การพัฒนาความไวของเทคนิค Nucleic Acid Lateral Flow (NALF) โดยศึกษาในไวรัส HIV-1

นายมงคล พงษ์สุชาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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STRATEGIES AND IMPROVE SENSITIVITY OF NUCLEIC ACID LATERAL FLOW (NALF) IN HIV-1 NUCLEIC ACID

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A Thesis Submitted in Partial Fulfillment of the Requirements

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มงคล พงษ์สุชาติ : การพัฒนาเพื่อเพิ่มความไวของเทคนิคNucleic Acid Lateral Flow (NALF) โดยศึกษาในไวรัส HIV-1. (STRATEGIES AND IMPROVE SENSITIVITY OF NUCLEIC ACID LATERAL FLOW (NALF) IN HIV-1 NUCLEIC ACID) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.เกียรติ รักษ์รุ่งธรรม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม :อ.ดร.นพ.อมรพันธุ์ เสรีมาศพันธุ์, 57 หน้า.

วิธีนิวคลิอิคแอซิดแลเทอรัลโฟล (Nucleic Acid Lateral Flow, NALF) สามารถตรวจวิเคราะห์สารพันธุกรรมที่ต้องการได้อย่างรวดเร็วโดยมีขั้นตอนการทำงานไม่ซับซ้อน ซึ่งเหมาะอย่างยิ่งในการวินิจฉัยโรคในกรณีที่มีเครื่องมือในห้องปฏิบัติการจำกัด อย่างไรก็ตามข้อด้อยของชุดตรวจชนิดนี้คือมีความไวต่ำงานวิจัยนี้จึงศึกษาการสร้างชุดตรวจและเพิ่มความไวให้กับชุดตรวจในการตรวจหาเชื้อเอชไอวี (HIV-1) ได้แก่วิธีการใช้รังสียูวีการติดเอนไซม์ลงบนผิวของอนุภาคทองคำ และการใช้วิธี Helicase Dependent Isothermal Amplification (HDA)

ผลการวิจัยได้พัฒนาชุดตรวจวินิจฉัยการติดเชื้อเอชไอวี (HIV-1) โดยวิธี NALF และทดสอบจนได้สภาวะที่เหมาะสมในการได้ความเข้มของสัญญาณสูงที่สุด จากนั้นได้ศึกษาวิธีต่างๆเพื่อเพิ่มความไวในการตรวจปริมาณ HIV-1 cDNA ได้ผลดังนี้วิธีแรกคือฉายรังสียูวีคู่กับการติดฉลาก streptavidin/biotin เพื่อเพิ่มการเกาะของ capture probes พบว่าสามารถเพิ่มความเข้มของสัญญาณเพิ่มขึ้น 30% ทั้งนี้การฉายรังสียูวีโดยไม่ติดฉลากโอลิโกนิวคลีโอไทด์ไม่ได้เพิ่มสัญญาณของการตรวจแต่อย่างใด วิธีที่สองคือการติดเอนไซม์ Horse Radish Peroxidase (HRP) บนผิวของอนุภาคทองคำ พบว่าสัญญาณที่ได้มีค่าสูงกว่ากลุ่มที่ไม่ได้ติดเอนไซม์ 3 เท่า หรือ 300 % ($p<0.05$) อย่างไรก็ตามทั้งสองวิธีสามารถให้ผลบวกในกรณีที่ตัวอย่างมีปริมาณ เชื้อHIV-1 คือ 2.5fmol หรือ $5.9x10^{11}$ copies/mL และวิธีสุดท้ายคือการเพิ่มปริมาณสารพันธุกรรมโดยวิธี HDA สามารถตรวจหา cDNA จากตัวอย่างที่ทราบปริมาณHIV-1 RNAได้ต่ำที่สุดที่ 445 copies/mL

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....
ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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MONGKOL PONGSUCHART : STRATEGIES AND IMPROVE SENSITIVITY OF NUCLEIC ACID LATERAL FLOW (NALF) IN HIV-1 NUCLEIC ACID. ADVISOR : PROF.KIAT RUXRUNGTHAM, MD, CO-ADVISOR : AMORNPUN SEREEMASPUN, MD, Ph.D., 57 pp.

Nucleic Acid Lateral Flow (NALF) is a rapid test for nucleic acid detection that is easy to perform and to interpret. This advantage is suitable for a pathogen diagnosis in low resource settings. However, the major weakness is it has limited sensitivity. Thus, this study aimed to setup and improve sensitivity of NALF to detect HIV-1 nucleic acid. The strategies including UV crosslink, enzyme link gold nanoparticles conjugate and Helicase Dependent Isothermal Amplification (HDA).

The NALF platform for HIV-1 detection were setup and optimized. UV exposure was performed to increase the capture probe adherence to the membrane. The results revealed that the streptavidin/biotin combined with UV crosslink method had increased approximately 30% of signal level when compare to streptavidin/biotin alone. Whereas, Non-labeled oligonucleotides combined with UV crosslinking gave a comparable result to the streptavidin/biotin method. HRP linked-gold nanoparticle approach increased approximately 3 folds of signal level or 300% ($p<0.05$) when compared to the conventional method. HDA amplification using HIV-1 cDNA from clinical samples with known viral load then deteced by the NALF system was able detect at least 445 copies/mL of HIV-1 RNA from plasma (pre-amplification template).

Field of Study : Medical Sciences
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Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
AuNP	Gold nanoparticles
bp	Base pair
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
g (centrifugation speed)	Gravity
h.	Hours
M	Molar
mM	Millimolar
mg	Milligram
mL	Milliliter
nM	Nanomolar
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase Chain reaction
rpm	Round per minutes
SDS	Sodium dodecyl sulfate
SSC	Saline-sodium citrate

CHAPTER I

BACKGROUND

1.1 HIV-1 diagnostic test

Mother to child of HIV-1 infection is widely epidemic in the last 10 years. In each year, there are about 500,000 children in developing country who inherited HIV-1 from their mother and about 20,000 infected HIV-1 per 1 million children in Thailand [1,2]. HIV-1 infected diagnostic method that use for infant can divide to 2 groups.

HIV-1 serology method: this method aim to detect HIV-1 specific antibody. Normally, this method will effective when infected the virus for 2-3 weeks. However, limitation is HIV-1 specific antibody were transmission from mother to child and remaining until 18 months after child birth. The alternative method is P24 antigen detection and HIV-1 viral culture is less sensitivity, high cost and time consume. HIV-1 genetic material detection: nucleic acid detection (DNA/RNA) such as Polymerase Chain Reaction (PCR) method can give result in 2-3 months after child birth and give best result in 4-6 months age.

In clinical practice PCR diagnostic was test after child birth in 24-48hours, 14-21 days, 1-2 months and 4-6 month [3]. Normally, HIV-1 DNA PCR diagnostic is the standard method for child case. Because of, this method is an effective, 96% sensitivity and 99% sensitivity of 28 days child[4]. Moreover, development of HIV-1 RNA PCR give better sensitivity but, reproducibility is decrease when viral load are lower than 10,000 copies/mL[5].

Recently, HIV-1 PCR diagnostic mainly use commercial kit such as QL RNA NASBA but cost is 2,000-2,500 Baht per test [5,6]. An in house test system was developed for cheaper but this

system is required to use special equipment, well training personnel and overall procedure is more than 7 days[7].

Nucleic Acid Lateral Flow (NALF) is the promising method for rapid diagnostic without special equipment and low cost per unit. However, poor sensitivity is the major problem for this method. The best sensitivity was demonstrate by Mao et al. at 1.25 fM[8], but still limit for detect small amount of nucleic acid. As a result, development of NALF sensitivity will help improve the system for low cost, rapid and simple for developing country.

1.2 Nanotechnology

Nanotechnology is the study of manage, construct, analyst and application of material that have diameter from 1 to 100 nanometers[9]. Nanotechnology can develop new material such as nanomaterial (nanotube, nanorod and nanoparticles) with different physical, chemical and biological properties. As a result, nanotechnology have been applied in various filed like microchip technology, medical and science research. Gold nanoparticles (AuNPs) is a nanomaterial that normally stay in red colloidal solution [10]. Generally, synthesis of AuNPs use $HAuCl_4$ solution (Au^{3+}) with a reducing agent to convert gold ion into Au^0, then further be supplied with a stabilizer to prevent aggregation by surrounding the gold nanoparticles. Sodium citrate is commonly used as both stabilizer and reducing agent [11].

Figure 1 Summary diagram for production of gold nanoparticles. Sodium citrate is used as the reducing agent and stabilizer[12].

AuNPs have a unique optical properties different form yellow gold. Size particles of gold nano smaller than wavelength made surface plasmon resonance (SPR). Charge density and synchronized oscillation of free electron on AuNPs surface and dielectric solution contribute to wavelength absorption at 520nm. Thus AuNPs will have ruby red colours as figure [13].

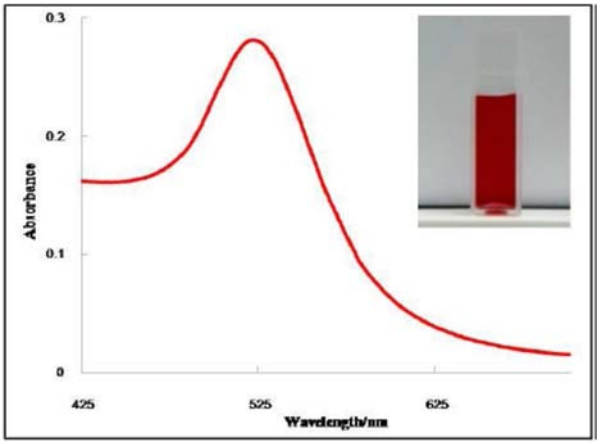

Figure 2 Optical properties of gold nanoparticles. Light absorption spectrum of gold nanoparticles at 520nm and the small picture showed gold nanoparticles.

AuNPs surface can modified for detection or tracking target molecules such as DNA, protein, carbohydrate, drugs and fluorescent dye [14-16]. Thus, AuNPs modified surface by specific binding with target molecules[17] is an effective system for detect target molecules.

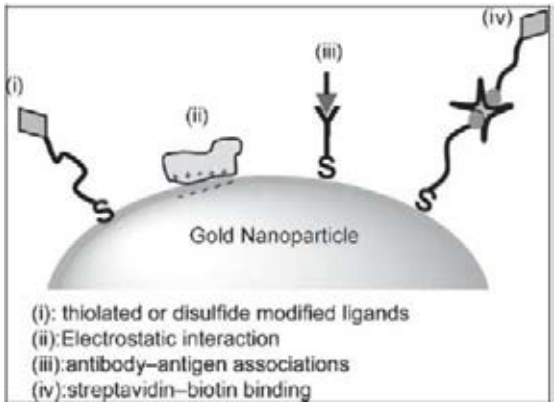

Figure 3 Application of gold nanoparticles. Schematic showed application of AuNPs in biology and medical filed by attach biomolecules on AuNPs surface[12].

CHAPTER II

LITERATURE REVIEW

2.1 HIV-1 diagnostic method

HIV-1 infected diagnostic method that use for infant can divide to 2 groups

1. HIV-1 serology method: this method aim to detect HIV-1 specific antibody. Normally, this method will effective when infected the virus for 2-3 weeks. However, limitation is HIV-1 specific antibody were transmission from mother to child and remaining until 18 months after child birth. The alternative method is P24 antigen detection and HIV-1 viral culture is less sensitivity, high cost and time consume.
2. HIV-1 genetic material detection: nucleic acid detection (DNA/RNA) such as Polymerase Chain Reaction (PCR) method can give result in 2-3 months after child birth and give best result in 4-6 months age.

In patient that have age under 1 years PCR is give better result because antibody detection method cannot identified between mother and child antibody that lead to false positive result[5]. In addition, PCR method is high sensitivity and specificity. The advantage and disadvantage in each method are summary in table 1[3].

วิธีการตรวจ	ข้อเด่น	ข้อด้อย
การตรวจวัดภูมิคุ้มกันที่จำเพาะต่อเชื้อเอชไอวี		
HIV antibody	เทคนิคการตรวจไม่ซับซ้อน มีใช้ในทุกโรงพยาบาล และราคาถูก	แปลผลบวกในทารกอายุ 6-12 เดือนได้ยาก ว่าเป็นผลบวกเนื่องจากภูมิคุ้มกันของเด็ก หรือจากมารดา
Semiquantitative HIV antibody	เทคนิคการตรวจไม่ซับซ้อน ใช้ในทารกช่วงอายุ 6-12 เดือนได้ การตรวจ 2 ครั้งขึ้นไปและดูแนวโน้ม การเปลี่ยนแปลงขึ้นหรือลงจะเพิ่ม ความแม่นยำมากขึ้น	ความจำเพาะ(แยกทารกที่ไม่ติดเชื้อ) ในทารกช่วงอายุ 6-12 เดือนอยู่ในช่วงร้อยละ 53-100 โดยมีความจำเพาะมากขึ้นเมื่ออายุเข้าใกล้ 12 เดือน
HIV IgA	ราคาไม่แพง	มีความไวของการทดสอบต่ำในทารกอายุต่ำกว่า 6 เดือน
การตรวจหาเชื้อไวรัสเอชไอวี		
Boosted ICD p24 antigen*	มีความไวและความจำเพาะร้อยละ 100 ตั้งแต่ทารกอายุ1- 2 เดือน รายงานผลเป็นแบบ quantitative	ยังไม่มีใช้แพร่หลายในประเทศไทย อยู่ในช่วงพัฒนาชุดทดสอบ
HIV DNA PCR*	มีความไวและความจำเพาะร้อยละ 100 ตั้งแต่ทารกอายุ1- 2 เดือน รายงานผลเป็นแบบ qualitative	เทคนิคการทำซับซ้อน ราคา 2000-2500 บาทต่อการทดสอบ
Plasma HIV RNA PCR	มีความไวและความจำเพาะร้อยละ 100 ตั้งแต่ทารกอายุ1- 2 เดือน รายงานผลเป็นแบบ quantitative	เทคนิคการทำซับซ้อน ยังไม่มีใช้ในโรงพยาบาลทั่วไป ราคา 3000-4000 บาทต่อการทดสอบ

* ศูนย์รวบรวมและวิเคราะห์เชื้อเอชไอวีแห่งประเทศไทยกำลังพัฒนาชุดทดสอบ โดยมีต้นทุนที่ราคา 200-300 บาท

Table 1 Summary of advantage and disadvantage of HIV-1 detection in child[18]

In 2001 S. Vongsheree et al. have been developed in house test using primers target on *Pol* gene. The results showed specificity and sensitivity is 100, 94.4% respectively. In the same year Puthanakij et al. were compared in house test kit with Amplicor HIV-1 commercial kit and results showed sensitivity and specificity is 90.9, 100.0 respectively.

In 2006 H. Thaisri et al. were compared between in house multiplex PCR and Amplicor HIV-1, in house kit was 100% sensitivity. The research group concluded in house kit is comparable to commercial kit but less expense 5 times[18].

In 2009 N. Mehta et al. were developed HIV-1 detection systems by using Real-time PCR and dried blood spot that have 100% sensitivity when compared with Roche Ultrasensitive assay. In addition, this system could report with quantitative result and limit of detection is 136 copies/mL[19].

2.2 Nanotechnology

Nanotechnology is the study of manage, construct, analyst and application of material that have diameter from 1 to 100 nanometers[9]. Nanotechnology can develop new material such as nanomaterial (nanotube, nanorod and nanoparticles) with different physical, chemical and biological properties. As a result, nanotechnology have been applied in various filed like microchip technology, medical and science research as described in table 2.

Nanoparticle	Example	Medical application	References
Metal nanoparticles	Quantum dots Gold nanoparticles Gold nanorods Gold nanoshells Gold nanocages	Diagnostics Biosensor Molecular imaging Drug delivery	Chen et al 2005; Loo et al 2005; Yeh et al 2005; Huang et al 2006; Baron et al 2007; Maysinger 2007; Oyelere et al 2007; Skrabalak et al 2007; Villalonga et al 2007; Cho et al 2008
Nanotubes and nanowires	Carbon-nanotubes	Biomolecular sensing Delivery of vaccines or proteins	Baron et al 2007; Maysinger 2007; Cho et al 2008
Dendrimers	Poly(amido) amine PAMAMs	Drug carriers Imaging agents Gene delivery	Rawat et al 2006; Maysinger 2007; Villalonga et al 2007; Cho et al 2008
Liposomes	(PEG)ylated immunoliposomes	Drug delivery Gene encoding	Rawat et al 2006; Villalonga et al 2007; Cho et al 2008
Polymeric micelles	[PEG-PAsp (DOX)] Doxorubicin conjugated to poly(ethylene glycol)-poly (α, β-aspartic acid)	Drug delivery of water-insoluble drugs	Sahoo and Labhasetwar 2003; Cho et al 2008
Ceramic nanoparticles	Silica-based nanoparticle entrapping photosensitizing anticancer drug, 2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide	Drug delivery	Sahoo and Labhasetwar 2003
Polymeric nanoparticles	PLGA (Poly(D, L-lactic-coglycolic acid)) PLA-PGA (Poly-L-glutamic acid)	Drug delivery Protein delivery Gene expression vector	Rawat et al 2006; Sahoo et al 2007; Villalonga et al 2007; Cho et al 2008
Polysaccharide nanoparticles	Cellulose nanocrystals	Targeted delivery Bioimaging	Dong and Roman 2007; Villalonga et al 2007
Magnetic nanoparticles	Superparamagnetic iron oxide	Magnetic Resonance Imaging contrast agents	Baron et al 2007; Lu et al 2007
Bionanoparticles (BNPs) – Protein-based nanosystems	Ferritin Viruses and virus-like particles Heat shock protein cages	Gene delivery Bioimaging Drug delivery Vaccine development	Lee and Wang 2006

Table 2 nanomaterial that use in different application for medical purpose[20]

Currently, nanomaterials are widely use in medical application and gold nanoparticles are the most frequency use. Gold nanoparticles (AuNPs) is the 1-100nm size metal particles with distinctive properties such as good conductivity, color depend on particles size and nontoxicity. Thereby, AuNPs were generally use as a reporter by attach biomolecules (DNA, RNA, antibody) on their surface, drug delivery system and photothermal therapy [21-23].

In 1996 Mirkin et al. were the first group that developed colorimetric gold nanoparticle assays for DNA detection [15,24,25]. By using color changing of AuNPs when aggregation, AUNPs labeled with oligonucleotide will bind with target DNA, lead to AuNPs aggregation and change color from red to purple as figure 4. This principle have been developed by the other groups as DNA, RNA and protein detection[26].

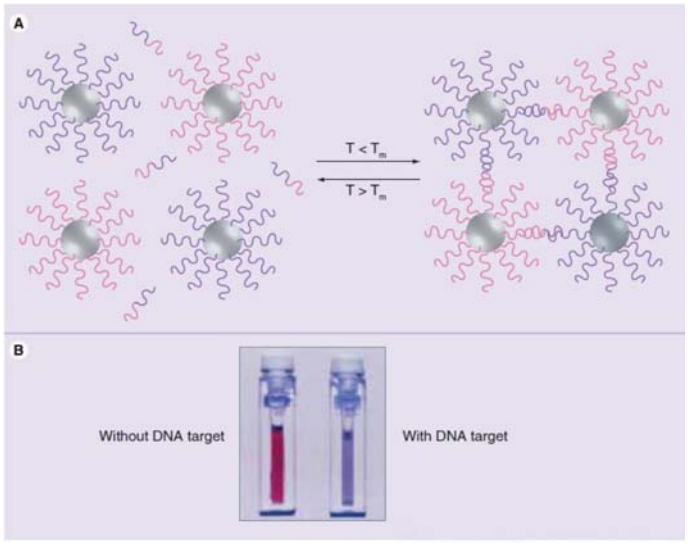

Figure 4 Principle diagram of colorimetric DNA detection using AuNPs probe for target DNA detection (4A) and color of AuNPs when present of target DNA and absence of target (4B)[26].

In 2004 Li et al. have been reported single strand DNA (ssDNA) and double strand DNA (dsDNA) have different absorption properties on AuNPs surface [27-29]. Short ssDNA can absorb on AuNPs surface and promote stability of particles. They developed colorimetric DNA detection system using unmodified gold nanoparticles and probe (ssDNA). The principle is ssDNA probe will attach with target DNA and contribute to dsDNA, this type of DNA was poorly absorption on AuNPs surface. When add salt buffer on this solution AuNPs will change color from red to purple by aggregation. In contrast, if ssDNA probe have not complementary sequences with sample DNA, still in single strand form the DNA have highly absorption on AuNPs surface. When add salt solution on the nanoparticles, the color will not change as figure 5.

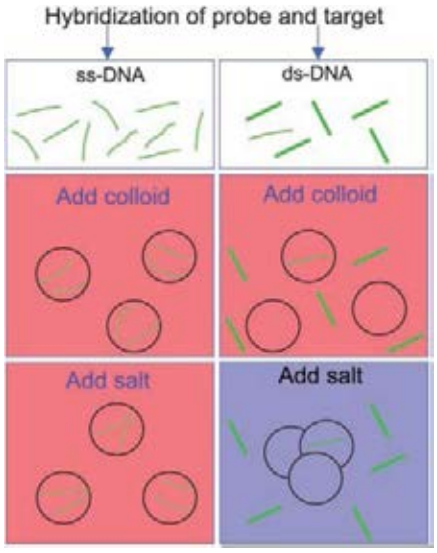

Figure 5 Diagram showed colorimetric DNA detection using unmodified AuNPs[28]

2.3 Nucleic acid lateral flow (NALF)

Nucleic acid lateral flow (NALF) is microchromatographic assay in a dipstick format has been developed with a promise to be used as a new alternative screening for wide applicability of rapid strip test. Current NALF platform has been proposed to detect the presence of the target nucleic acid analyte. This provides a faster, cheaper, and easier procedure than those of Northern and Southern techniques [8,30-32]. Generally, the platform of NALF strip has been described elsewhere [33,34]. In brief, NALF is composed of a test line and a control line on nitrocellulose membrane (Figure 6). The test line and the control line contain different biotin-modified nucleic acid probes grafted on streptavidin-functionalized nitrocellulose membrane support. A tested DNA sample will be dropped on a sample pad and mixed with conjugate probes (complementary probes to DNA of interest attached on gold nanoparticles). Target DNAs of the same sequence that have been hybridized with the conjugate probes will be further sandwich-hybridized with specific biotin-modified probes at the test line while the excess conjugate probes will be trapped with another specific biotin-modified probes at the control line.

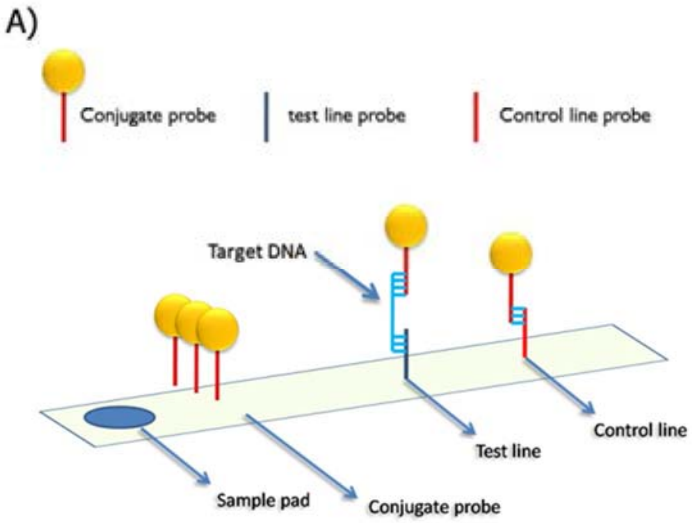

Figure 6 Diagram illustrated the principle of Nucleic Acid Lateral Flow Strip Test (NALF). A figure represents core components of NALF platform, including sample pad, conjugate pad and conjugate probe, test line and control line

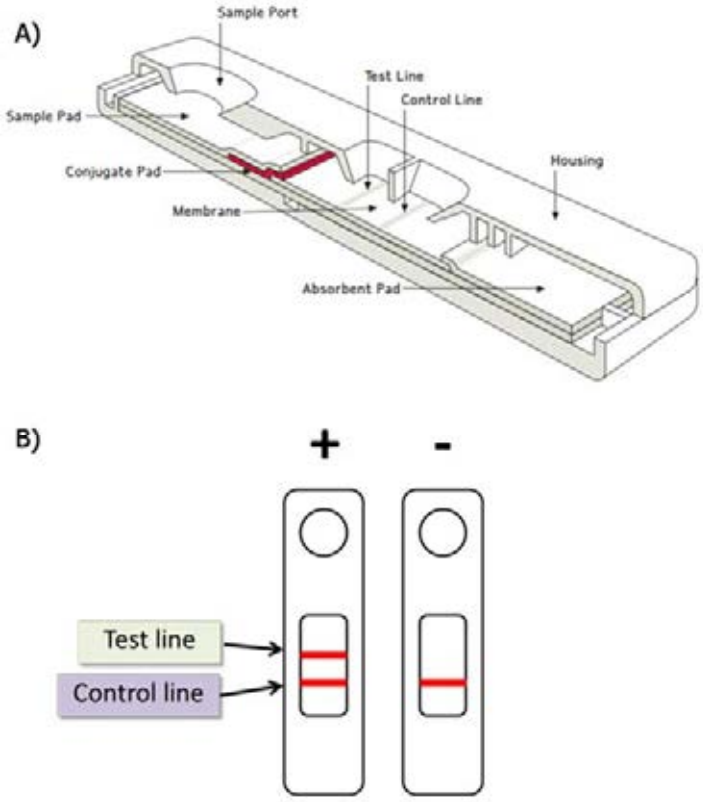

Figure 7 Nucleic Acid Lateral Flow construction and result read. Schematic view of a lateral flow strip test showed individual assemble on single platform form left side: sample pad(Sample

applied here), conjugate pad containing DNA labeled gold nanoparticles, membrane that have test line and control line, then finish with absorbent pad for absorb excess reagent. (A) Lateral flow strip interpreted, left is positive result that can observed red band on test line and control line, right is negative due to observed only control line band. (B) [35]

NALF platform have the same disadvantage of lateral flow immuneassay such as background signal from single step running without washing step, low sample volume etc. In addition, NALF need amplification process before applied on the platform due to low sensitivity of the system[36]. There are several reported method for enhance sensitivity such as enzymatic reaction, silver nanoparticles enhance, PCR amplification etc.[8,37-39].

In 2009 Mao et al. have were developed simple diagnostic system using gold nanoparticle probe and lateral flow principle which detect genomic DNA without amplification process. Genomic DNA was hybridization by sandwich hybridization method then appears the results at test line and control line, a red band that can observe by naked eyes. This method have lowest limit of detection at 2.5ug/mL or 1.25fM[8]. Currently, several research groups were developed NALF platform for rapid diagnostic system that target on virus, bacteria even genotyping that can summary in table 3

Analyte	Method	Application	Sensitivity	Reference
Specific RNA after isothermal amplification	Polystyrene dyed microsphere labeled specific oligonucleotide	Detection of *Bacillus anthracis*	2 *B. anthracis* cells	[40]
PCR amplified product with dA tail of 23S ribosomal RNA	Colloidal gold labeled oligo dT strands	Bacterial infections in arthroplasty	10 cells of S. aureus	[41]
Cryptosporidium species oocytes in water	Dye-entrapped and oligonucleotide-labeled liposomes	Contamination of Cryptosporidium	1 oocyst in 10 μL	[42]
4 dengue serotypes after isothermal nucleic acid based amplification in serum	Dye-entrapped liposome DNA probe	Detection of virus infection	50–50,000 copies of RNA molecules in serum	[43]
PCR amplified product	Dye-entrapped and oligonucleotide-labeled	Contamination of *Cryptosporidium parvum*	10 oocysts in 0.5 mL water	[44]
Human genomic DNA	Colloidal gold labeled specific oligonucleotide and HRP	Detection of Human genomic DNA	2.5ug/mL (1.25fM)	[8]
PCR amplified product of *Staphylococcus aureus*	Dye-entrapped and oligonucleotide-labeled	*Staphylococcus aureus* detection	50 CFU	[45]

Table 3 Overview of Nucleic Acid Lateral Flow publication. detection method and sensitivity (modified from [36]).

2.4 Helicase Dependent Isothermal Amplification (HDA)

The Polymerase Chain Reaction (PCR) is widely used in biomedical research and pathogen diagnostics [46]. The method relies on thermal cycling, cycles of repeated heating and cooling of the reaction for DNA annealing and enzymatic replication of the DNA. The disadvantage of the conventional PCR is the high cost of thermal cycler which is not suitable for low resource setting laboratory and field diagnostics. Thus, isothermal amplification methods have been established[47].

There are different isothermal amplification methods such as Loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), Nucleic acid sequence based amplification (NASBA) [48-50]. However, these isothermal amplification methods have their limitations. Most of them have complicated reaction systems. In addition, they are unable to amplify DNA targets to sufficient length to be useful for research and diagnostic applications[51].

Properties	PCR	NASBA	LAMP	HDA	RCA
DNA amplification	+	+	+	+	+
RNA amplification	+	+	+	+	+
Temperature (s)	94, 55–60, 72	37–42	60–65	60–65	45, 50
Number of enzyme (s)	1	2-3	1	2	1
Primer design	simple	Simple	complex	simple	simple
Tolerance to biological Components	-	-	+	+	-
Need template Denaturation	+	+	-	-	+
Denaturing agent (s)	Heat	RNase H	Betaine	Helicase	Φ29 DNA polymerase

Table 4 Properties of various isothermal amplification methods and PCR modified from[52].

Helicase Dependent Isothermal Amplification (HDA) was derived by mimicking DNA replication. In nature, DNA helicase is used to separate two complementary DNA strands during DNA replication by breaking hydrogen bonds between annealed nucleotide bases. By this principle, DNA helicase generates single-stranded DNA templates for primer hybridization and subsequent extension. As the DNA helicase unwinds dsDNA chemically, the initial heat denaturation step by thermocycler is no longer needed. In addition, engineered Taq polymerase optimized for working at 65°C and a pair of primers that were designed for annealing DNA template at 65°C were used together with the helicase. Thus, HDA provides a simple DNA amplification method at a constant

temperature from the denaturation step through the extension step. For typical HDA-PCR assay time taken is 90 minutes for 30-40 reaction cycles.

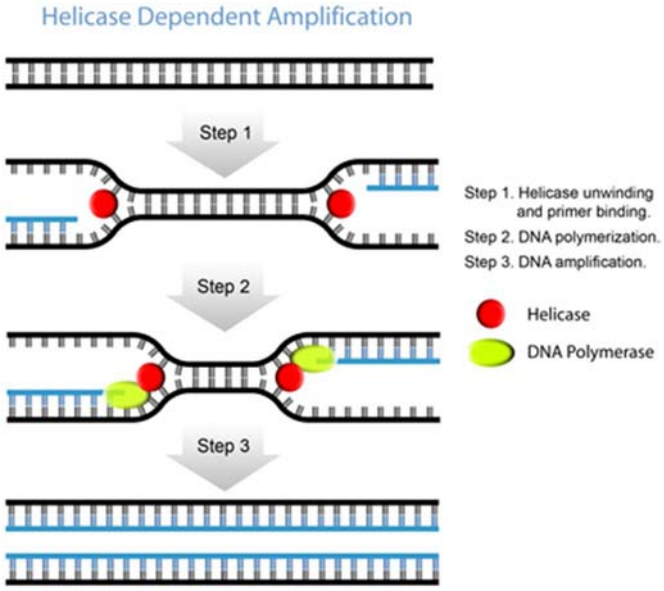

Figure 8 Helicase Dependent Isothermal Amplification principles. At first, double stranded DNA templates are unwound by DNA helicase and the resulted single stranded DNA templates are allowed to bind with primers. DNA polymerase then extends the 3' ends of each primer using free deoxynucleotides (dNTPs) to produce two DNA replicates. The two replicated DNAs independently enter the next cycle of HDA, resulting in exponential amplification of the target sequence.

In 2008, Goldmeyer et al. used HDA and disposable nucleic acid lateral flow device targeting on *nuc* and *mecA* genes for Methicillin-Resistant *Staphylococcus aureus* detection. The limit of detection of the system was 50 CFU per reaction and the specificity was 100% [53]. In the same year, Chow et al. reported application of HDA for *Clostridium difficile* detection. The limit of detection of this system was 20 copies per reaction and the sensitivity was 100% which is higher than that of EIA assay (90.9%). In 2011, Tang et al. reported application of HDA-PCR with HIV-1 detection system by targeting on *Gag* gene. The limit of detection was reported at 50 copies per reaction [54]. In addition, Kim et al. demonstrated herpes simplex virus types 1 and 2 detection system using HDA-PCR and disposable nucleic acid lateral flow device. The sensitivity of this assay was 5.5 and 34.1 copies per reaction for HSV-1 and HSV-2 respectively[55].

CHAPTER III

MATERIALS AND METHODS

3.1 Equipment

1.	-80°C Deep Freezer model MDF-U32V	Sanyo, Japan
2.	-20°C Freezer	Sanyo, Japan
3.	4°C Refrigerator	Sanyo, Japan
4.	Autoclave	Hirayama, Japan
5.	Centrifuge	Sorvall, Germany
6.	Cuvette	Hellma, Germany
7.	Gel Document and Quantity One 4.4.1	Biorad, USA
8.	Microcentrifuge	Eppendorf, Germany
9.	Microcentrifuge tube 0.5 mL and 1.5 mL	Axygen Scientific, USA
10.	Micropipette P1, P10, P100 and P1000	Biohit, Finland
11.	Microwave	LG, Korea
12.	Spectrophotometer	Biorad, USA
13.	Stirrer Hot Plate	BEC Thai, Thailand
14.	Water bath	Memmert GmbH&Co., Germany
15.	Zetasizer machine	Marvern, USA

3.2 Chemical and kits

1. 100 bp DNA ladder	Fermentas, Canada
2. 6x Loading dye solution	Fermentas, Canada
3. Agarose gel	Research Organics Inc., USA
4. DL-Dithiothreitol	Sigma Aldrich, USA
5. Ethidium bromide	Sigma Aldrich, USA
6. Horse Radish Peroxidase	Sigma Aldrich, USA
7. Hydrogen tetracholoaurate (III) trihydrate	Sigma Aldrich, USA
8. Hydrochloric acid	Merck, USA
9. illustra NAP-10 Columns	GE Healthcare, USA
10. Nitric acid	Merck, USA
11. RNase, DNase free water	Invitrogen, USA
12. Sodium Chloride	Sigma Aldrich, USA
13. Sodium dodecyl sulfate	Sigma Aldrich, USA
14. Sodium hydroxide	Sigma Aldrich, USA
15. Sodium phosphate dibasic	Sigma Aldrich, USA
16. Sodium phosphate monobasic	Sigma Aldrich, USA

3.3 Synthesis and characterization of gold nanoparticles (AuNPs)

Citrate reduction method was first proposed by Turkevich et al. in 1951 [56]. In a typical standard citrate reduction procedure, 45 mL of deionized water was heated to 90-100 °C on a stirrer hot plate under refluxing condition. While stirring vigorously, 2 mL of 1% hydrogen tetrachloroaurate ($HAuCl_4$) and 3.76 mL of 38.8 mM trisodium citrate, a reducing agent, were quickly added resulting in color change from yellow to grey, purple and red, respectively. This solution was stirred for 2 hours. Finally, the observation of deep red color indicates the formation of gold nanoparticles. The solution of gold nanoparticles was kept at 100 °C for 15 minutes and subsequently cooled to room temperature. The final gold nanoparticles are approximately spherical and have negatively charged surface. The concentration of the synthesized gold nanoparticles is 200μg/mL. Particle size and zeta potential of gold nanoparticles were measured by zetasizer machine (Malvern). Light absorption was measured by UV spectrophotometer at wavelength 400-800nm.

3.4 Conjugation of thiolated probes on AuNPs

Thiolated probes that are specific to the sequence of *RT* gene of HIV-1 virus (in the sequences below) have been conjugated on surface of gold nanoparticles. This process was previously described by Hill and Mirkin [57].

1. 50 μL of thiolated DNA probes (conjugate probe in Table 5) and 7.7mg of Dithiothreitol (DDT) were mixed and incubated for 30 minutes.
2. Prepared NAP-10 column by washing with 2 mL of distilled water 3 times
3. Activated thiolate DNA probes were applied to NAP-10 column, add 400 μL of distilled water to the column and allow it to flow through uncollected.
4. Then add 950 μL distilled water to the NAP-10, and collect the flow through at the ninth drop to the last drop from column in 1.5 mL microcentrifuge tubes.
5. Activated thiolated probes were mixed with 1 mL of AuNPs and incubated for 24 hours at room temperature.

6. Added phosphate adjustment buffer to the conjugated solution to obtain a final phosphate concentration of 9 mM. By calculation: 1,000 µL of AuNPs + *x* µL DNA = Total volume in µL (Total volume 1 in µL)/10 = *y* µL phosphate adjustment buffer needed.
7. Added 10% SDS (wt/vol) solution to obtain a final SDS concentration 0.1% (wt/vol). For helps to keep the particles from aggregating. Calculation: 1000 µL of AuNPs + *x* µL DNA + *y* µL phosphate adjustment buffer = Total volume 2. Finally, SDS to add = (total volume 2 x 0.1)/10.
8. After shaking for 30 minutes, Calculated the volume of salting buffer needed to obtain a final concentration of 0.3 M NaCl. Calculation: (Total volume 2 × 0.3 M)/(2M) = volume of salting buffer needed in microliters. The amount per addition is equal to the volume of salting buffer divided by 6.
9. After the last salt addition and overnight incubation, the solution was centrifuged at 12,000 rpm for 12 minutes. The pellets was collected and dispersed in 1 mL eluent buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Tween and 10 % sucrose)

Additional step: for AuNP-DNA conjugated with Horse Radish Peroxidase (AuNP-DNA-HRP)

10. 1 mL of finished salting AuNP-DNA was centrifuged at 12,000 rpm for 12 minutes. Removed supernatant and disperse the pellet in 1 mL of distilled water
11. Added 25 µL of 5 ug/mL of HRP solution and incubated for 1 hour.
12. After incubation for 1 hour, the solution was centrifuged at 12,000 rpm for 12 minutes. The pellet was dispersed in 1 mL of eluent buffer.

Name	Sequence
Conjugate probe	5'-Thiol-CTGTACCAGTAACATTAAAGCCAGG-3'
Capture probe (test line)	5'- GTTAAACAGTGGCCATTGACAGAA GA-Biotin-3'
Control probe (control line)	5'-Biotin-CCTGGCTTTAATGTTACTGGTACAG-3'
Capture probe (non-biotin) (test line)	5'- GTTAAACAGTGGCCATTGACAGAAGA-3'
Control probe (non-biotin)	5' -CCTGGCTTTAATGTTACTGGTACAG-3'
HIV-1 *RT gene* Target sequence	5'-TCTTCTGTCAATGGCCACTGTTTAACCTTTGGT CCATCCATTCCTGGCTTTAATGTTACTGGTACAG-3'
HIV-1 *Protease gene* Non-target sequence	5'-GACATGGTCTTAGTAATTTCGGTCCTTACCTAC CTGGTTTCCAATTTGTCACCGGTAACTGTCTTCT-3'

Table 5 DNA sequences that are used in NALF platform.

3.5 Lateral flow strip test construction

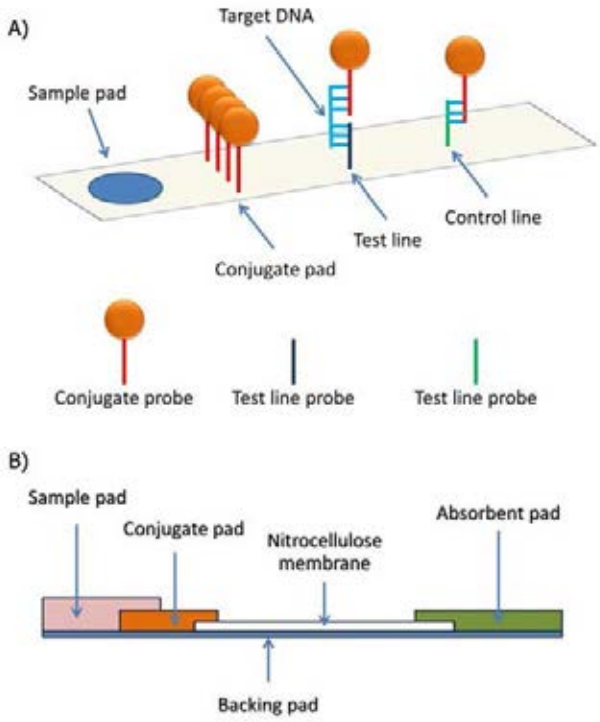

Figure 9 Platform components and layout. A) Nucleic acid lateral flow platform schematic, demonstrates function of each components. B) Diagram shows main components and arrangement of the module.

Method to construct the lateral flow test strips was slightly modified from the method previously described by Mao et al. [8].

3.5.1 Nitrocellulose membrane preparation

1. 60 µL of 1 mM of DNA probe (control or test line probe) was mixed with 140 µL PBS and 1.67 mg/mL of streptavidin solution (300 µL) and incubated for 1 hours
2. Removed excess streptavidin by centrifugal filter at 6,000 rpm for 20 minutes, washed with 1mL of PBS solution for 3 times and eluted in 500 µL of PBS buffer.
3. Then dropped the solution on nitrocellulose membrane (Millipore HF240) as test and control lines and allowed the lines to dry for 1 hour.

4. **Additional step for Streptavidin/Biotin with UV crosslink:** nitrocellulose membranes will be exposed to UV light by UV crosslink machine at 125mJ/cm^2 for 60 seconds

3.5.2 Nitrocellulose membrane preparation for non-labeled oligonucleotide and UV crosslink method

1. Dropped 1 μL of 100uM non-labelled oligonucleotide on nitrocellulose membrane (Millipore HF240) as test and control line
2. Nitrocellulose membranes will be exposed to UV light by UV crosslink machine at 125mJ/cm^2 for 120 seconds

3.5.3 Conjugate pad preparation

1. Cut fiberglass membrane to 0.5x1 cm.
2. Dropped 10 μL of conjugate probes (AuNP-DNA) on fiber glass membrane and stored in desiccator for 24 hours.
3. After conjugate pad completely dried, stored at 4°C

3.6 Sample assay procedure

3.6.1 for synthetic DNA experiments

1. 1 μL of 100nM HIV-1 synthetic DNA was mixed with 50 μL of running buffer (4x SSC buffer supplement with 0.5% SDS) and applied on sample pad.
2. Added 50 μL running buffer every 5 minutes
3. After running for 30 minutes, each nucleic acid lateral flow strip was analyzed by image analyzer software.

3.6.2 PCR product from clinical sample experiments

1. Incubated PCR product in boiling water for 5 minutes, then incubated on ice for 15 minutes.
2. Applied 50 µL of denatured PCR product on sample and added 50 µL of 4X SSC buffer supplement with 0.5% SDS.
3. Added 50 µL running buffer every 5 minutes
4. After running for 30 minutes, each nucleic acid lateral flow strip was analyzed by image analyzer software.
5. For enzyme-linked gold nanoparticles (DNA-AuNP-HRP): after running NALF platform for 30 minutes, applied 90 µL of signal enhancement solution that contained 0.05% 3-amino-9-ethyl-carbazole (AEC), 0.015% H_2O_2 in 0.05 M sodium acetate buffer (pH 5.5) on sample pad.
6. After 10 minutes of enzymatic reaction, platform was washed by 50 µL of 4X SSC buffer supplement with 0.5% SDS for 2 times.

3.7 Helicase dependent Isothermal Amplification (HDA-PCR)

HDA-PCR reaction was prepared following the protocol from manufactory leaflet. Primers in this experiment were primer sequences has been validated and used routinely at the HIV-1 drug resistance service, ChulaMRC. (see Table 6).

primers	sequences
HDA-FWD (29bp)	5’ GGTTGTACTTTAAATTTCCCAATTAGTCC 3’
HDA-FWD (26bp)	5’ TTTCTTCTGTCAATGGCCACTGTTTA 3’

Table 6 Primer sets for detected HIV-1 by HDA-PCR

1. After preparing master mix, 5 µL of cDNA clinical sample was added to master mix reaction
2. Added 25 µL mineral oil on top layer of PCR reaction to prevent evaporation.
3. Incubated in 65°C water bath or heat block for 90 minutes.

4. After that, 50 µL of PCR product was dropped on sample pad of NALF platform.

reagent	**reaction**	
	1	unit
H2O	25.5	µL
10x buffer	5	µL
MgSO4	2	µL
NaCl	4	µL
dNTP	3.5	µL
fw primer	0.75	µL
rw primer	0.75	µL
Enz mix	3.5	µL
Template	5	µL

Table 7 PCR components in reaction. PCR components in all of HDA-PCR experiments used the same setup based on Biohelix corp. leaflet.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characterization of Gold Nanoparticles (AuNPs)

The sodium citrate-stabilized gold nanoparticles were characterized by UV-visible spectrophotometry and Zetasizer machine. The absorption spectrum of gold nanoparticles is shown in figure 10. The plasmon wavelength (λ_{max}) indicates the size distribution of AuNPs that are in their unique properties. In this study, λ_{max} is approximately 520 nm. These values are in accordance with the colors of AuNP solution observed by naked eyes. These absorption values of gold nanoparticles are similar with other different synthetic methods, as reviewed by Daniel [58].

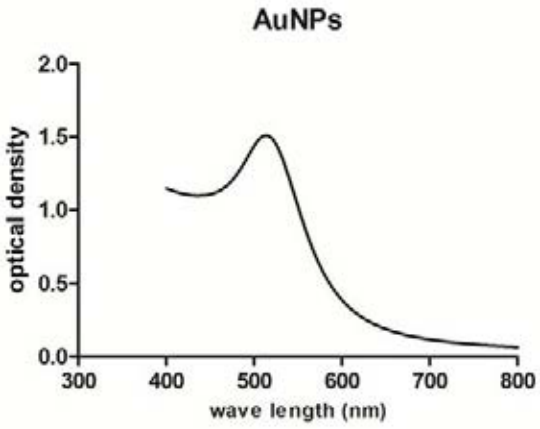

Figure 10 Optical properties of gold nanoparticles. Spectrophotometry results reveal the shift of maximal absorption peak to 540 nm after coating the gold nanoparticle with glutathione, while the citrate-capped AuNPs show 520 nm maximal absorption peak.

For further characterization of gold nanoparticles, zeta potential and particles size measurement were done by the Zetasizer machine. The average gold nanoparticle size is approximately 9.2 nm (Figure 10A) and zeta potential value is -26.8mV (Figure 10B). The zeta potential showed the stability of colloidal gold in solution. According to the guideline [59], optimum line between stable and unstable colloidal solution is generally taken either more than +30mV or less than -30mV. Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable.

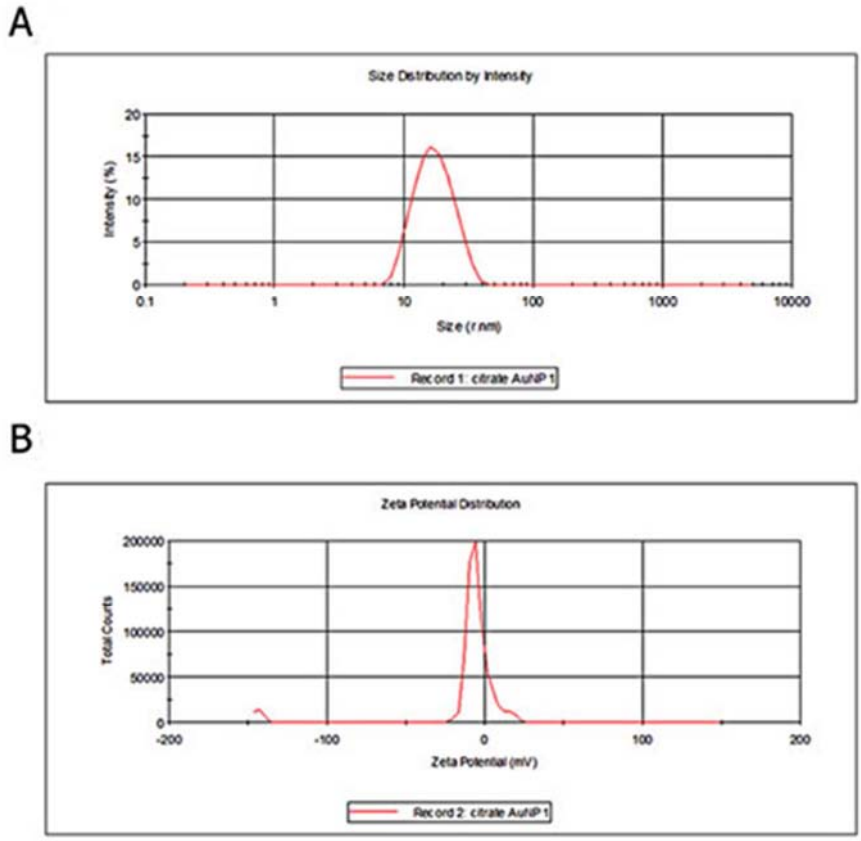

Figure 11 Zeta potential and size measurements. A) Zetasizer reveals that average size distribution of gold nanoparticles is 9.2 nm. B) zeta potential results showed that the average zeta potential is -26.8mV; that zetapotential value was acceptable for use.

4.2 Nucleic acid lateral flow platform setup

Nucleic acid lateral flow platform have been setup using these set of probe. The HIV-1 probe sequences target the *RT* gene. The sequences were based on those that have been used in Chula VCI HIV-1 drug resistance service and were well validated for specificity before use.

Components	Probe sequences
Conjugate probe	**5'**-Thiol-TGTACCAGTAACATTAAAGCCAGG3'
Test line	5'- GTTAAACAGTGGCCATTGACAGAA GA-Biotin-3'
Control line	5'-Biotin-CCTGGCTTTAATGTTACTGGTACAG-3'

Table 8: Probe sequences that use in NALF platform.

The platform was tested with HIV-1 synthetic target DNA for specificity and reproducibility. 3 sets of platform were prepared and ran with 100nM of synthetic target DNA, 100uM non-target DNA and mixture of synthetic target and non-target DNA in the ratio 1:1000. After running with 4x SSC buffer supplement with 0.5% SDS for 30 minutes, all groups were analyzed for signal level by image J software. Signal levels from groups, target and mixture of target and non-target DNA were positive in test and control line. In contrast, for non-target groups, only control line can be observed. This confirms the specificity of the system.

In addition, mixture of target and non-target DNA mimicked the actual clinical sample [60]. Significant change in signal level in mixture of DNA groups could indicate that non-specific DNA might easily disrupt the system. Results from this experiment revealed that HIV-1 *RT* primers and target sequences selected from the HIV-1 drug resistance service are suitable for setting up this NALF system.

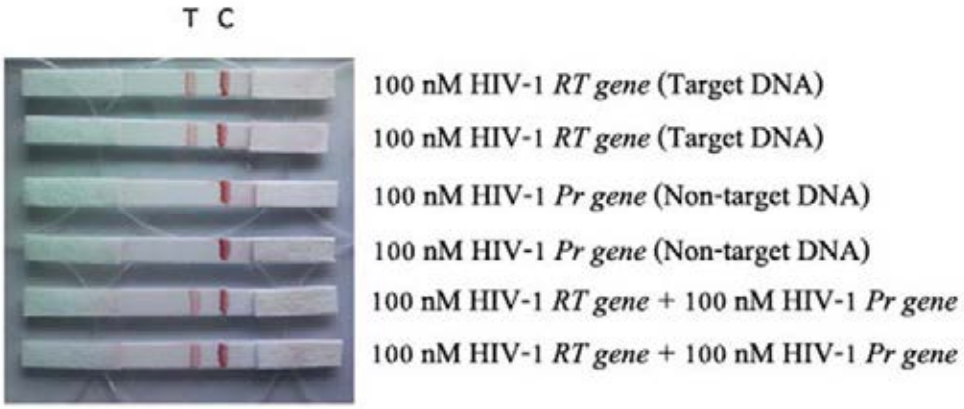

Figure 12 Validation of Nucleic acid lateral flow platform. NALF signal visualization and hybridization specificity: 3 groups of dipsticks are set up for result comparison by using 100nM HIV-1 synthetic target DNA, 100nM non-target DNA and mixture of 100nM HIV-1 synthetic target and 100nM non-target DNA as the tested samples on each dipstick. Photos of all groups were taken in duplicate strips.

4.3 Effect of lateral flow components to signal level

4.3.1 Running buffer concentration

SSC buffer has been tested with lateral flow strip by various concentrations ranging from 1x to 6 x SSC buffer concentrations. All groups are ran with 100nM synthetic DNA and washed by 4x SSC buffer supplement with 0.5% SDS. The running time is 30 minutes. After running with synthetic DNA at desired time, lateral flow strip are being analyzed by image analyzer software. Test line signal from image analyzer software suggested an increase of test line signal level that varied with the buffer concentration. The best signal levels are found in 6x SSC buffer. This finding is in accordance to previous study[8]. However, for practical use, 4x SSC buffer is selected because 6x SSC buffer induces SDS precipitation and increases running time significantly.

Salt concentration in SSC buffer played a major role in hybridization of NALF platform. Higher salt concentrations will cause a decrease in stringency but an increase in background and non-specific signal. In contrast, lower salt concentrations in hybridization buffer will cause an increase in stringency, an increase in specificity but a decrease in signal level[8,61]. Thus, running

buffer concentration level is required to be tested before use. There should be a balance between signal level and specificity.

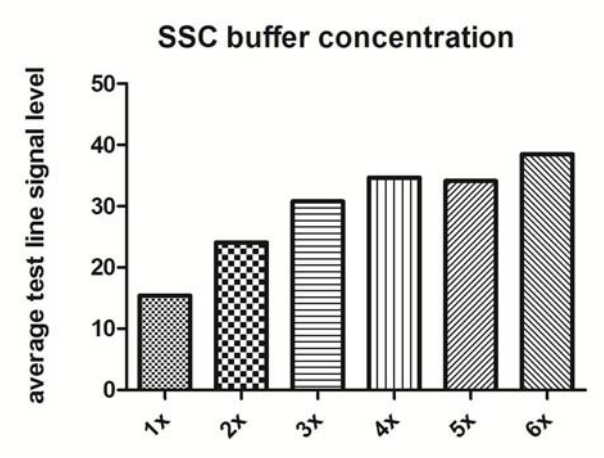

Figure 13 Effect of running buffer concentration on signal level. Platform is ran with 100nM synthetic target DNA and washed by 4X SSC buffer supplement with 0.5% SDS. After running platform for 30 minutes, test line signals were analyzed by image analyzer. Result revealed the best signal level was 6X SSC buffer.

4.3.2 Nitrocellulose flow rate

Nitrocellulose membrane played an important role on signal level of the platform. Nitrocellulose membrane in each series was characterized by flow rate which is the time to hold the analyst sample to appropriate hybridization before going to test line zone. Low amount of DNA sample are more suitable for low flow rate membrane. In contrast, large amount of DNA or protein detection platform were needed for faster flow rate[35]. The optimal nitrocellulose experiments have been performed by duplicate sample. The experiment setups are 2 set of nucleic acid lateral flow with different nitrocellulose membrane. The 2 different membranes are HF240 and HF180. Each platform used the same parameters. They are run with 1uL of 100nM synthetic DNA with 4x SSC supplement with 0.5% SDS for 30 minutes. The results suggested that HF240 nitrocellulose membrane gave higher signal when compared to HF180 membrane. This experiment supported Xun Mao et al.'s results[8]. The nitrocellulose membrane manufacturer gave the explanation of this

result. Using membrane with different pore sizes and properties affects the flow rate. Slow flow rate will provide more time for DNA hybridization with conjugate than fast flow rate membrane[35].

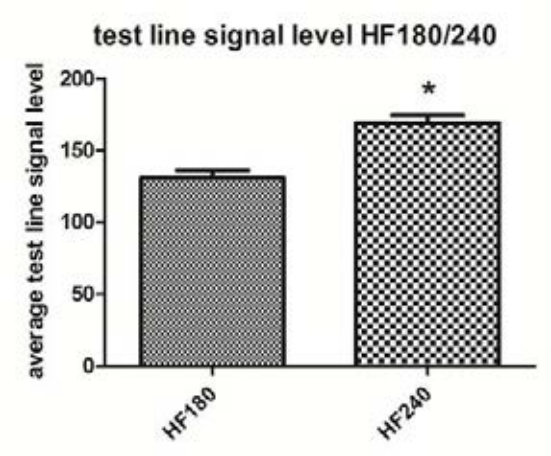

Figure 14 Membrane flow rate comparison. Platform was ran with 100nM synthetic target DNA and washed by 4X SSC buffer supplement with 0.5% SDS. After running platform for 30 minutes, test line signals were analyzed by image analyzer. Result revealed the best signal level was 6X SSC buffer. The different between two groups of platform is flow rate of nitrocellulose membrane. The results suggested HF240 increase signal level significantly.

4.3.3 Dispensing volume on Conjugate probe

The dispensing volume of the conjugate solution controlled the amount of AuNP-DNA conjugating on the conjugate pad. Dispensing time is dependent on the system preference. Thus, users have to determine appropriate dispensing volume by trial and error based on previous literatures. Three different volumes of conjugate probe were tested based on earlier reports; 10, 15, 30 ul of AuNP-DNA conjugate were assembled on NALF platform in duplicates [8,13,15,43,45]. Each NALF strip was ran with 1ul of 100nM synthetic DNA and 4X SSC buffer supplement with 0.5% SDS. After 30 minutes, each strip was measured for signal level by image analyzer software.

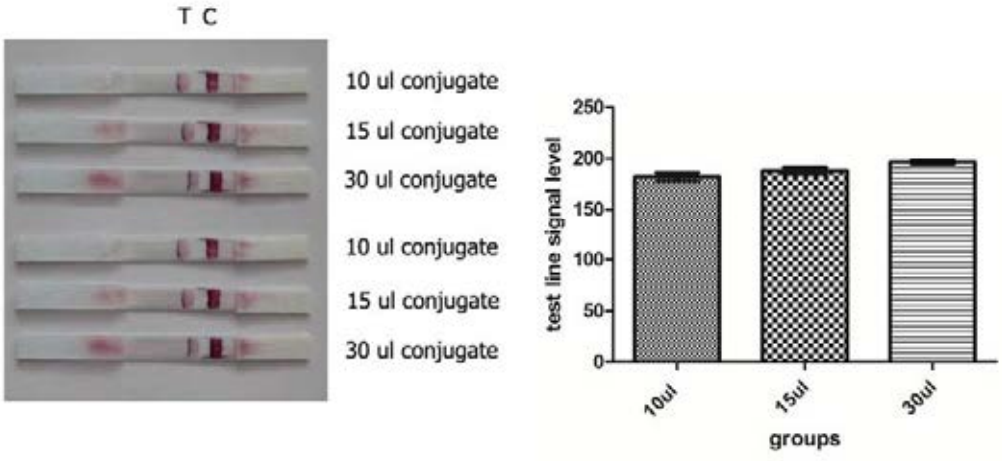

Figure 15 Effect of dispensing volume on test signal level. 3 different dispensing volumes were tested with 100nM HIV-1 synthetic target DNA ran with 4X SSC buffer supplement with 0.5% SDS. The results suggested test line signal level slightly increase when dispensing volume rise.

The results suggested that 10 μL conjugate DNA is enough for running a positive result with clear signal. Dispensing at 15 and 30 μL does not enhance signal significantly as compared to 10 μL platform. Practically, increasing the dispensing volume will increase cost per unit of platform at the rate of about 1 Baht per microliter and also increase time in washing step after running the platform.

4.4 Improve NALF platform sensitivity using UV crosslink

4.4.1 Select UV exposure time

UV exposure has been performed in 2 different groups of NALF dipsticks: 50mJ/cm^2 for 30, 60, 120 seconds, and 125mJ/cm^2 for 30, 60, 120 seconds. The result showed that both UV-exposed groups have clear observable signals at test lines, whereas the control group (no UV treatment) displayed no signal detectable by naked eyes (Figure 15A, 15B). Image analyzer also confirmed that the signal intensifies when more exposure time is increased in both groups. (Figure 15C). In particular, these figures suggest that UV usage on NALF helps to ease naked-eye detection of the low-signal test strip.

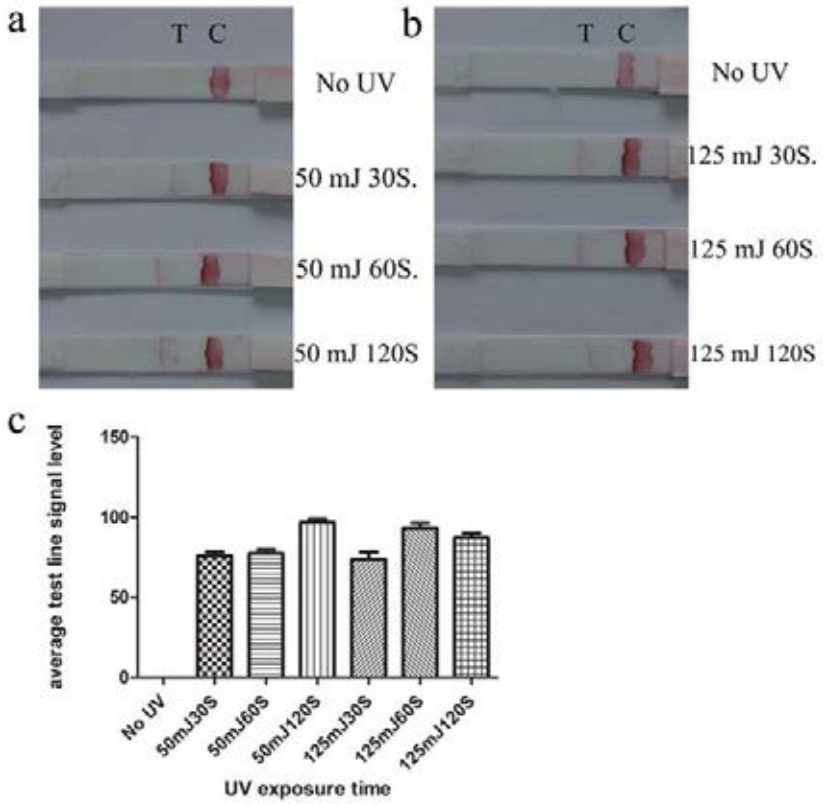

Figure 16 Signal level of NALF with different UV intensities and exposure time. (A) 1 μL of 1nM synthetic target DNA run in each strip using 4X SSC buffer supplement with 0.5% SDS. Test and control line's signals from 50mJ/cm^2 UV-treated NALF at 30, 60, 120 second exposure times was compared with no UV-treated NALF. (B) Same condition but change UV intensity to 125mJ/cm^2 NALF signals from 125mJ/cm^2 UV intensity at 30, 60, 120 second (C) Quantitate signals of image analyzer from each platform.

4.4.2 Selectivity test

NALF test strips were made and exposed to 50 mJ/cm^2 of UV for 30, 60 and 120 seconds respectively. They were compared to NALF with no UV exposure in the same platform. 100nM synthetic target DNA sample was run for 20 minutes on conventional platform strip. The result confirmed that UV-exposed NALF group increased the signal as seen on both the test line and control line (Figure 16A).

For better understanding, it is investigated if the signal outcome after UV treatment is affected by hybridization specificity of the probe-target sequences. Three groups of extracted DNA were set as samples for UV-treated NALF platforms: i) well-matched synthetic target DNA; ii) non-complementary synthetic DNA; iii) mixture of the target and non-target DNA. After 20 min of reaction, the NALF photos were taken. The results revealed that the first and the third groups displayed red bands from control line and test line but the second group has only one band at the control line (Figure 16B). Image analyzer has also indicated that the first and the third groups contain comparable signal intensities at the test line but the second group shows no observable signal from the test line (Figure 16C).

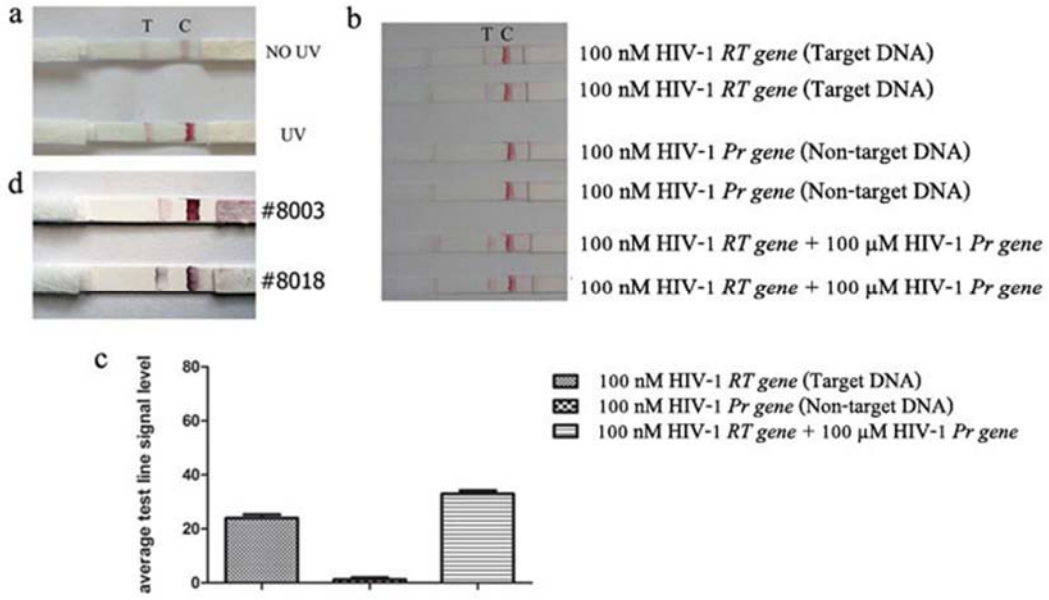

Figure 17 Validation of UV treatment and influencing parameters. (A). Test with different sequences: Signal of NALF platform that is used in *Orientia Tsutsugamushi* detection, UV-exposed group showed stronger band of test line and control line than those with no UV-treated group. (B) *NALF signal visualization and hybridization* specificity: 3 groups of dipsticks with HIV-1 synthetic target DNA, non-target DNA and mixture of target and non-target DNA as tested samples respectively. Photos of all groups are taken in duplicated strips. (C) Test line signal level from dipstick showed no signal in non-target groups and the same level in target and target+non-target groups. (D) Test with long length DNA samples: The signal from dipstick running with 1.3 kb HIV-1 PCR product. Strip No.8003 and No.8018 were performed with the same condition.

UV crosslink is one of the methods for fixing DNA to the membrane. Church G.M. and Gilbert, W. had demonstrated the use of ultraviolet (UV) light to covalently bind nucleic acids (DNA or RNA) to nitrocellulose or nylon type membranes [62]. Mechanism of the crosslinking is that, thymine, when exposed to UV, becomes highly reactive and forms covalent linkages with amine groups on the surface of the membrane. This procedure causes the formation of a stable bond between DNA and nitrocellulose membrane, permitting highly stringent assay conditions without loss of substrate molecules[63]. UV crosslink was used widely in southern blot hybridization to greatly improve sensitivity [64]. In conclusion, UV crosslink might reduce loss of DNA at the test and control lines due to high salt concentration buffer that may cause the formation of bond between the lines and the membrane. In the later experiment, it has been proven that this concept and the results agree with previous literature that UV crosslink can prevent loss of DNA on nitrocellulose membrane[63,64].

However, calculation of copies number revealed that NALF platform required a very high copies number of target DNA to give a positive result [65,66]. For 1μL of 100nM of HIV-1 synthetic DNA (60bp), it is estimated that about $5.9x10^{13}$ copies/mL are needed. The result suggested that this system is not practical for the diagnosis or for treatment monitoring due to the lack of sensitivity.

4.5 Strategies to improve NALF platform sensitivity using Non-labeled oligonucleotides

4.5.1 Optimization UV exposure time

Five different oligonucleotides from specific regions within the RT gene of HIV-1 were used to make the control and test lines. Different UV exposure times were tested: 30, 60, 120, and 300 seconds. Test strip that was not exposed to UV was used as control. To determine the signal level of the platform, an image analyzer software (Scion Image©) was used. Pixel intensity from each test line area was recorded. It is observed that when the UV exposure time was increased to 120 seconds, the intensity of the signal also increased. However, when the UV exposure time was

more than 120 seconds, the intensity of the signal decreased (figure 17). The tests showed that the best UV exposure time in this system was 120 seconds at 125mJ/cm^2.

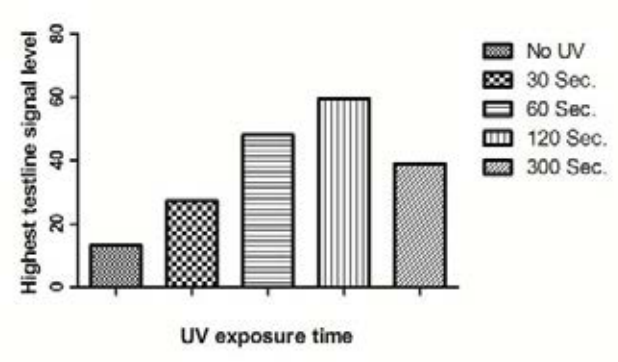

Figure18 Different UV exposure times were tested using our UV crosslink platform. Platform running with 100nM HIV-1 synthetic target DNA. Measurements of the signal were done on the test lines. Data was obtained from image analyzer software. The intensity of the signal level increased when the UV exposure time was increased up to 120 seconds. However, when the UV exposure time was beyond 120 seconds, the intensity of the signal decreased significantly.

This indicates that it is important to find the optimal UV exposure time in order to obtain a strong signal from the test line [67]. It also showed that the intensity of the signal was highly dependent upon the optimal UV exposure time[67]. Sambrook and Russel mentioned that it is imperative to find the appropriate UV exposure time in a series of preliminary experiments[61]. In this study, the result is consistent and reproducible with data obtained from a previous experiment.

4.5.2 UV crosslink efficiency

Next, the efficiency of the UV crosslink in fixing the test and control lines on to the nitrocellulose membrane is tested. In order to assess this, UV exposed and unexposed nitrocellulose membranes with test and control lines were washed in high salt concentration SSC buffer for 3 times. Each test was duplicated with a control (blank nitrocellulose membrane). Total amount of DNA lost from the nitrocellulose membranes were measured by a Nanodrop Machine. Nitrocellulose membranes that were not exposed to UV lost 51.21% of the DNA from the test line whereas those that were cross-linked by UV had a loss of 19.51%. (Figure 18) These results proved that UV crosslinking can firmly fix DNA onto the nitrocellulose membrane and reduce the

concentration of DNA lost to high salt concentration buffer. In addition, discussion about this experiment has been described in section 4.4.2 already.

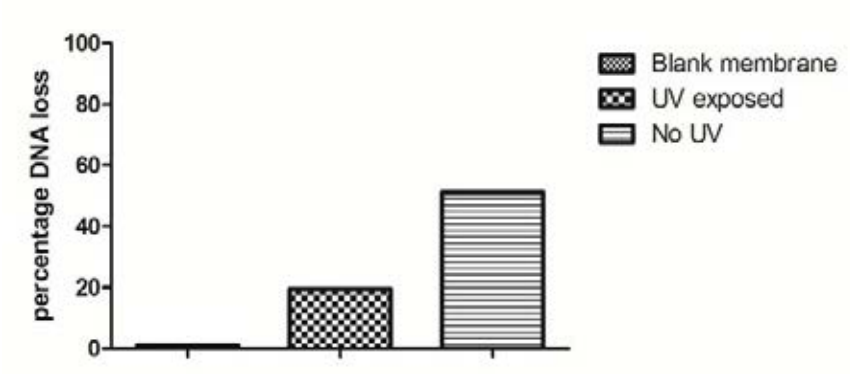

Figure 19 Comparison between UV exposed and unexposed nitrocellulose membranes with test and control lines. The results showed that the UV exposed group can firmly fix oligonucleotides on to the membrane and prevent loss of DNA when washed in high salt concentration running buffer. The UV exposed group had 19.51% loss of DNA compared to 51.21% in the unexposed UV group.

4.5.3 Specificity test

After proving that the test and control lines were firmly attached onto the nitrocellulose membranes, the platform's ability to detect different subtypes of HIV-1 was tested. All specificity tests were done in quadruplicates using mixtures of DNA to stimulate a real life scenario. Each test was composed of three lateral flow strips with 100 nM of target DNA (Figure 19A.a), 100 nM non-target DNA (Figure 19A.b), and DNA mixture (Figure 19A.c; 100nM target DNA and 100uM non-target DNA).

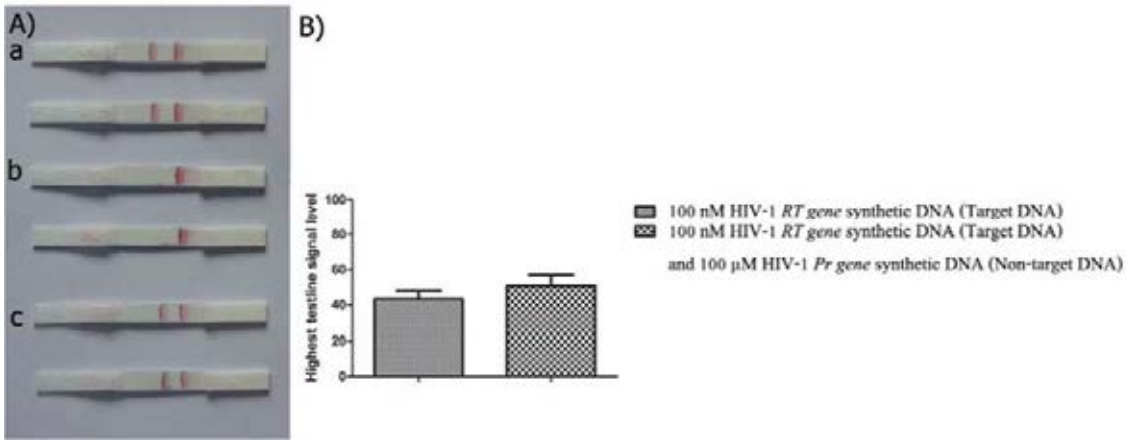

Figure 20 Specificity test of UV crosslink platform using target and non-target synthetic DNA. 3A) Specificity tests were done in quadruplicates. However, the picture here shows results of only 2 replicates from each group tested: a) HIV-1 synthetic target DNA b) non-target DNA and c) mixture of target and non-target DNA (ratio 1:1000). For Figure3B, the bar graph shows the intensity of the signal from the test line measured from the target DNA and DNA mixture groups.

After running the UV cross-linked test strips for 20 minutes, only signals for the test line were detected in the target DNA and mixture groups. In Figure 19A.b, signal for test line is not detected in the non-target DNA group. The intensity of the signal from the test line was measured by the signal analyzer software. The intensity of the signal from the test line was compared between target DNA and DNA mixture groups by using a paired samples t-test (Figure 19B). Signal intensity between both groups were not significantly different ($P>0.05$). This suggested that the non-target DNA mixture does not reduce the test strip's specificity, and this platform has good specificity in detecting HIV-1.

4.5.4 Signal comparison between the direct UV crosslink and streptavidin-biotin method

After analyzing the specificity of the UV crosslink platform, we compared it to the conventional method which used the streptavidin-biotin system. This was done by measuring the

intensity of the signal from the test line of each system. All comparison tests were done in quadruplicates. All the tests used 100 nM of target DNA, 4x SSC running buffer supplemented with 0.05% SDS, and running time of 20 minutes (figure 20A). After 20 minutes of running the tests, the pictures were analyzed by image analyzer software.

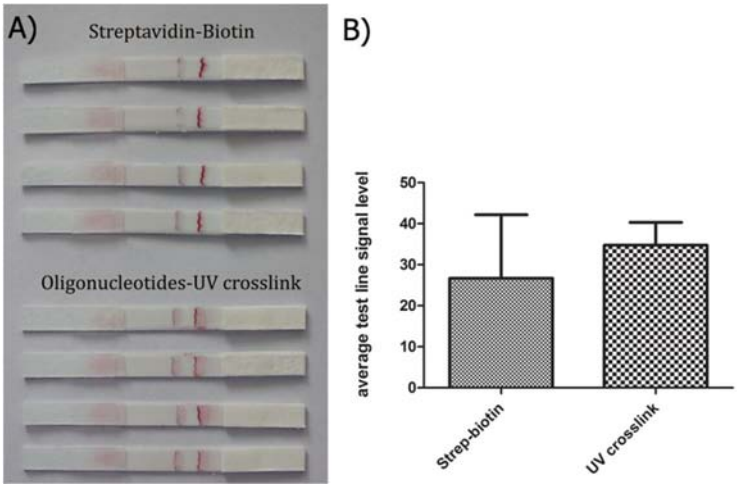

Figure 21 Comparison between the intensity of the streptavidin-biotin (conventional method) and the oligonucleotides-UV crosslink platforms. Figure 20A. A picture of 4 replicates from both platforms using 100fmol of synthetic DNA. From the naked eye, the intensity of the signal from the test line (far left) from both systems yielded similar results. Fig4b. The intensity of the signal from the test line was measured by image analyzer software and plotted as a bar graph. The intensity of the signals from both platforms was comparable.

According to the image analyzer software, the intensity of the signals for the test line from both platforms was comparable. The signal level scores of each replicate were calculated by a paired samples t-test. The signal levels between the streptavidin-biotin and UV crosslink systems were not significantly different with a p-value >0.05 (Figure 20B). Therefore it can be confidently stated that the UV crosslink system can be reliably used as an alternative, cost-effective platform in detecting HIV-1.

In conclusion, a new alternative method for NALF production by using UV light-treated DNA probe without biotin labeling is proposed. This results in reduction of fabrication time and

cost for the test strips. UV treatment for 120 seconds at 125mJ/cm^2 is necessary and sufficient to fix DNA on to the nitrocellulose membrane and this yielded very good intensity and specificity in the HIV-1 NALF model. Cost per unit for chemical part in UV crosslink method was 13.08 Baht and streptavidin/biotin system was 64.68 Baht. UV crosslink method, being cheaper by a whopping 5 times, is able to produce comparable signal level and specificity. Thus, UV crosslink system could be an alternative way for reducing chemical cost in NALF platform.

4.6 Enzyme-linked gold nanoparticle-DNA conjugate (AuNP-DNA-HRP)

Signal enhancement effect on NALF platform

2 sets of AuNP-DNA conjugates with HRP and another 2 sets of AuNP-DNA conjugates without HRP were prepared. Each set was assembled to NALF platform with the same parameters (running buffer concentration, running time, target DNA concentration). 2.5µL of 1nM HIV-1 synthetic target DNA (2.5 fmol) was applied on sample pad in each group. After running with 4x SSC supplemented with 0.5% SDS for 60 minutes, the platforms that used enzyme-linked AuNP-DNA conjugate were impregnated in a signal enhance solution. After 15 minutes, enzyme reaction running buffer rinsed with running buffer for removal of background signal.

Each platform was analyzed by image analyzer software. The results revealed that the signals from AuNP-DNA-HRP platforms gave a significantly greater signal level when compared with AuNP-DNA platforms ($P<0.05$). The test line signal level was increased from 7.3 in non-labeled conjugate to 29.9 in HRP-labeled conjugate. This amounted to approximately 75.58% increase in signal level. The result supported the experiment reported by Xun Mao et al. [8] which showed that enzyme linked gold nanoparticles could increase sensitivity of the nucleic acid detection system. Copy number calculator was used to calculate number of copies in DNA template. 2.5 fmol HIV-1 synthetic DNA was equivalent to 5.9×10^{11} copies/mL.

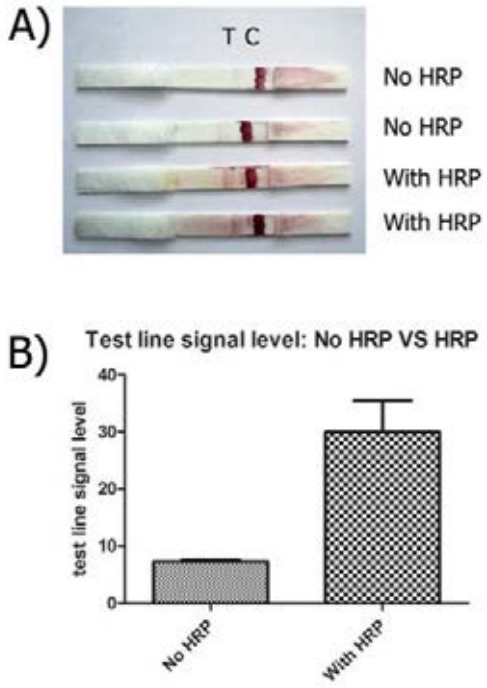

Figure 22 Signal enhancement by HRP labeled gold nanoparticles. This test was done in quadruplicates. However, the picture here shows results of only 1 set from each group tested. Average signal levels between conjugate nanoparticles with and without HRP. Approximately 2.5 fmol of HIV-1 *RT* synthetic target DNA was tested in a quaduplicate sets.

4.7 Helicase Dependent Isothermal Amplification (HDA)

4.7.1 Amplification sensitivity

Helicase Dependent Isothermal Amplification (HDA) PCR reaction set was purchased from Biohelix corp and used following the main protocols from the enclosed leaflet. Primers were designed according to the leaflet criteria as described in table 9. The PCR product region was designed to cover target DNA from previous experiments and meets the criteria of the HDA.

parameters	minimum	optimal	Maximum
Product size (bp)		80-120	
Product Tm (°C)	68	71	75
Primer size (bp)	24	27	33
Primer Tm (°C)	60	68	74
Primer GC%	35	44	60

Table 9 Recommended parameter settings for HDA-PCR reaction.

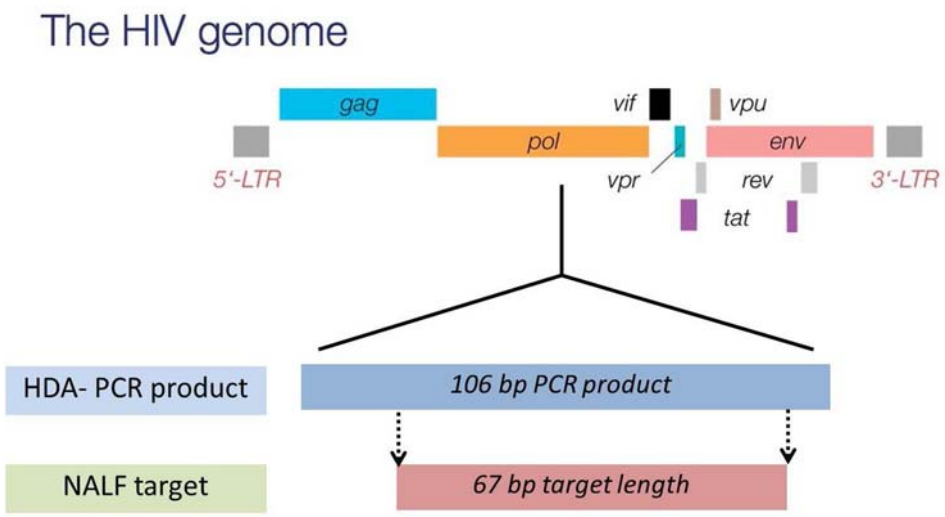

Figure 23 Diagram of slected HDA-PCR product region and target location for NALF.

HIV-1 cDNA clinical samples from Chula VCI HIV-1 drug resistance were used in HDA-PCR for test runs. cDNA sample no. 8712 and 8677 were used for testing system. The sample no.8712 and 8677 had HIV-1 RNA approximately 52,180 and 10,129 copies/mL respectively. A total of the 5 µL of cDNA template was added to the PCR reaction and incubated in waterbath at 65 ºC for 90 minutes. After incubation, 5µL PCR product was detected by gel elctrophoresis by running at 80 Volts for 40 minutes in 2% agarose gel. The results revealed that both HDA-PCR reactions can amplify the HIV-1 cDNA template correctly (Figure 24A). The reaction from sample 8712 has a sightly smear band above 100 bp that may be resulted from high number of DNA templates which lead to non specific amplification[68]. As a result, cDNA sample no. 8712 was

diluted 10 times before bring added to PCR reaction. 5μL of diluted of cDNA sample No. 8712 was added to HDA-PCR reaction and incubated for 90 minutes. The results show only taget band without DNA smear as seen in Figure 24B.

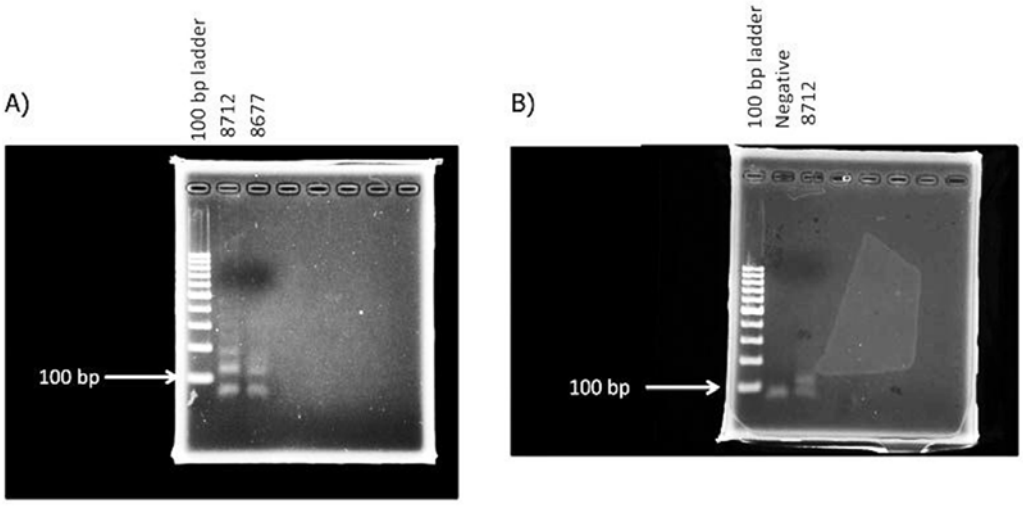

Figure 24 HDA-PCR running with HIV-1 cDNA sample. A) 8712, 8677 cDNA clinical sample that have viral load 52,180 and 10,129 copied/mL were amplified using HDA-PCR which observed 100bp PCR product band. 8712 PCR product has a smear band above 100 bp band. B) After diluting cDNA template sample No.8712 for 10 folds, the PCR product had correct band size and smear band was no longer oberved.

3 sets of HDA-PCR were prepared. Each set used template from HIV-1cDNA clinical samples that were obtained from HIV-1 drug resistance service (sample No.8613). Each set was run with 10 fold series of viral load from $1x10^5$ to $1x10^1$ copies/mL. In addition, the clinical sample input of HIV-1 RNA was approximately 512, 51.2, 5.12, 0.5 and 0 copies per reaction respectively. After the PCR reaction has finished, each tube was run on NALF platform and observed after 60 minutes running. The result revealed that the limit of detection of the system is 1,000 copies/mL.

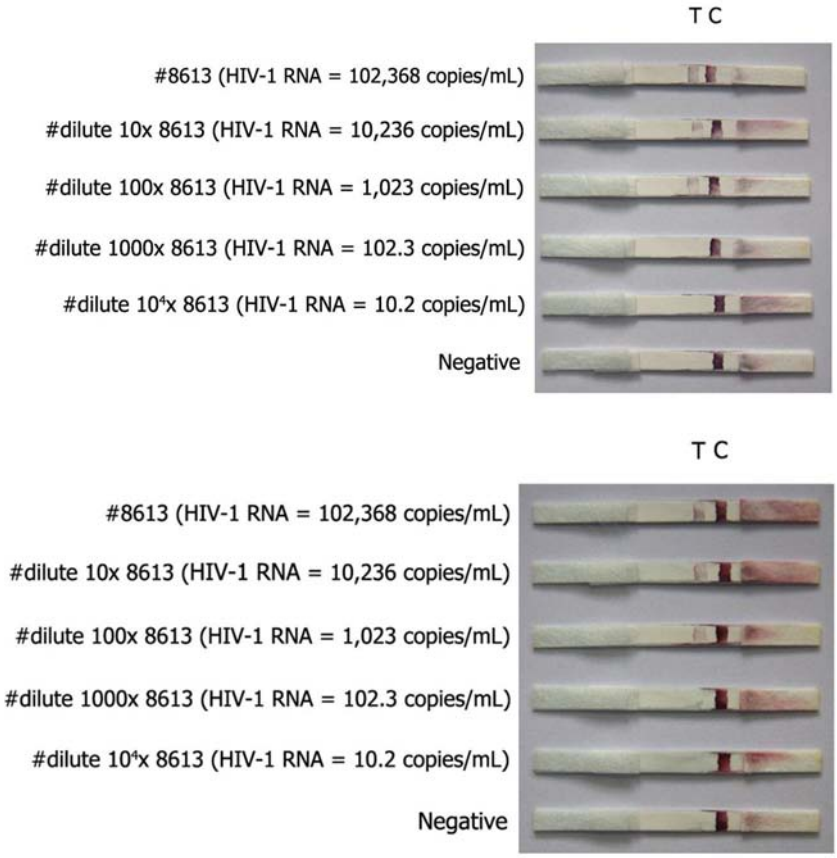

Figure 25 Sensitivity test in cDNA clinical sample. 2 sets of the platforms that showed limit of detection of this system at 10^3 copies/mL. (This is the number of HIV-1 RNA copies that the cDNA derived from.)

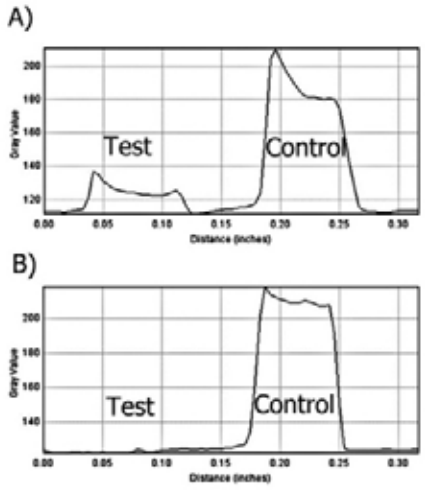

Figure 26 signal from test line and control line from image analyzer software. A) Signal from 10^3 copies/mL, the figure showed two peaks that represent intensity of the signals. The first peak is

test line (note with Test) and control line is the second peak (Control). B) Signal from negative groups revealed signal peak in control line only.

4.7.2 Clinical sample experiments

The other experiment was performed using known viral load cDNA that was obtained from drug resistance service groups in vaccine and cellular immunology laboratory. Viral loads of the samples were described in table 7.

Sample No.	Plasma HIV-1 RNA (copies/mL)
8715	10,129
8734	4,450
8734 (Diluted 10X)	~ 440
8734 (Diluted 100X)	~ 44

Table 10 HIV-1 cDNA information. The table describes cDNA samples that were obtained from drug resistant service and viral load data.

HDA-PCR was performed on cDNA sample by using the protocol described above. After 90 minutes PCR reaction, the PCR products were treated following the protocol from Mao et al. [8] by boiling in water for 5 minutes and cooling on ice for 10 minutes. Then, 50 μL PCR products were dropped on sample pad in each NALF strip and run with 4X SSC buffer supplemented with 0.5% SDS.

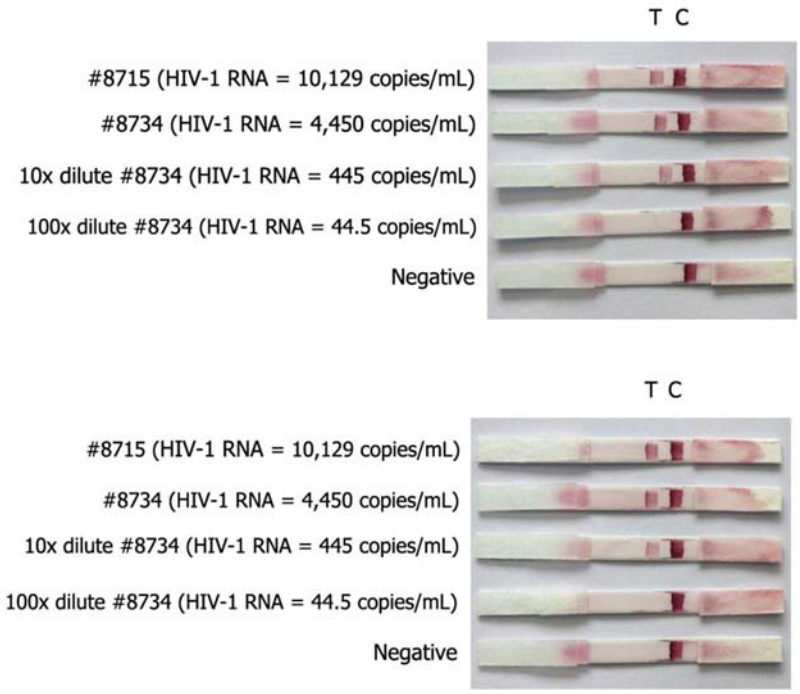

Figure 27 HIV-1 clinical sample tests. Results after running for 60 minutes reveal positive bands in #8715, 8734 and 10X dilution of #8734 and control band only appeared in 100X dilute of #8734 and negative groups.

The results suggested that HDA-PCR in combination with NALF platform can dramatically increase sensitivity to approximately 445 copies/mL or 2.23 copies per reaction of HIV-1 RNA template.

In NALF platform alone, the sensitivity is reported to be 1.25fM by Mao et al. [8] which means that more than million copies are needed to get positive band and that is not practical for real use. The result from these experiments supported the previous data from Tang et al. [54]. They designed HIV-1 DNA detection system which targeted gag region and reported the limit of detection to be 50 copies, 75% of the sample was positive.

CHAPTER V

CONCLUSION

In brief, this study reported that UV crosslinking combined with streptavidin/biotin test line system can increase signal by 30% when compared to the system without UV crosslinking. In addition, UV crosslinking also did not interfere with specificity of the platform. These results suggested that UV crosslinking method could be performed as an additional step to increase sensitivity in streptavidin/biotin test line system.

UV crosslinking and oligonucleotide system can be used for coating of test line and control line probe on HFB membrane firmly with a loss of about 20% of DNA when the lines were washed in 2M NaCl buffer. Specificity of the system was good. No false positive signal in mix DNA type condition was observed. However, this method could not increase test line signal level significantly when compared to streptavidin/biotin system.

Horse Radish Peroxidase linked–oligonucleotide conjugated gold nanoparticles (HRP-AuNP-DNA) can significantly increase the signal level by 3 folds or 300% when compared to non HRP-conjugated groups ($p<0.05$). Finally, Helicase Dependent Isothermal Amplification (HDA) can improve sensitivity of the NALF platform to approximately 445 copies/mL or 2.23 copies per reaction in HIV-1 RNA template.

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APPENDIX

APPENDIX

REAGENTS AND PREPARATIONS

1. Stock 1% hydrogen tetrachloroaurate (100mL)

$HAuCl_4.3H_2O$	1	g
Deionized water	100	mL

2. Stock 38.8 mM sodium citrate (100 mL)

$C_6H_5Na_3O_7.2H_2O$	1.1411	g
Deionized water	100	mL

3. Phosplate buffer saline (PBS)

KCl	0.2	g
KH_2PO_4	0.2	g
NaCl	8.0	g
Na_2HPO_4	1.15	g

Adjust volume to 1,000 mL with distilled water and sterilized by autoclaving for 15 min at 15 psi. pH was adjusted to 7.0-7.4

4. 20X Saline-sodium citrate buffer (SSC)

NaCl	175.3	g
$C_6H_5Na_3O_7.2H_2O$	88.2	g

Adjust volume to 1,000 mL with distilled water and sterilized by autoclaving for 15 min at 15 psi. pH was adjusted to 7.0-7.4

5. NALF Running Buffer (100 mL)

20X SSC buffer	20	mL
Deionized water	75	mL
10% SDS	5	mL

6. 0.1M Phosphate Buffer (100 mL)

1M K_2HPO_4	61.5	mL
1M KH_2PO_4	38.5	mL

7. Salting buffer (100 mL)

Na_2HPO_4	0.0562	g
NaH_2PO_4	0.0125	g
NaCl	5.844	g
Deionized water	100	mL

8. Eluent buffer (100 mL)

Na_3PO_4	0.327	g
BSA	5	g
Sucrose	10	g
Tween 20	0.25	mL
Deionized water	100	mL

9. 0.1M Sodium acetate buffer (100 mL)

1M acetic acid	2.882	mL
0.3M sodium acetate	27.33	mL
Deionized water	100	mL

10. 10X TBE Buffer (1,000 mL)

Tris Base	108	g
Boric acid	55	g
EDTA (pH 8.0)	40	mL
Deionized water	960	mL

11. 10X TE (Tris-EDTA) Buffer (1,000 mL)

1 M Tris-HCl pH 7.5	100	mL
500 mM EDTA pH 8.0	20	mL
Deionized water	880	mL

12. 3-amino-9-ethyl-carbazole (AEC) stock solution

AEC	0.02	g
DMSO	1	mL

13. Signal enhance solution

AEC stock solution	2	mL
30% Hydrogen peroxide	2	mL
0.1M Sodium acetate buffer	96	mL

14. Borate buffer (100 mM borate buffer (pH = 9.5)

H_3BO_3	3.091	g
Deionized water	500	mL

15. Coupling buffer

Na_2HPO_4	4.392	g
NaH_2PO_4	2.291	g
NaCl	5.884	g
Deionized water	500	mL

16. Passivation buffer

Na2HPO4	10.119	g
NaH2PO4	0.449	g
NaCl	4.383	g
Deionized water	500	mL

BIOGRAPHY

Mongkol Pongsuchart was born in Bangkok, Thailand on 12th October, 1985. He graduated with the Bachelor Degree of Science (Biology) from Faculty of Sciences, Srinakarinwirot University in 2007. She enrolled in Master Degree of Medical Science, Faculty of Medicine at Chulalongkorn University in 2007.