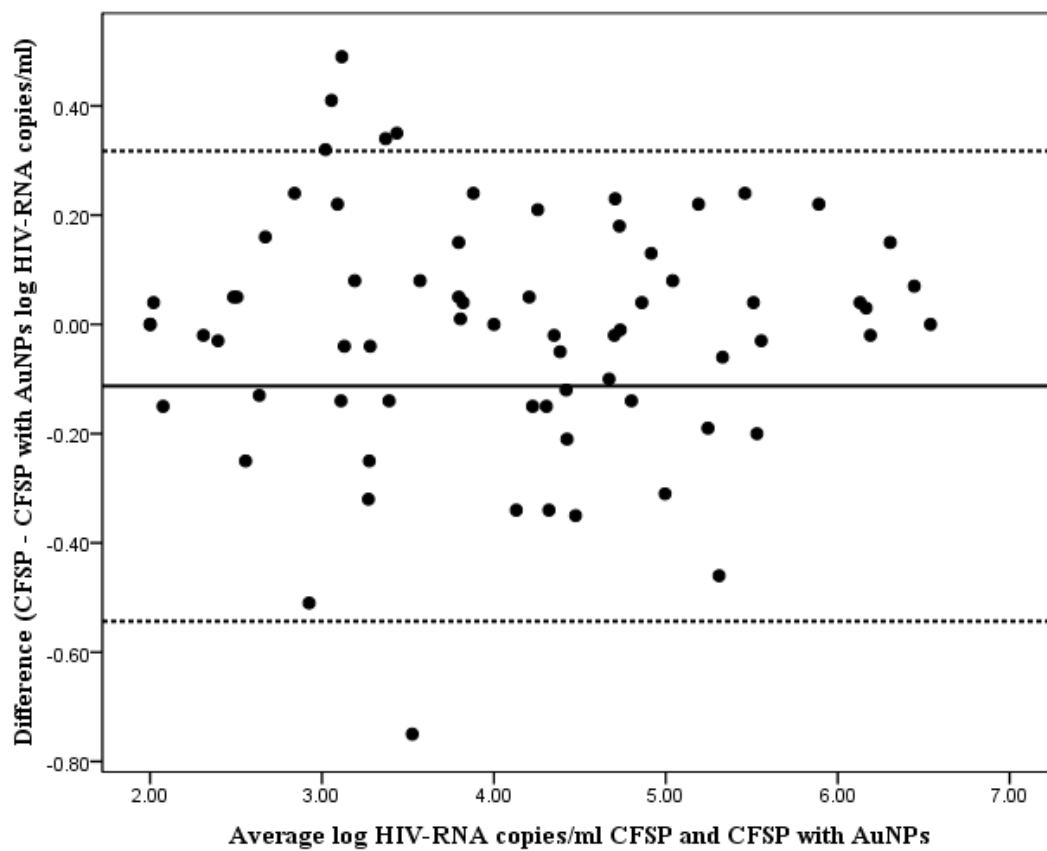
Figure 4-5 Bland-Altman plot of HIV viral load between cellulose fiber sample pad and cellulose fiber sample pad with gold nanoparticle.
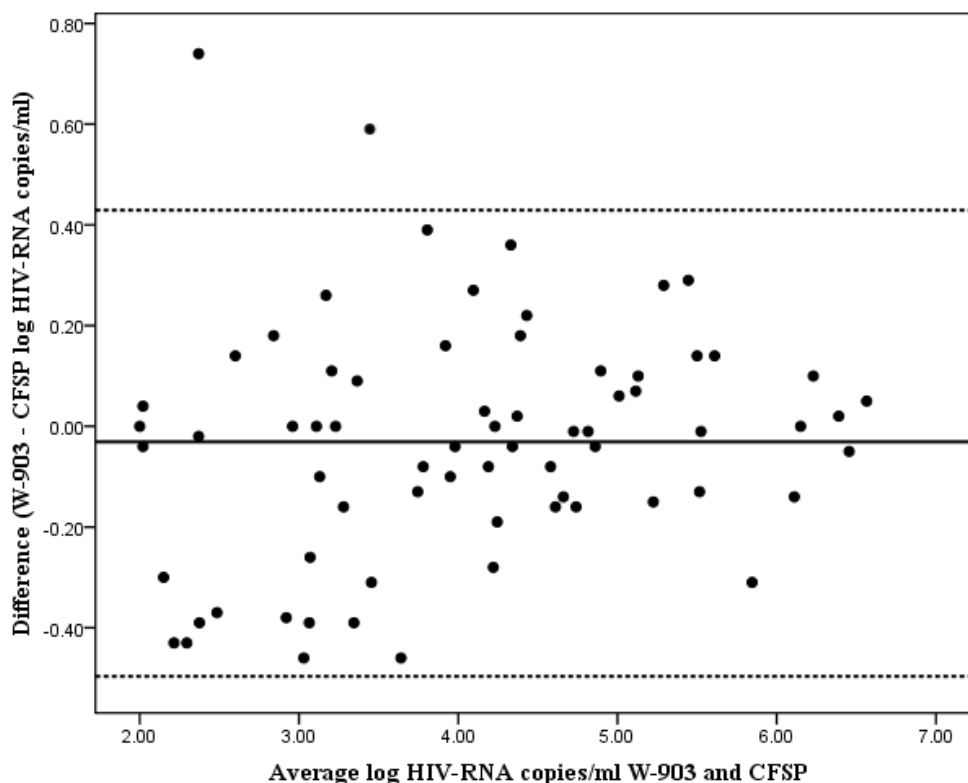
Figure 4-6 Bland-Altman plot of HIV viral load between Whatman 903 and cellulose fiber sample pad.

Figure 4-4 showed Bland-Altman analysis of viral load in Whatman 903 compared Whatman 903 with gold nanoparticle. The mean log copies/ml (± standard deviation) for Whatman 903 and Whatman 903 with gold nanoparticle were -0.01 ± 0.37. Mostly of the data points were within 95% limits of agreement.

Figure 4-5 showed Bland-Altman analysis of viral load in cellulose fiber sample pad compared cellulose fiber sample pad with gold nanoparticle. The mean log copies/ml (± standard deviation) for Whatman 903 and Whatman 903 with gold nanoparticle were -0.11 ± 0.43. Mostly of the data points were within 95% limits of agreement.

Figure 4-6 showed Bland-Altman analysis of viral load in Whatman 903 sample pad compared cellulose fiber sample pad. The mean log copies/ml (± standard deviation) for Whatman 903 and Whatman 903 with gold nanoparticle were -0.03 ±

0.47. Most of samples were within 95% limits of agreement, except only two samples were not.

4.8.3 One-way repeated measure (ANOVA)

One-way repeated measure (ANOVA) was used to compare means of four groups (Whatman 903, Whatman 903 with gold nanoparticle, cellulose fiber sample pad and cellulose fiber sample pad with gold nanoparticle). As shown in Table 4-9, the results showed that no significant (P>0.05) difference mean between four types of filters paper for all range of viral load.

Table 4-9 Difference means of four types filter paper by One-way repeated measure (ANOVA)

Viral load range (copies/ml)	F	P-value
Below 2,000	0.33	0.80*
2,001 – 10,000	0.05	0.99*
10,001–100,000l	0.42	0.74*
100,001–1,000,000	0.05	0.99*
Higher than 1,000,00	0.07	0.98*
All	0.02	1.00*

*P>0.05 no significant.

4.8.4 Wilcoxon signed-rank test

Wilcoxon signed-rank test was used to compare two groups. Aanalysis of Pearson's correlation coefficient and Bland-Altman analysis found that Whatman 903 and cellulose fiber sample pad were correlation and good agreement. The results of HIV viral load from Whatman 903 and cellulose fiber sample pad shown in Table 4-10. So, we chose two types (Whatman 903 and cellulose) of filter paper to analyze by Wilcoxon signed-rank test. The results showed that out of 37 from 70 samples (52.86%) had HIV viral load level of cellulose fiber sample pad more than Whatman 903 but no difference of mean (Z = -1.227 , P-value = 0.22) by Wilcoxon signed-rank test.

Table 4-10 HIV Viral load detection by Whatman 903 and cellulose fiber sample pad (copies/ml).

Viral load range (copies/ml)	sample	HIV Viral load (copies/ml)		
		Whatman 903	Cellulose fiber sample pad	Mean difference
Below 2,000	013	100	110	-10
	026	100	100	0
	044	100	200	-100
	053	100	270	-170
	024	110	100	10
	007	120	320	-200
	005	150	370	-220
	057	200	470	-270
	066	230	240	-10
	069	470	340	130
	027	540	1,300	-760
	023	550	100	450
	031	630	1,800	-1,170
	051	740	1,800	-1,060
	063	850	560	290
	016	870	1,600	-730
	006	910	920	-10
	061	1,200	1,500	-300
	040	1,300	1,300	0
	025	1,400	3,500	-2,100
	030	1,600	2,300	-700
	001	1,700	1,700	0
	004	1,800	1,400	400
2,001 – 10,000	002	2,000	4,100	-2,100
	058	2,000	1,100	900
	021	2,600	2,100	500
	037	2,600	7,400	-4,800
	020	4,800	6,500	-1,700
	008	5,500	6,600	-1,100
	068	5,500	1,400	4,100
	032	7,900	10,000	-2,100
	010	9,100	10,000	-900
	035	9,900	4,100	5,800
	065	9,900	6,900	3,000

Table 4-10 HIV Viral load detection by Whatman 903 and cellulose fiber sample pad (copies/ml) (Cont).

Viral load range (copies/ml)	sample	HIV Viral load (copies/ml)		
		Whatman 903	**Cellulose fiber sample pad**	**Mean difference**
10,001–100,000l	038	12,000	23,000	-11,000
	012	14,000	17,000	-3,000
	022	14,000	22,000	-8,000
	011	15,000	14,000	1,000
	067	17,000	9,100	7,900
	070	17,000	17,000	0
	017	21,000	23,000	-2,000
	049	24,000	23,000	1,000
	043	30,000	20,000	10,000
	056	32,000	14,000	18,000
	042	34,000	49,000	-15,000
	045	35,000	42,000	-7,000
	046	35,000	21,000	14,000
	039	39,000	54,000	-15,000
	050	46,000	66,000	-20,000
	033	52,000	54,000	-2,000
	041	65,000	66,000	-1,000
	047	69,000	76,000	-7,000
	054	89,000	69,000	20,000
100,001–1,000,000	048	110,000	96,000	14,000
	055	140,000	200,000	-60,000
	062	140,000	120,000	20,000
	009	150,000	120,000	30,000
	003	270,000	140,000	130,000
	059	280,000	380,000	-100,000
	028	330,000	340,000	-10,000
	060	370,000	270,000	100,000
	018	390,000	200,000	190,000
	014	480,000	350,000	130,000
	029	490,000	1,000,000	-510,000
Higher than 1,000,00	034	1,100,000	1,500,000	-400,000
	015	1,400,000	1,400,000	0
	064	1,900,000	1,500,000	400,000
	019	2,500,000	2,400,000	100,000
	036	2,700,000	3,000,000	-300,000
	052	3,900,000	3,500,000	400,000

Table 4-11 HIV Viral load detection by Whatman 903 and cellulose fiber sample pad (log).

Viral load range (copies/ml)	sample	HIV Viral load (copies/ml)		
		Whatman 903	Cellulose fiber sample pad	Mean difference
	013	2.00	2.04	-0.04
	026	2.00	2.00	0.00
	044	2.00	2.30	-0.30
	053	2.00	2.43	-0.43
	024	2.04	2.00	0.04
	007	2.08	2.51	-0.43
	005	2.18	2.57	-0.39
	057	2.30	2.67	-0.37
	066	2.36	2.38	-0.02
	069	2.67	2.53	0.14
Below 2,000	027	2.73	3.11	-0.38
	023	2.74	2.00	0.74
	031	2.80	3.26	-0.46
	051	2.87	3.26	-0.39
	063	2.93	2.75	0.18
	016	2.94	3.20	-0.26
	006	2.96	2.96	0.00
	061	3.08	3.18	-0.10
	040	3.11	3.11	0.00
	025	3.15	3.54	-0.40
	030	3.20	3.36	-0.16
	001	3.23	3.23	0.00
	004	3.26	3.15	0.11
	002	3.30	3.61	-0.31
	058	3.30	3.04	0.26
	021	3.41	3.32	0.09
	037	3.41	3.87	-0.45
2,001 – 10,000	020	3.68	3.81	-0.13
	008	3.74	3.82	-0.08
	068	3.74	3.15	0.59
	032	3.90	4.00	-0.10
	010	3.96	4.00	-0.04
	035	4.00	3.61	0.38
	065	4.00	3.84	0.16

Table 4-11 HIV Viral load detection by Whatman 903 and cellulose fiber sample pad (log) (Cont).

Viral load range (copies/ml)	sample	HIV Viral load (copies/ml)		
		Whatman 903	**Cellulose fiber sample pad**	**Mean difference**
10,001–100,000l	038	4.08	4.36	-0.28
	012	4.15	4.23	-0.08
	022	4.15	4.34	-0.20
	011	4.18	4.15	0.03
	067	4.23	3.96	0.27
	070	4.23	4.23	0.00
	017	4.32	4.36	-0.04
	049	4.38	4.36	0.02
	043	4.48	4.30	0.18
	056	4.51	4.15	0.36
	042	4.53	4.69	-0.16
	045	4.54	4.62	-0.08
	046	4.54	4.32	0.22
	039	4.59	4.73	-0.14
	050	4.66	4.82	-0.16
	033	4.72	4.73	-0.02
	041	4.81	4.82	-0.01
	047	4.84	4.88	-0.04
	054	4.95	4.84	0.11
100,001–1,000,000	048	5.04	4.98	0.06
	055	5.15	5.30	-0.15
	062	5.15	5.08	0.07
	009	5.18	5.08	0.10
	003	5.43	5.15	0.29
	059	5.45	5.58	-0.13
	028	5.52	5.53	-0.01
	060	5.57	5.43	0.14
	018	5.59	5.30	0.29
	014	5.68	5.54	0.14
	029	5.69	6.00	-0.31
Higher than 1,000,00	034	6.04	6.18	-0.13
	015	6.15	6.15	0.00
	064	6.28	6.18	0.10
	019	6.40	6.38	0.02
	036	6.43	6.48	-0.05
	052	6.59	6.54	0.05

4.9 Drug resistance genotyping detection.

As mentioned in previous section, the cellulose fiber sample pad without using gold nanoparticles showed the viral load detection performance related to Whatman 903. Then, we performed drug resistance genotyping to investigate its performance of cellulose fiber sample pad compared to plasma and Whatman 903. We randomly selected 5 , 5 , 3 and 3 samples from range of viral load 2,000 – 10,000 copies/ml, 10,001 – 100,000 copies/ml, 100,001 – 1,000,000 copies/ml and higher than 1,000,001 copies/ml, respectively. The result showed that plasma, Whatman 903 and cellulose fiber sample pad can used to detect the drug resistance. It showed the same nucleotide mutation (Table 4-12) and drug interpretation (Table 4-13 – Table 4-16).

Table 4-12 Drug resistance mutations by ViroSeq® HIV-1 Antiretroviral drug resistance Genotyping kit form plasma, Whatman 903 and cellulose fiber sample pad.

Viral load range (copies/ml)	Sample	Plasma			Whatman903			Cellulose fiber sample pad		
		NRTI	NNRTI	PI	NRTI	NNRTI	PI	NRTI	NNRTI	PI
2,000-10,000	021	-	-	-	-	-	-	-	-	-
	008	-	-	L10V	-	-	L10V	-	-	L10V
	032	-	-	-	-	-	-	-	-	-
	035	-	-	V11I	-	-	V11I	-	-	V11I
	010	M184I	-	L10V	M184I	-	L10V	M184I	-	L10V
10,001-100,000	012	-	V179D	L10V	-	V179D	L10V	-	V179D	L10V
	049	T69N	-	-	T69N	-	-	T69N	-	-
	039	-	K103N	-	-	K103N	-	-	K103N	-
	050	-	K103N	L10V	-	K103N	L10V	-	K103N	L10V
	041	-	V179D	L10V	-	V179D	L10V	-	V179D	L10V
100,001-1,000,000	062	-	-	-	-	-	-	-	-	-
	003	-	-	L10V	-	-	L10V	-	-	L10V
	018	K65R, K70E, M184V	L100I, K103N, V108I, M230L, K238T	L10V	K65R, K70E, M184V	L100I, K103N, V108I, M230L, K238T	L10V	K65R, K70E, M184V	L100I, K103N, V108I, M230L, K238T	L10V
>1,000,001	015	-	-	-	-	-	-	-	-	-
	019	K65R, M184I	K103N, M230L	L10I, L10V	K65R, M184I	K103N, M230L	L10I, L10V	K65R, M184I	K103N, M230L	L10I, L10V
	064		K101Q	-		K101Q	-		K101Q	-

Table 4-13 The result of HIV-1 Antiretroviral drug resistance (viral load range 2,000 – 10,000 copies/ml)

NO.	Sample	NRTI							NNRTI				PI								
		3TC	FTC	AZT	ddI	d4T	ABC	TDF	DLV	EFV	NVP	ETR	APV	FOS	IDV	SQV	LPV	DRV	NFV	ATV	TPV
021	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
008	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
032	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

(N=None, PR=Possible Resistance, R= Resistance)

Table 4-13 The result of HIV-1 Antiretroviral drug resistance (viral load range 2,000 – 10,000 copies/ml). (Cont.)

NO.	Sample	NRTI							NNRTI				PI								
		3TC	FTC	AZT	ddI	d4T	ABC	TDF	DLV	EFV	NVP	ETR	APV	FOS	IDV	SQV	LPV	DRV	NFV	ATV	TPV
035	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
035	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
035	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
010	Plasma	R	R	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
010	W-903	R	R	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
010	CFSP	R	R	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

(N=None, PR=Possible Resistance, R= Resistance)

Table 4-14 The result of HIV-1 Antiretroviral drug resistance (viral load range 10,001 – 100,000 copies/ml).

NO.	Sample	NRTI							NNRTI				PI								
		3TC	FTC	AZT	ddI	d4T	ABC	TDF	DLV	EFV	NVP	ETR	APV	FOS	IDV	SQV	LPV	DRV	NFV	ATV	TPV
012	Plasma	N	N	N	N	N	N	N	PR	N	PR	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	PR	N	PR	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	PR	N	PR	N	N	N	N	N	N	N	N	N	N
049	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
039	Plasma	N	N	N	N	N	N	N	R	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	R	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	R	N	N	N	N	N	N	N	N	N	N	N	N

(N=None, PR=Possible Resistance, R= Resistance)

Table 4-14 The result of HIV-1 Antiretroviral drug resistance (viral load range 10,001 – 100,000 copies/ml). (Cont.)

NO.	Sample	NRTI							NNRTI				PI								
		3TC	FTC	AZT	ddI	d4T	ABC	TDF	DLV	EFV	NVP	ETR	APV	FOS	IDV	SQV	LPV	DRV	NFV	ATV	TPV
050	Plasma	N	N	N	N	N	N	N	R	R	R	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	R	R	R	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	R	R	R	N	N	N	N	N	N	N	N	N	N
041	Plasma	N	N	N	N	N	N	N	PR	N	PR	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	PR	N	PR	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	PR	N	PR	N	N	N	N	N	N	N	N	N	N

(N=None, PR=Possible Resistance, R= Resistance)

Table 4-15 The result of HIV-1 Antiretroviral drug resistance (viral load range 100,001 – 1,000,000 copies/ml)

NO.	Sample	NRTI							NNRTI				PI								
		3TC	FTC	AZT	ddI	d4T	ABC	TDF	DLV	EFV	NVP	ETR	APV	FOS	IDV	SQV	LPV	DRV	NFV	ATV	TPV
062	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
003	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
018	Plasma	R	R	N	PR	N	R	R	R	R	R	PR	N	N	N	N	N	N	N	N	N
	W-903	R	R	N	PR	N	R	R	R	R	R	PR	N	N	N	N	N	N	N	N	N
	CFSP	R	R	N	PR	N	R	R	R	R	R	PR	N	N	N	N	N	N	N	N	N

(N=None, PR=Possible Resistance, R= Resistance)

Table 4-16 The result of HIV-1 Antiretroviral drug resistance (viral load more than 1,000,001 copies/ml)

NO.	Sample	PI									NNRTI				NRTI						
		TPV	ATV	NFV	DRV	LPV	SQV	IDV	FOS	APV	ETR	NVP	EFV	DLV	TDF	ABC	d4T	ddI	AZT	FTC	3TC
015	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
019	Plasma	N	N	N	N	N	N	N	N	N	N	R	R	R	PR	R	N	PR	N	R	R
	W-903	N	N	N	N	N	N	N	N	N	N	R	R	R	PR	R	N	PR	N	R	R
	CFSP	N	N	N	N	N	N	N	N	N	N	R	R	R	PR	R	N	PR	N	R	R
064	Plasma	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	W-903	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	CFSP	N	N	N	N	N	N	N			N	N	N	N	N	N	N	N	N	N	N

(N=None, PR=Possible Resistance, R= Resistance)

4.10 HIV drug resistance genotyping detection through dried plasma spot (DPS) using cellulose fiber sample pad.

In this section, we investigated the performance of drug resistance genotyping detection by using plasma instead of whole blood by using the cellulose fiber sample pad as the filter paper because in some laboratories can only detect the viral load from plasma. Therefore, it might be a good option, if dried plasma spot can be used to detect drug resistance genotyping. Moreover, it is also easy to transport. The results showed that drug resistance genotyping detection of dried plasma spot and dried blood spot form cellulose fiber sample pad were the same which showed same nucleotide mutation (Table 4-17) and drug interpretation (Table 4-18). Therefore, we can conclude that either dried plasma spot or whole blood on the cellulose fiber sample pad can be used to detect the virus mutation.

Table 4-17 Drug resistance mutations by ViroSeq® HIV-1 Antiretroviral drug resistance Genotyping kit of dried plasma spot and dried blood spot form cellulose fiber sample pad

Viral load range (copies/ml)	Sample	Dried blood spot			Dried plasma spot		
		NRTI	NNRTI	PI	NRTI	NNRTI	PI
2,000 – 10,000	035	-	-	V11I	-	-	V11I
10,001- 100,000	049	T69N	-	-	T69N	-	-
100,001- 1,000,000	018	K65R, K70E, M184V	L100I, K103N, V108I, M230L, K238T	L10V	K65R, K70E, M184V	L100I, K103N, V108I, M230L, K238T	L10V
>1,000,001	019	K65R, M184I	K103N, M230L	L10I, L10V	K65R, M184I	K103N, M230L	L10I, L10V

Table 4-18 *The result of HIV-1 Antiretroviral drug resistance on cellulose fiber sample pad.*

NO.	Sample	3TC	FTC	AZT	ddI	d4T	ABC	TDF	DLV	EFV	NVP	ETR	APV	FOS	IDV	SQV	LPV	DRV	NFV	ATV	TPV
				NRTI					NNRTI				PI								
035	DBS	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	DPS	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
049	DBS	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	DPS	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
018	DBS	R	R	N	PR	N	R	R	R	R	R	PR	N	N	N	N	N	N	N	N	N
	DPS	R	R	N	PR	N	R	R	R	R	R	PR	N	N	N	N	N	N	N	N	N
019	DBS	R	R	N	PR	N	R	PR	R	R	R	N	N	N	N	N	N	N	N	N	N
	DPS	R	R	N	PR	N	R	PR	R	R	R	N	N	N	N	N	N	N	N	N	N

(N=None, PR=Possible Resistance, R= Resistance)

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CHAPTER V
DISCUSSION

Currently, CD4+ cell count and HIV viral load are used for monitoring HIV therapy. HIV viral load is used to determine level of virus after antiretroviral treatment. There are several molecular techniques used to detect HIV viral load which are approved by FDA such as Roche cobas taqman HIV-1 test, Abbott real time HIV-1 amplification and bioMerieux Nucleisens HIV-1 QT [65]. All those methods are recommended to use plasma for detection. Each technique required a difference volume of plasma but mostly around 1 ml. In developing countries, plasma transportation by cold-chain system is still a problem. An alternative specimen collection by dried blood spot is used to detect HIV proviral DNA in newborn and HIV viral load [66]. Dried blood spot has benefit over traditional method in term of avoiding the cold-chain system and only a small volume of whole blood is used. WHO recommended to use dried blood spot for HIV viral load detection in developing countries [11] but only a few countries used dried blood spot paper as an optional method for sample collection. Specimen collection by using dried blood spot may be used to determine HIV viral load. When compared dried blood spot and plasma for HIV viral load detection, the result showed that they had a good correlation according to the study of Garrido C and colleague [14]. They compared dried blood spot and plasma for detecting HIV viral load by Nuclisens EasyQ and Abbott real time. The R^2 values of automatic machines showed 0.87 and 0.70, respectively [14].

Thus, this study aimed to develop method to enhance sensitivity of HIV viral load detection in dried blood spot by using gold nanoparticle and anion polymer-coated magnetic beads. Nucleisens EasyQ was used in this study because it was the method that approved for using with dried blood spot. According to the study of Shawky SM et al [67], gold nanoparticle can bind to nucleic acid which was used for direct detection of unamplified hepatitis C virus RNA extracted from clinical samples [67]. We hypothesized that gold nanoparticle might bind and concentrate virus from dried blood spot paper. We optimized the condition of gold nanoparticle binding by varying size

(10, 20, 40 nm) and volume (6.25, 12.5, 25.0, 50.0 µl) and these conditions were applied to determine HIV viral load in Whatman 903 by using HIV-1 reference strain. Results showed that optimum condition for gold nanoparticle was 20 nm in size and 50.0 µl in volume. Moreover, the result of HIV viral load detection by gold nanoparticle in dried blood spot was comparable to supernatant HIV-1 reference strain (100 µl). Another material, anion polymer-coated magnetic beads was used to enhance sensitivity because of its properties in binding to many viruses including HIV [52, 68-73]. We optimized the condition of anion polymer-coated magnetic beads by varying bead volume and extraction step. Result showed that HIV viral load from dried blood spot and HIV supernatant cannot be reported because there was some leftover beads which can interfere HIV viral load detection.

To reduce the cost of paper, we evaluated three kinds of paper; Whatman 903, nitrocellulose membrane and cellulose fiber sample pad. Whatman 903 was used in this study because it was approved by WHO for HIV viral load detection [11]. Nitrocellulose membrane had ability to bind with nucleic acid [56], we hypothesized that it might bind to virus. Furthermore, the cost of nitrocellulose membrane is cheaper than Whatman 903. Cellulose fiber sample pad had ability to absorb serum and whole blood which commonly used in immunochromatography test [60]. We hypothesized that this paper can capture virus for HIV viral load detection. Result showed that only two types of papers can be used. Whole blood cannot be absorbed by nitrocellulose membrane so it might be not suitable for HIV viral load detection. Cellulose fiber sample pad showed ability to absorb whole blood and used for HIV viral load detection.

Moreover, we determined the ability of gold nanoparticle with the optimal condition (20 nm in size and 50.0 µl in volume) by adding gold nanoparticle on filter paper compared with filter paper without gold nanoparticle for HIV viral load detection. Results showed that with gold nanoparticle on Whatman 903 and without gold nanoparticle were correlated (R^2=0.97) and with gold nanoparticle on cellulose fiber sample pad and without gold nanoparticle were correlated (R^2=0.96). Therefore, with gold nanoparticle on filter paper and without gold nanoparticle had not significant difference (P>0.05) for HIV viral load detection. Because of gold nanoparticle did not enhance the binding of virus, so we compared ability of cellulose fiber sample pad with

standard paper (Whatman 903). The result of HIV viral load from cellulose fiber sample pad was comparable to Whatman 903 and showed good correlation (R^2=0.96) which were within 95% limit agreement (-0.03 ± 0.46 log copies/ml) by Bland-Altman agreement analysis. Other studies in Botswana, Ahlstrom grade 226 and Munktell TFN also showed a strong concordance in quantitative viral load analysis that were within 95% limit agreement by Bland-Altman analysis between Whatman 903 and Ahlstrom grade 226 (-0.034 ± 0.246 log copies/ml) and Whatman 903 and Munktell TFN (-0.028 ±0.186 log copies/ml) [74]. However, Ahlstrom grade 226 and Munktell TFN can be used instead of Whatman 903 but it is not available in general laboratory. Nevertheless, the cost of Whatman 903, Ahlstrom grade 226 and Munktell TFN are more expensive than cellulose fiber sample pad. As a result of this study showed that cellulose fiber sample pad can be determined the HIV viral load comparable to Whatman 903, Ahlstrom grade 226 and Munktell TFN ($R^2 > 0.95$). Therefore, cellulose fiber sample pad may be an alternative option to determining HIV viral load for our country.

After patients received antiretroviral drugs, they were monitored an efficiency of treatment by HIV viral load. If level of HIV viral load showed more than 1,000 copies/ml, the patients should be investigated drug resistance genotype to alter an appropriate antiretroviral drug formula [6]. To determine drug resistance genotype, it commonly used plasma specimen. There were the other specimen for drug resistance genotype as same as HIV viral load such as dried blood spot [11]. Genotype efficiency between plasma and dried blood spot showed 100% acoording to the study of Rottinghaus E and colleague [16], and the same as the study of Masciotra S and colleague demonstrated high concordance between these two sample types [25]. In this study, we performed drug resistance genotype by cellulose fiber sample pad compared to Whatman 903. Result showed that genotype efficiency presented 100% between cellulose fiber sample pad and Whatman 903. So cellulose fiber sample pad might be performed instead of Whatman 903.

Our study showed that cellulose fiber sample pad can be used to determined HIV viral load and drug resistance genotype comparable to Whatman 903. However, the cost of Whatman 903 (38 baht, GE Healthcare) is more expensive than cellulose fiber sample pad (4.8 baht, Merck Millipore Corporation). It is benefit to use cellulose

fiber sample pad instead of Whatman 903 to reduce cost. According to the report of National Health Security Office (NHSO) in 2014, they found that they had 138,528 tests of HIV viral load and 4,202 tests of HIV drug resistance genotype [75]. When we calculated the cost of cellulose fiber sample pad compared to Whatman 903, using of cellulose fiber sample pad can reduce cost of HIV viral load and drug resistance genotype down to 4,599,126.6 baht and 139,506.4 baht, respectively.

From this study, we showed an advantage of dried blood spot for determining HIV viral load and drug resistance genotype. It is easy to collect specimen by fingerprick. According to Fajardo E, et al, they showed good correlation between plasma and fingerprick-dried blood viral load levels, with 95% limits of agreement by Bland-Altman analysis [76]. Moreover, dried blood spot is convenient to transport because it avoided the cold-chain system and used a small volume of whole blood. Therefore, cellulose fiber sample pad will be useful for HIV viral load detection and drug resistance genotype in resource-limited countries.
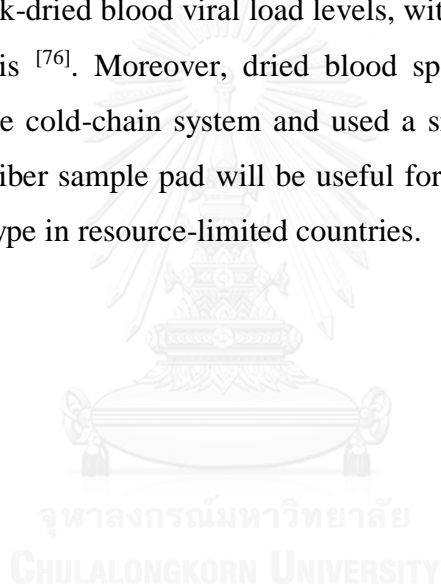
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APPENDIX

VITA

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