การตรวจหาโปรตีน HPV 16 E6 และ HPV 16 L1 ด้วยวิธี Lateral flow

นางสาวพิชญา หุนานนทศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DETECTION OF HPV 16 E6 AND HPV 16 L1 PROTEINS BY LATERAL FLOW ASSAY

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พิชญา หุนานนทศักดิ์ : การตรวจหาโปรตีน HPV 16 E6 และ HPV 16 L1 ด้วยวิธี Lateral flow (DETECTION OF HPV 16 E6 AND HPV 16 L1 PROTEINS BY LATERAL FLOW ASSAY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ภาวพันธ์ ภัทรโกศล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. นพ. อมรพันธุ์ เสรีมาศพันธุ์, รศ. นพ. สมชัย นิรุตติศาสน์, 79 หน้า.

มะเร็งปากมดลูกเป็นโรคมะเร็งที่คร่าชีวิตของผู้หญิงเป็นอันดับที่ 4 ของผู้หญิงทั่วโลก โดยสัมพันธ์กับการคงอยู่ของการติดเชื้อไวรัสแปปิโลมา (Human Papillomavirus; HPV) โดยเฉพาะชนิด 16 ซึ่งพบได้มากกว่าร้อยละ 50 ของผู้ป่วย ในการศึกษานี้พบ HPV DNA ในตัวอย่างป้ายปากมดลูกจำนวน 185 ตัวอย่าง (ร้อยละ 71.71) จาก 258 ตัวอย่างซึ่งแบ่งระดับความผิดปกติเป็น CIN I (ร้อยละ 66.18),CIN II (ร้อยละ 69.57), CIN III (ร้อยละ 81.25) และมะเร็ง (ร้อยละ 79.10) โดยพบความชุกของ HPV 16 ต่อตัวอย่างที่ติดเชื้อร้อยละ 28.11 (52/185) และความชุกสูงสุดพบในตัวอย่างมะเร็งร้อยละ 49.06 (26/53) ปัจจุบันการตรวจคัดกรองโรคมะเร็งปากมดลูกใช้วิธีตรวจทางเซลล์วิทยาและการตรวจหาเชื้อ HPV ซึ่งใช้เวลานาน ต้องการแพทย์ผู้เชี่ยวชาญ และเครื่องมือพิเศษ ระหว่างการพัฒนาเป็นมะเร็งนั้น จะมีการแสดงออกของโปรตีนก่อมะเร็ง E6 ในปริมาณสูง ในขณะที่การพบโปรตีน L1 บ่งบอกถึงระยะการสร้างอนุภาคใหม่ของไวรัส ดังนั้นการตรวจหาโปรตีน HPV 16 E6 น่าจะช่วยในการบ่งชี้ระยะแรกของการพัฒนาเป็นมะเร็ง และการตรวจหาโปรตีน L1 ช่วยในการบ่งชี้ระยะการติดเชื้อของไวรัสได้ เนื่องจากวิธี lateral flow assay เป็นวิธีที่ทำได้ง่าย รวดเร็ว ราคาถูก และไม่ต้องการเครื่องมือในการศึกษาครั้งนี้จึงทำการพัฒนาวิธี lateral flow assay โดยอาศัยหลักการ sandwich immunochromatography ร่วมกับอนุภาคนาโนทองคำที่เชื่อมต่อกับแอนติบอดีเพื่อตรวจหาโปรตีน HPV 16 E6 และ HPV 16 L1 โดยทำการผลิตโปรตีน HPV 16 E6 และ HPV 16 L1 ขึ้นเพื่อเป็นตัวอย่างควบคุมชนิดบวก การพัฒนาวิธี lateral flow assay เพื่อตรวจหา HPV 16 L1 ล้มเหลวเนื่องจากการมีอยู่ของ urea จึงพัฒนาเฉพาะวิธีการตรวจหา HPV 16 E6 เท่านั้น ทำการทดสอบหาสภาวะที่เหมาะสมของแอนติบอดีที่ใช้เชื่อมต่อกับอนุภาคนาโนทองคำ (1-10 µg/ml gold), ขนาดของอนุภาคนาโนทองคำ (10 และ 40 นาโนเมตร), ชนิดของแอนติบอดี (polyclonal และ monoclonal) และสารละลาย blocking (3% PEG และ 10% BSA) สุดท้ายพบว่าใช้แอนติบอดีชนิด mouse monoclonal anti-HPV 16 E6 เชื่อมต่อกับอนุภาคนาโนทองคำที่ความเข้มข้น 10 µg/ml gold, แอนติบอดีชนิด rabbit polyclonal anti- HPV 16 E6 ตรึงบริเวณเส้น test line และแอนติบอดีชนิด goat polyclonal anti-mouse ตรึงบริเวณเส้น control line สามารถสังเกตผลได้ด้วยตาเปล่าภายใน 15 นาที นอกจากนี้พบว่าอนุภาคนาโนทองคำขนาด 40 นาโนเมตรให้ความไวของการทดสอบ (5 µg) มากกว่าขนาด 10 นาโนเมตร (20 µg) และไม่พบความจำเพาะต่อ HPV 18, 39 และ 45 แต่อย่างไรก็ตาม วิธีที่ได้พัฒนาขึ้นนี้มีความไวต่ำมากและไม่สามารถนำไปใช้ตรวจสอบกับตัวอย่างผู้ป่วยได้ จึงเป็นความท้าทายที่จะพยายามพัฒนาวิธีให้มีความไวเพิ่มขึ้นต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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PHITCHAYA HUNANONTHASAK: DETECTION OF HPV 16 E6 AND HPV 16 L1 PROTEINS BY LATERAL FLOW ASSAY. ADVISOR: ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D., CO-ADVISOR: ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D., ASSOC. PROF. SOMCHAI NIRUTHISARD, M.D., 79 pp.

Cervical cancer, the fourth leading cause of cancer death in women worldwide, is associated with the persistence of Human Papillomarus (HPV) infection especially type 16 which covered more than 50% of the cases. In this study, HPV DNA was detected in 185 (71.71%) of 258 cervical swab samples with CIN I (66.18%), CIN II (69.57%), CIN III (81.25%) and SCC, (79.10%). The prevalence of HPV 16 among HPV DNA positive samples was 28.11% (52/185) and the highest prevalence was found in SCC 49.06% (26/53). Nowadays, the screening methods for cervical cancer are based on cytology and HPV detection which are time consuming, require pathologist and special equipments. During cancer development, high expression of E6 oncoprotein is demonstrated while the presence of L1 protein implies the productive stage. Thus detection of HPV 16 E6 might be helpful for early detection of cancer development and that of L1 protein helps in determining the stage of viral infection. Since the lateral flow assay is simple, rapid, cost-effective and no instrument requirement, in this study, a lateral flow assay based on sandwich immunochromatography combining gold nanoparticle-antibody conjugates was developed to detect HPV 16 E6 and L1 proteins. Recombinant HPV 16 E6 and L1 proteins were expressed and used as positive controls in the lateral flow assay. The lateral flow assay for detecting HPV 16 L1 was unsuccessfully developed because of the presence of urea. The developed HPV 16 E6 protein test has been only established. The antibody conjugated with AuNPs (1-10 µg/ml gold), sizes of AuNPs (10 and 40 nm), type of antibodies (polyclonal and monoclonal), and blocking solution (3% PEG and 10% BSA) were optimized. Finally, the mouse monoclonal anti-HPV 16 E6 conjugated AuNPs at the concentration of 10 µg/ml gold, rabbit polyclonal anti- HPV 16 E6 at the captured test line and goat polyclonal anti-mouse at the control line demonstrated the optimal condition providing the signal within 15 min observed by naked eyes. The 40-nm AuNPs gave better sensitivity of detection (5 µg) than the 10-nm AuNPs (20 µg). No cross reactions were found with HPV18, 39, and 45. Although, this developed assay showed very low sensitivity and was unable to test with clinical specimens, the challenge to improve the sensitivity should be further attempted.

Field of Study: Medical Microbiology

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Student's Signature .....

Advisor's Signature .....

Co-Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
ASC	Atypical squamous cells
ATCC	American Type Culture Collection
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
bp	Base pair
CaCx	Cervical carcinoma
°C	Degree Celsius
CFTR	Cystic fibrosis transmembrane conductance regulator
CIN	Cervical intraepithelial neoplasia
cm	Centimeter
DDW	Deionized distilled water
DI	Deionized
DNA	Deoxyribonucleic acid
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme linked immunoassay
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration
GST	Glutathione S-transferase
h	Hour
$HAuCl_4$	Chloroauric acid
HBV	Hepatitis B virus
HCl	Hydrocolic acid
hCG	Human chorionic gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HPV	Human Papillomavirus

HSIL	High-grade squamous intraepithelial lesions
IgG	Immunoglobulin G
IPTG	Isopropylthiogalactoside
KCl	Potassium chloride
kDa	Kilodalton
KH_2HPO_4	Dipotassium hydrogen phosphate
LB	Luria-Bertani
LCR	Long control region
LSIL	Low-grade squamous intraepithelial lesions
M	Molar
mRNA	Messenger ribonucleic acid
min	Minute
ml	Milliliter
mM	Millimolar
MWCO	Molecular-weight cutoff
mV	Millivolt
Na_2HPO_4	Sodium phosphate
NCM	Nitrocellulose membrane
nm	Nanometer
NMSCs	Non-melanoma skin cancers
OD	Optical density
ORF	Opening reading frame
Pap	Papanicolaou-stained
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma protein
rcf	Relative centrifugal force
RFLP	Restriction fragment length polymorphism

RIA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
sec	Second
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris buffer saline
TTBS	Tris Buffered Saline and Tween 20
TEMED	Tetramethylethylenediamine
µl	Microliter
µg	Microgram
URR	Upstream regulatory region
WCL	Whole cell lysate

CHAPTER I

INTRODUCTION

Human Papillomavirus (HPV) belongs to the *Papillomaviridae* family. The viral genome consists of a circular double-stranded DNA that contains several functional early genes (E1-E7), two late structural genes (L1-L2) and noncoding upstream regulatory region. Early genes encode proteins that involve in regulating viral replication, transcription and transformation. The late genes encode the major (L1) and minor (L2) capsid proteins. The E5, E6 and E7 proteins are the oncoproteins that play important role in malignant transforming of the infrectected cells. The E6 and E7 proteins can bind and inhibit function of tumor suppressor proteins p53 and pRB, respectively (1, 2). Several studies indicated that HPV is a causative agent of cervical cancer since Zur Hausen identified the association between HPV infection and cervical cancer in the 1970s (3). HPV DNA is found in more than 90% of cervical cancer (4-6). Up to date, over 170 types of HPV are recognized which 30-40 types are associated with anogenital infection. Approximately 30 types of anogenital HPV are in high risk group especially HPV 16 and 18, which are the most prevalent types at least 70-80% found in cervical cancer (7, 8).

Cervical cancer is the third most common diagnosed cancer and is the fourth leading cause of cancer death in women worldwide. There are more than 528,000 new cases of cervical cancer and approximately 266,000 deaths due to cervical cancer each year. More than 85% of the global burden occurs in developing countries, including Thailand (9). Nowadays, the diagnostic method of cervical cancer is based on the detection of cervical cell dysplasia and HPV DNA or mRNA. The clinical appearance of cervical cancer is observed for changing in cells of transform zone of cervix by cytology and histology. However, this method has some limitations. The results take time and false negative rate as high as 20-30% has been reported due to clumping cells. In contrast to HPV DNA detection, the method has high sensitivity and specificity but requires special instruments, experience technician and very expensive (10, 11). Moreover, the presence

of HPV DNA does not always correlate to the cancer development. Previous studies strongly indicated that HPV E6 oncoprotein is overexpressed in cervical cancer cells (12); therefore a test to detect HPV E6 mRNA has been developed for early detection of cancer development stage. The recent FDA approval of Aptima HPVE6/E7 RNA test is a significant application of E6/E7 as specific markers for cervical cancer screening (13). However, RNA is inclined to degrade and its detection requires expensive instrument and complicated procedures. Besides mRNA, the protein is also another target for cancer screening test. The screening test based on the direct detection of E6 oncoprotein may have advantages for early detection of cancer development. In addition to L1 protein detection, it implies the productive stage of infection.

According to Kamonwan (2009), the immunogoldagglutination assay was developed for detecting HPV16 E6 and L1 protein directly from clinical specimen and the result was visibly detected by an agglutinate of the reaction. With nanotechnology, the assay used gold nanoparticle (AuNPs) conjugated with either HPV16 E6 or HPV16 L1 polyclonal antibodies(14). However, this assay has low sensitivity and takes time (overnight). Thus, this study aims to improve the sensitivity and time of the detection by using the lateral flow assay instead of agglutination assay.

The lateral flow assay based on the principle of immunochromatography exists for a wide array of target analytes. The first test was made for the detection of human chorionic gonadotropin (hCG) (15). This method is simple, rapid, cost-effective and possibly future to be point-of-care test especially combining with gold nanoparticles, which are the most frequently used for developing biomarker platforms especially in clinical diagnostics. Gold nanoparticles have high surface areas and unique physiochemical properties. Several approaches are based on color changes of AuNPs upon aggregation. In addition, it can be tailored with a wide variety of surface functionalization to selectively bind biomarkers and also be coupled with metal deposition for signal enhancement (16-18).

The purpose of this study was to develop a lateral flow assay using AuNPs-conjugated antibodies against either HPV 16 E6 or L1 proteins.

CHAPTER II

OBJECTIVE

The objective of this study is to develop the gold nanoparticles lateral flow strip assay for detection of human papillomavirus type 16 E6 and L1 proteins.

CHAPTER III

REVIEW OF LITERATURE

Virology of Human papillomaviruses

HPV is a small DNA virus belonging to the *Papillomaviridae* family. The virus is a non-enveloped, icosahedral symmetric virus about 55 nm in size. The HPV genome is a circular double-stranded DNA approximately 8 kb in length and divided into three regions, i.e., Long control region (LCR), early region, and late region (Figure 1). A noncoding upstream regulatory region (URR) or long control region (LCR) contains the origin of replication and transcription factor binding sites for repressors and activators that regulate DNA replication by controlling the transcription of opening reading frame (ORF). An early region, encoding of early proteins (E1, E2, E4, E5, E6, and E7), plays role in viral replication, transcription and oncogenesis especially E5-E7. A late region encodes two structural proteins for the viral capsid, i.e., major capsid protein (L1) and minor capsid protein (L2). The function of HPV genes are listed in Table 1 (1, 2).

HPV can be classified into various types based on the degree of genetic relatedness. HPV type is defined as having less than 90% nucleotide sequence homology of the L1, E6 and E7 ORFs to any other HPV types. Up to date, more than 170 types of HPV have been classified on the basis of DNA sequence. An updated phylogenetic analysis using metagenomic sequencing including all 170 HPV types, as well as single animal papillomaviruses, is presented in Figure 2 (7).

Besides typing by genetic similarity, HPV can also be classified according to their tissue tropism, resulting in two groups: cutaneous and mucosal HPV groups. The cutaneous HPV group (such as HPV 2, 5, 8, 24, 27, 57 and 93) (19) is commonly found in several skin lesions such as benign skin warts (20), non-melanoma skin cancers (NMSCs) (21), and also commonly detected on healthy skin (22). The mucosal HPV group is found in the oral mucosa, respiratory tract, conjunctiva, and anogenital tract. Among those anogenital HPVs, they can be divided by their association with cancer in two groups (23).

First, non-oncogenic or low-risk HPV types such as HPV 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73 and 81, can cause benign or low-grade abnormalities of cervical cells, anogenital warts, and a disease of the respiratory tract called recurrent respiratory papillomatosis (RRP). Second, oncogenic or high-risk HPV types including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 can cause intraepithelial neoplasia of the anogenital region, including cervical, vulvar, vaginal, penile, and anal cancers. HPV type 16 and 18 are the most closely associated with cervical carcinoma (1, 7, 23).

The progression of the life cycle of HPV depends on the differentiation of host cell. HPV infects the basal layer of squamous epithelium cells via micro-abrasion and interacts with heparin sulphate proteoglycans (24, 25) as a specific viral receptor on host cell surface. At the initially infected state, the nonproductive stage is usually found, the viral DNA is maintained in the nuclei of infected basal epithelial cells as a low copy number (50 ～ 100 copies per cell) (26). After leaving the basal membrane, the infected cells initiate the differentiation program. The E1 and E2 viral proteins are essential and play role in maintenance the viral DNA as episome. In the upper layers of the stratified epithelium, the viral genome is replicated to a high copy number per cell (27). When host cells proliferate to terminal differentiation, the synthesis of capsid proteins is triggered. The genomes are encapsidated to form progeny virions. Finally, the virions release out of the cells by cell lysis. The shed virions can re-infect the basal epithelium or spread to new hosts (Figure 3) (6, 28, 29).

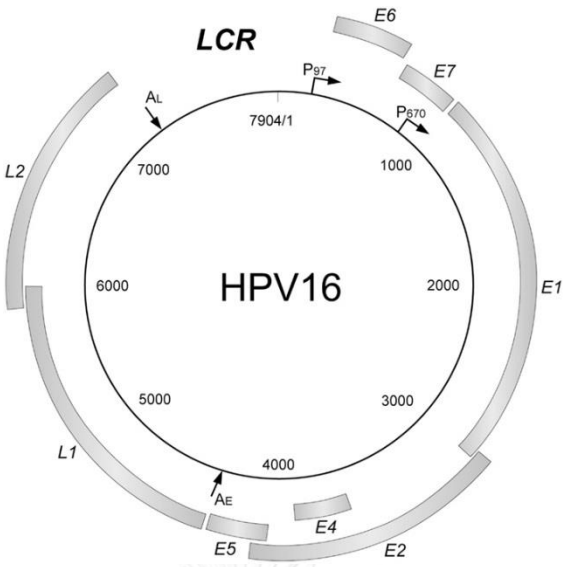

Figure 1 Map of HPV type 16 genome. LCR: Long control region; E1-E7: Early region; L1-L2: Late region (30).

Table 1 Human papillomavirus gene functions

gene	Function
E1	Helicase, ATPase, ATP-binding protein essential for viral DNA replication
E2	Viral transcription factor Binding E1 to facilitate initiation of viral DNA replication important in genome encapsidation
E4	Interacts with cytoskeletal proteins, allows viral assembly
E5	Weak transforming activity, up-regulates growth factor receptors
E6	Binds p53, directs p53 ubiqutin-mediated degradation with E7 immobilizes primary keratinocytes
E7	Binds retinoblastoma protein, deregulates the G1/S checkpoint
L1	Major capsid protein
L2	Minor capsid protein

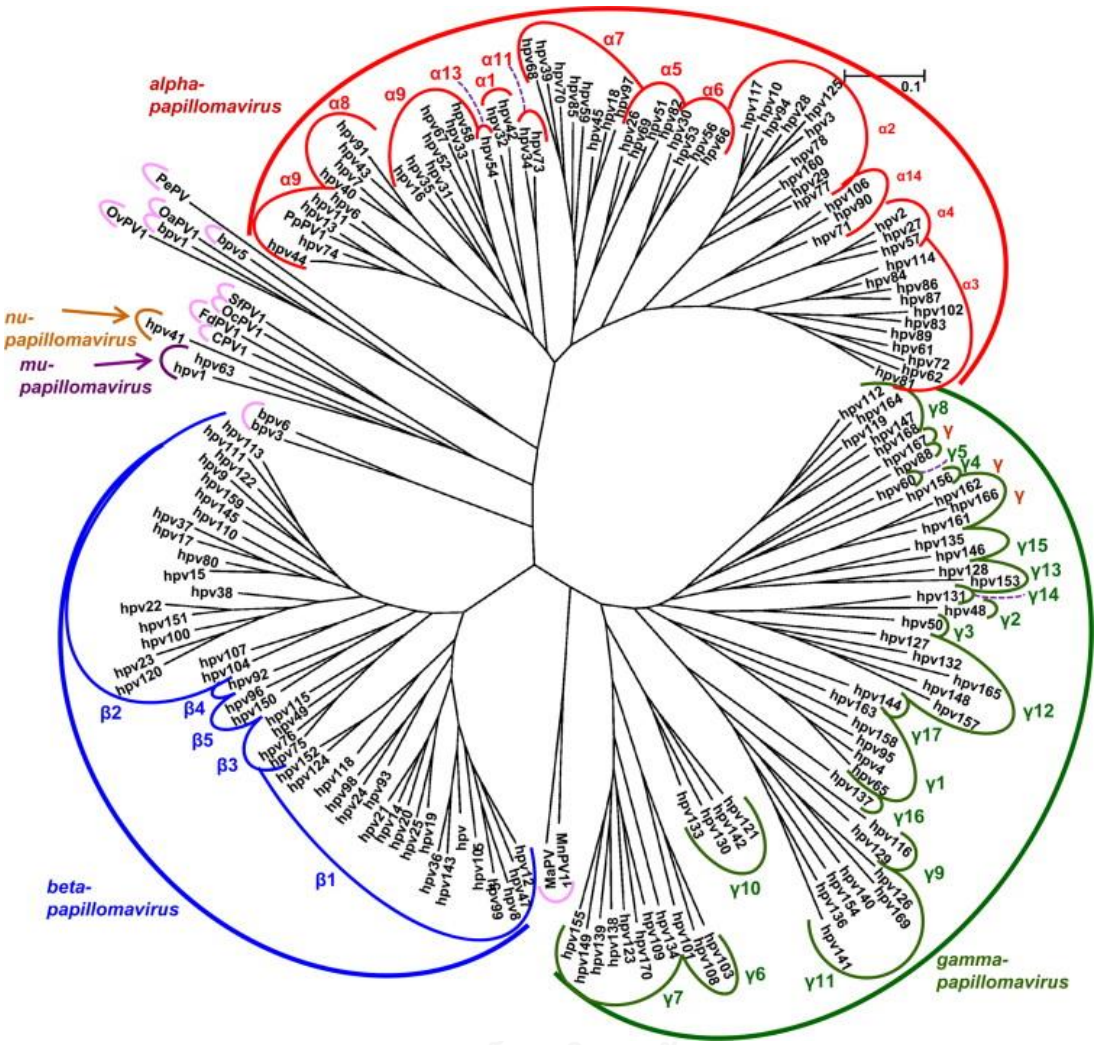

Figure 2 Phylogenetic tree containing the sequences of 170 HPV types, single animal papillomaviruses and newly identified human papillomaviruses using metagenomic sequencing (7).

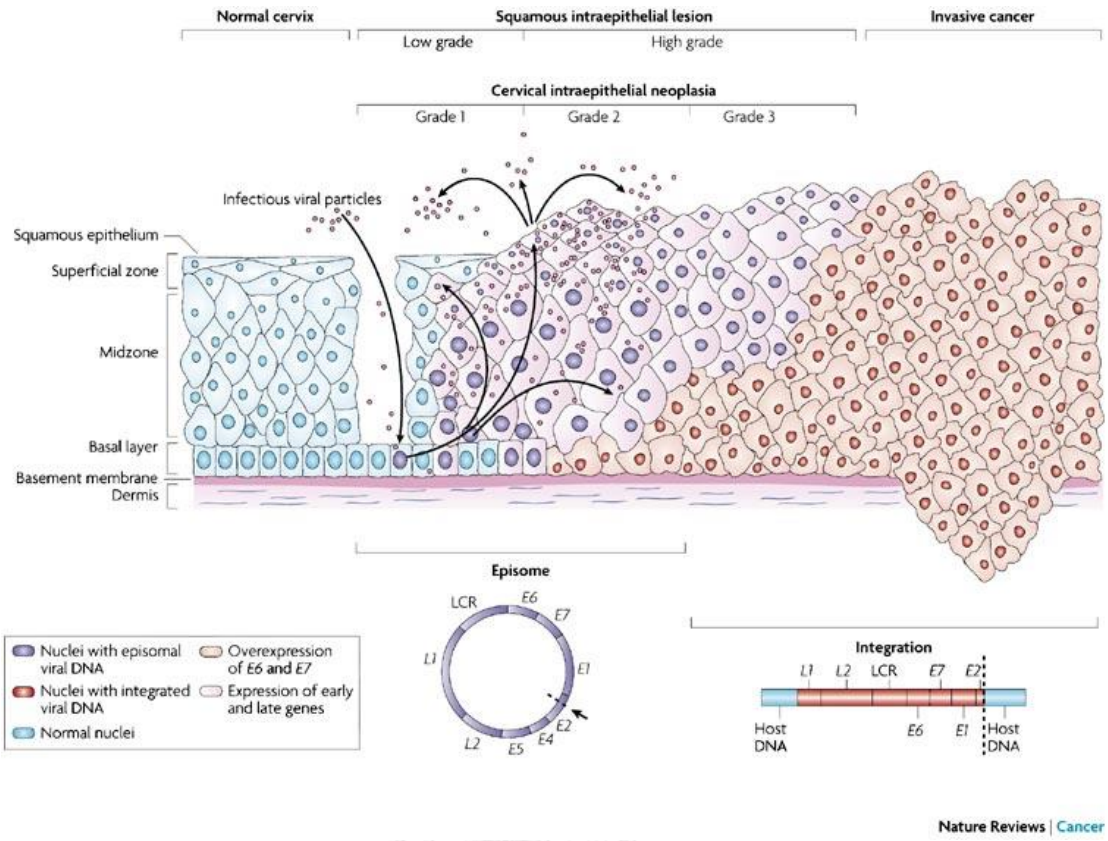

Figure 3 HPV mediated progression to cervical cancer. Uninfected epithelium is shown on the left and HPV infected epithelium is shown on the right. HPV is thought to access the basal cells through micro-abrasions in the cervical epithelium. In the nonproductive stage, early genes are expressed and viral DNA replicates from episome in the lower layers of epithelium. The productive stage occurs in the upper layer (the mid and superficial zone). The late genes are expressed and immensed DNA replication. L1 and L2 encapsudated newly synthesized viral genomes. The virus that is shed can re-infect the basal epithelium or spread to new hosts (6).

Pathogenesis of HPV infection

Transmission of HPV occurs primarily by skin-to-skin contact. All HPV types have a high degree of tissue specificity. Squamous epithelial cells are target of HPV infection. The beginning of HPV replication begins with viral entry through to require mild abrasion or microtrauma of the epidermis. In the basal layers, the viral replication is considered and established itself by using the host DNA replication. The early proteins are expressed and the viral DNA replicates from episomal DNA. In the upper layer, the virus synthesizes late proteins, as the capsid proteins, and viral assembly occurs. HPV infection has been associated with benign and malignant lesion. In benign lesion, HPV DNA is not integrated to the host DNA but rather persists as extrachromosomal (episomal) DNA, whereas in most malignant lesion, the HPV DNA integrate into the host cell chromosome. Most of integration occurs in E1/E2 region of HPV genome leading to loss function of E2 protein which plays important role in regulating the expression of E6 and E7 proteins. Under this condition, the deregulation of E6 and E7 genes result in increased E6 and E7 proteins expression leading to cellular transformation (Figure 3). The E6 and E7 oncoproteins play a central role in HPV dependent malignant transformation. The E6 and E7 proteins are able to bind and inactivate tumor suppressor proteins, p53 and pRB respectively, and also cell cyclins and cyclin-dependent kinase (1, 31, 32). In high-risk HPV types, the E6 and E7 proteins have a high affinity for p53 and pRB resulting in disruption the normal functions of these cellular proteins leading to an increased proliferation rate, cellular immortalization and genomic instability (Figure 4) (33, 34). In addition, potential mechanisms contribute to transformation in cellular pathway including methylation of viral and cellular DNA, telomerase activation, hormonal and immune factors (34, 35). Clinical and histopathologic evidences of HPV infection usually develop one to eight months after initial exposure. However, the progression and outcome of HPV infection depend on HPV type, location of infection, and tissue tropism (4).

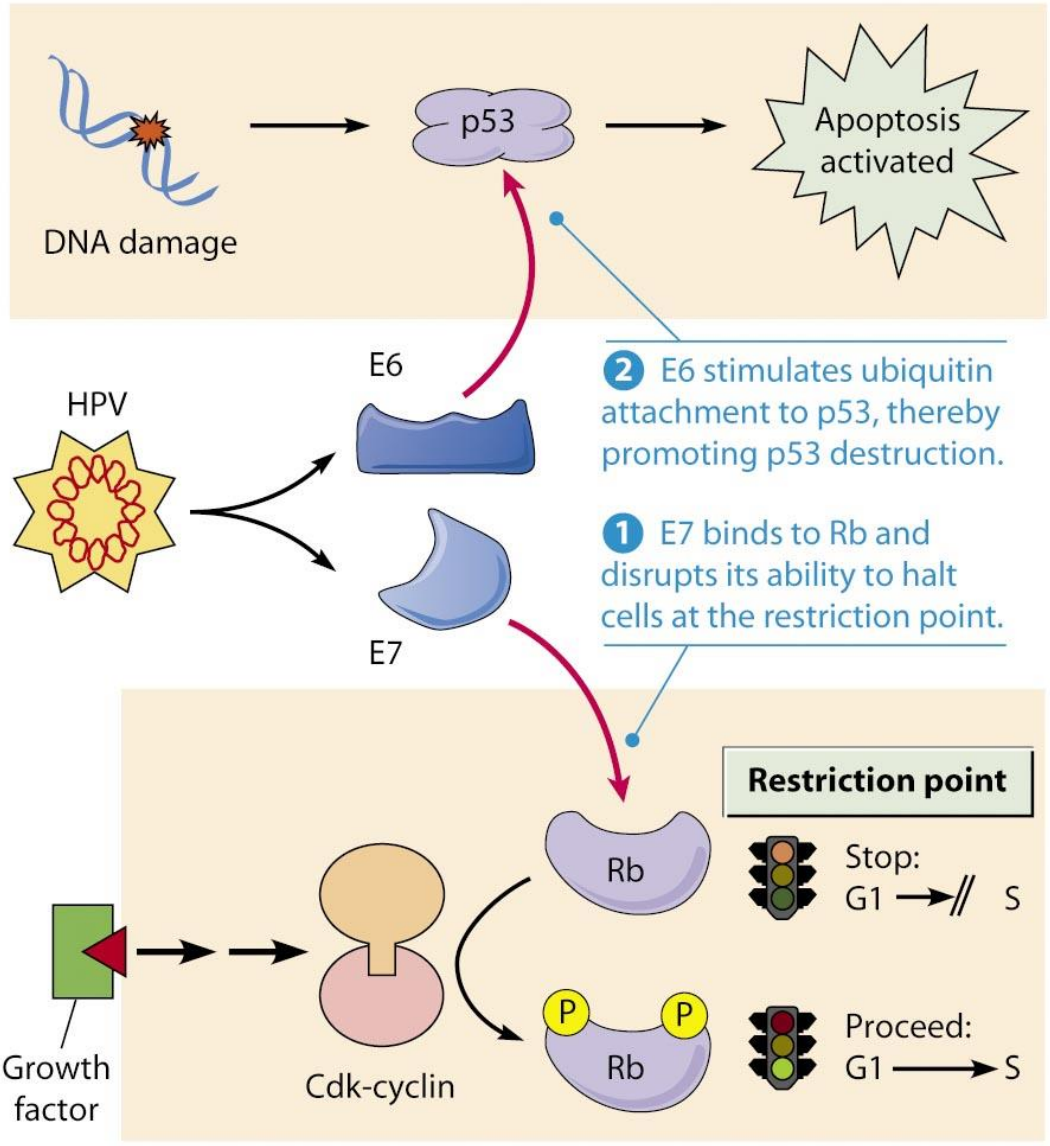

Figure 4 The E6 protein of HPV can bind with p53 and accelerate degradation of p53 via ubiquitin protease system. The E7 protein can disrupt the complex between pRB and E2F. The complex of E7/pRB releases the E2F to free form and continues its function as a transcription factor to activate DNA synthesis and cell proliferation. (From http://liveonearth.livejournal.com/612978.html accession date July 16, 2015)

Diseases and clinical manifestations

Most HPV infections are asymptomatic and result in no clinical disease. Clinical manifestations of HPV infection are associated with a wide range of disease processed, from benign verrucae vulgares, respiratory laryngeal papillomatosis, and condylomata acuminata to the malignancies of the cervix, anus, vagina, vulva, penile, and some head and neck. These variety of skin manifestations depend on HPV type (Table 2) (36). Diseases associated with HPV can be divided into skin and mucosal lesions of the genital and extragenital regions.

Anogenital HPV infection is recognized as the cause of anogenital warts which are usually found at penis, scrotum, pubic region, under the prepuce, glans and coronae sulcus, and rectal area in men, while in women are on vulva, vagina introitus, perineal area and cervix. Mostly isolated types are HPV 6, 11, 16 and 33 which are in low-risk group. The precancerous lesions represent dysplasia and atypical mitoses of basal cells which correlated with high-risk HPV infection in 90% of cases. Moreover, HPV infection plays role in the development of anogential cancers. The most common HPV types that associate with cervical cancer are high-risk HPV types 16 and 18 (34, 36-38).

Nongenital HPV infection is caused by cutaneous and mucosal HPV types. The most commonly seen cutaneous HPV lesion is the common wart or verruca vulgaris, caused by several low-risk HPV types, such as HPV 1, 2, 4, 27, and 57. Warts can occur anywhere on the skin surface. Other types of lesions include Epidermodysplasia verruciformis (EV), autosomal-recessive genetic disorder characterized by impaired cellular immunity to HPV infection. More than 90% of cases contain HPV types 5 or 8 (39). In addition, HPV infection can induce malignant mucosal lesions of oral cavity, respiratory tract, esophagus, and eyes. The HPV type 1-4, 6 and 11 are most commonly found in benign skin lesions, while type 16 and 18 are most found in cancers. (36-38, 40)

Table 2 Clinical manifestations associated HPV types

Manifestation	HPV types
Anogenital lesions	
Genital wart (condylomata acuminata)	6, 11, 30, 42, 43, 44, 45, 51, 52, 54
Bowenoid papulosis	16, 18, 34, 39, 42, 45
Bowen disease	16, 18, 31, 34
Gigantski kondilom (Buschke-Löwenstein)	6, 11
Low-grade intraepithelial neoplasias	6, 11, 43
High-grade intraepithelial neoplasias	16, 18, 31, 33, 35, 39, 42, 44, 45, 51, 52,
Invasive cancer	53, 56, 58, 59, 62, 66
Nongenital skin lesions	
Common wart (verrucae vulgaris)	1, 2, 4, 26, 27, 29, 41, 57, 60, 63, 65
Plantar wart	1, 2, 4, 63
Flat wart (verrucae plana)	3, 10, 27, 28, 29, 38, 41, 49
Mosaic wart	2, 27, 57
Epidermodysplasia verruciformis	3, 4, 5a, 5b, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, 49, 50, etc.
Nonmelanoma skin cancer (basal cell carcinoma, squamous cell carcinomas, Bowen's disease)	8, 15, 20, 23, 36, 38
Dysplastic melanocytic naevi and melanoma	2, 16, 33
Nongenital mucous lesions	
Recurrent laryngeal papillomatosis	6, 11
Squamous cell lung cancer	6, 11, 16, 18
Laryngeal cancer	16, 18
Focal epithelial hyperplasia (Heck disease)	13, 32
Conjuctival papillomas	6, 11
Oral warts	2, 4
Oral condyloma	6, 11
Florid oral papillomatosis	6, 11

Cervical cancer and HPV infection

Cervical cancer is a disease in which malignant neoplasm cells develop in tissues of the cervix. It may begin as asymptomatic pre-cancerous lesions and develops into invasive cancer over many years. According to the data from Globocan 2012, cervical cancer is the fourth most common cancer in women worldwide, with approximately 528,000 new cases and about 266,000 deaths in each year. More than 85% of the global burden occurs in the developing countries including Thailand (Figure 5) (9). It is now clear that HPV persistent infection is required for the development and maintenance of cervical cancer, and HPV DNA is found in almost all cervical cancers. These cancer-associated HPVs are classified as high-risk HPV types, which HPV16 and 18 are responsible for about 70% of the cervical cancers. HPV16 is the most prevalent high-risk HPV in the general population, and is responsible for approximately 50% of all cervical cancers (8).

The clinical appearance of cervical cancer is based on cytological and colposcopic examination. Cervical intraepithelial neoplasia (CIN) system is classified according to the abnormalities of cervical epithelium detected by histological examination of tissue biopsies into three grades; grades I to III are used to describe the proportion of the thickness of abnormal epithelium cell. CIN I is referred to mild dysplasia and CIN II/III are referred to moderate or severe dysplasia (Table 3) (10, 41). The Bethesda System is classified as grades by cytological examination of cervical smear indicating how much the cervical epithelium is affected and how abnormal cells appear, i.e., atypical squamous cells (ASC), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and carcinoma (Table 3) (10, 41)

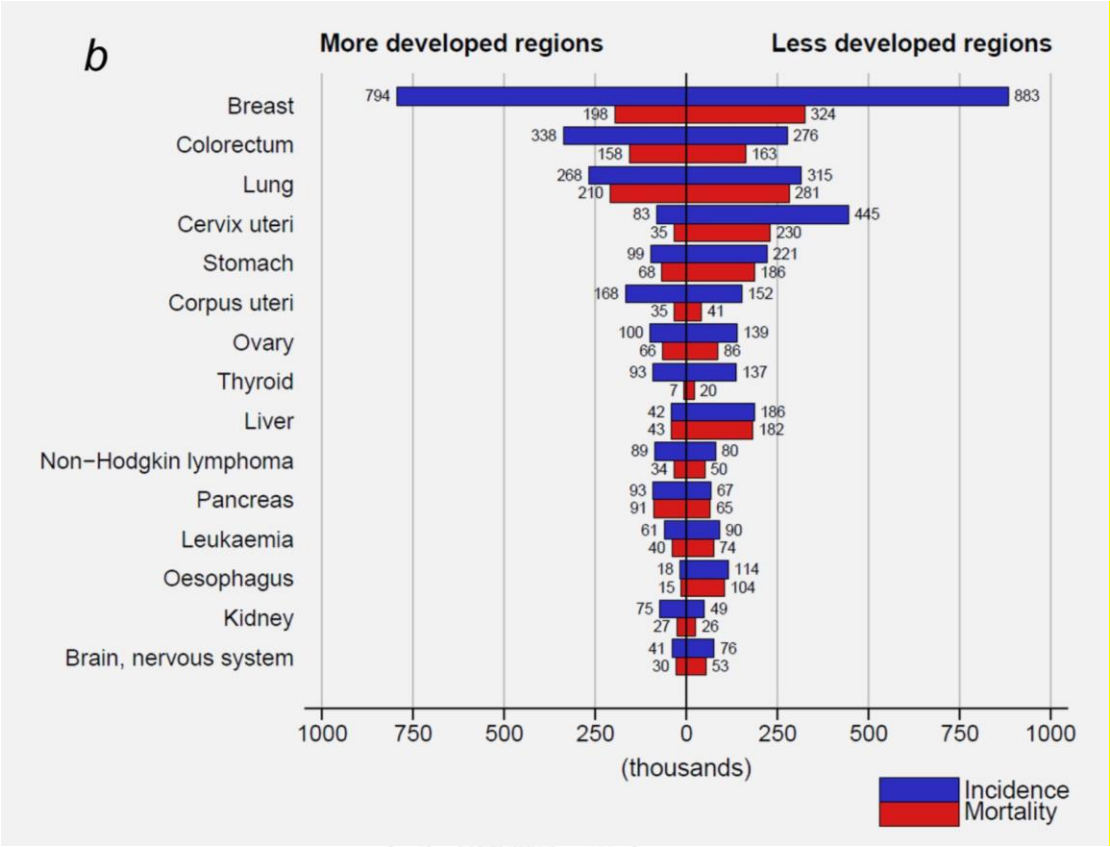

Figure 5 Data from Globocan 2012, cervical cancer is the fourth most common cancer in women worldwide. Estimated numbers (thousands) of new cancer cases (incidence) is shown in blue bar and deaths (mortality) in red bar of women in more developed and less developed regions.

Table 3 The Bethesda Classification System for cervical squamous cell dysplasia (10)

Bethesda System	CIN System	Interpretation
Negative for intraepithelial lesions or malignancy	Normal	No abnormal cells
ASC		
ASC-US (atypical squamous cells of undetermined significance)		Squamous cells with abnormalities greater than those attributed to reactive changes but that do not meet the criteria for a squamous intraepithelial lesion
ASC-H (atypical squamous cells, cannot exclude HSIL)		
LSIL (low-grade squamous intraepithelial lesions)	CIN I	Mildly abnormal cells; changes are almost always due to HPV
HSIL (high-grade squamous intraepithelial lesions with features suspicious for invasion)	CIN II/III	Moderately to severely abnormal squamous cells
Carcinoma	Invasive squamous cell carcinoma, Invasive glandular cell carcinoma (adenocarcinoma)	The possibility of cancer is high enough to warrant immediate evaluation but does not mean that the patient definitely has cancer

Diagnosis of HPV and cervical cancer

The majority cause of cervical cancer is associated with the persistent infection of HPV high-risk types; thus, the good marker for diagnosis progress of cervical neoplasia is HPV detection. Because HPV cannot be cultured from clinical specimens, molecular method for HPV nucleic acid detection is necessary to confirm the presence of HPV infection. The conventional method for detection of cervical cancer is based on cytology and histology and methods for diagnosis of HPV infection are HPV DNA or mRNA detection, immunocytochemistry for detection of HPV proteins, electron microscopy, and HPV serology (10).

Conventional Cytology

The Papanicolaou-stained (Pap) smear has been cytology formed the conventional method for screening. This method detects cell dysplasia of transformation zone of the cervix. The specimen was spread directly on the microscope slide by hand. The report of Pap smear is based on the classification of the Bethesda system or CIN system. However, this method has some limitations. False negative rates as high as 20-30% have been reported due to the clumping of cells (10).

Monolayer Cytology

Monolayer Cytology is a method of collection and processing of specimens for Pap smears. It was developed to reduce the number of false-negative results. The preservative solution has to be used to collect the specimen. In this method, the cellular structure is better preserved because the cells are immediately fixed. The FDA has approved the devices that used in automatically staining. The results showed statistically significant improvement in the use as diagnostic test with increased sensitivity in detecting the epithelial cell abnormality.

Histopathology

In histopathology, patients with abnormal Pap smear findings who do not have a gross cervical lesion are usually evaluated by colposcopy and colposcopy-directed biopsy. Low-grade and high-grade dysplasias have been detected by colposcopy, but do not detect microinvasive disease. Biopsy can be used to confirm most diagnosis by observing epithelial hyperplasia (10).

HPV nucleic acid detection

The nucleic acid of HPV can be detected in specimens by various methods. The methods can be divided into non-amplified techniques and amplified techniques. Non-amplified techniques such as hybridization assay which is based on the use of labeled probes that specifically hybridize to intracellular HPV DNA. The probes are labeled with radioactive or non- radioactive compounds. However, the sensitivity of this method is limited and this technique is time-consuming (42). Southern blot or dot spot hybridization can identify HPV genotypes directly from specimen using type-specific probes in multiple *in situ* hybridization experiments. Nevertheless, the technique is low sensitivity, labor intensive and unsuitable for high throughput screening (43).

Nucleic acid amplification methods have been developed such as type specific primer polymerase chain reaction (PCR) and general primers PCR. Type specific PCR assays are based on the E6 and E7 genes of HPV subtypes. The sensitivity of these assays is 10-200 HPV copies per sample depending on HPV type. General primers PCR using consensus primers amplify a broad spectrum of HPV types in PCR amplification. These primers target conserved regions of the HPV L1 capsid gene. Various methods have been used to identify HPV genotypes after amplification with general and consensus primers such as sequence analysis, restriction fragment length polymorphism (RFLP), and hybridization with type-specific probes. Up to date, several new HPV detection assays have been commercialized incluing Cobas™ HPV test, real-time PCR and CLART HPVDNA genotyping test (Genomica). The Hybrid Capture II

method is a signal amplified technique which uses probe hybridization and hybrid capture. The Hybrid Capture II method hybridizes full-length stabilized RNA probes of high-risk HPV types with the denatured target DNA followed by detection with antibodies and chemiluminescence. In addition, HPV mRNA testing is another promising option with potentially higher specificity and HPV mRNA test (Aptima) is now commercial available (10, 13, 44). The molecular methods for detecting of HPV nucleic acid have high sensitivity and high specificity. However, the method requires special instruments, experience technician and very expensive cost.

HPV Antigen detection

Detection of HPV antigen usually detected the late structural proteins by immunocytochemistry that can be applied to any tissue biopsies. It can confirm the presence of HPV induced morphological changes. These methods are highly specific for L1 capsid protein in superficial epithelial cells, but the sensitivity is less than HPV nucleic acid detection methods (10).

HPV antibody detection

Determination of sero-reactivity against targets other than the viral capsid proteins, such as E6, E2, E4 and E7 proteins, have been established in different formats, including Western blot, enzyme linked immunoassay (EIA), and radioimmunoprecipitation assay(RIA) (45). Although several studied showed HPV antibodies are generally more detectable in patients with HPV associated lesion than in control, HPV antibodies are not detectable in all patients with HPV pathology(10).

Nanotechnology and gold nanoparticles

Nanotechnology is the study of controlling of matter on the nanometer-length scale. It is creation and utilization of materials, devices, and systems within various applications such as in life sciences, medicine, electronic and energy production. Various nanotechnologies and their applications in life sciences have been used in molecular diagnostics under term nanodiagnostics. Molecular diagnostic technologies are used in biological research, detection of bioterrorism agents, clinical diagnostics, drug discovery and development, as well as in monitoring of treatment including novel methods such as gene therapy and RNA interference. The potential applications in molecular diagnostics are listed in Table 4. Nanotechnology is approached to manufacturing nanomaterials at the atomic and molecular level such as nanoparticles. It has a size from 1 to 100 nm in at least one dimension that has high surface area and unique physiochemical properties and easily tuned. It makes them useful tools in diagnostics and ideal candidates for producing biomarker platforms. Gold nanoparticles may improve technique suitable for application in clinical diagnosis with increased sensitivity at lower cost (16). The colloid gold which is directly visible to naked eye can be useful for developing rapid test. The tailoring of gold nanoparticle's surface by fabricating monolayer-protected nanoparticles has been employed in the rational design of peptide capping ligands (17). The proposed combinatorial approach enables the synthesis of exceptionally stable AuNPs with properties in aqueous media that are modulated by the amino acid sequence of the appended cysteine-terminated penta-peptide (18). Conjugation of gold nanoparticles with bio molecule, such as proteins, is synthesized based on the negative charge on the colloidal gold, providing an affinity for proteins that are positively charged as shown in Figure 6A. Some proteins may be attracted to gold surface by hydrophobic binding (Figure 6B) or conducting electrons of nitrogen and sulfur atoms of protein (Figure 6C) (46). We plan to conjugate antibodies against HPV16 E6 and L1 proteins with AuNPs to be used as a reagent for detection of HPV16 E6 and L1 by Lateral flow assay.

Table 4 Nanotechnologies with potential applications in molecular diagnostics (47)

Nanotechnology on a chip
Microfluidic chips for nanoliter volumes: NanoChip
Optical readout of nanoparticle labels
NanoArrays
Protein nanoarrays
Nanoparticle technologies
Gold particles
Nanobarcodes
Magnetic nanoparticles: ferrofluids, supramagnetic particles combined with MRI
Quantum dot technology
Nanoparticle probes
Nanopore technology
Measuring length of DNA fragments in a high-throughput manner
DNA fingerprinting
Haplotyping
Cantilever arrays
Multiple combined tests (such as protein and DNA) to be performed on the same disposable chip
Prostate specific antigen binding to antibody
Resonance light scattering technology
Nanosensors
Living spores as nanodetectors
Nanopore nanosensors
Nanosensor glucose monitor
Optical biosensors: surface plasmon resonance technology
Probes Encapsulated by Biologically Localized Embedding (PEBBLE) nanosensors
Photostimulated luminescence in nanoparticles
Quartz nanobalance DNA sensor
Sensing of Phage-Triggered Ion Cascade

A. ionic interaction B. hydrophobic attraction C. dative binding

Figure 6 Schematic picture of protein adsorption onto colloidal gold particles (46), A: ionic interaction between the negatively charged nanoparticle and the positively charged sites on the protein, B: hydrophobic attraction between the protein and the metal surface and C: dative binding between the metal and the conducting electrons of nitrogen and sulphur atoms of the protein.

Lateral flow assay

Lateral flow assay, also called immunochromatographic assay or strip test, is a technology that is currently widely applied. The test is initially developed as a pregnancy test (15) and has become important for diagnostic purpose, e.g. to determine pregnancy, failure of internal organ (heart attack, renal failure or diabetes), infection or contaminate with specific antigens, presence of toxic compounds in food, feed or environment and abuse of drug (48). The lateral flow assay combining colloidal gold for detection of infectious agents in samples has been published on several applications including bacteria and viruses. Table 5 presents the method application and its sensitivity. The lateral flow device contains labeled detection proteins, such as nanoparticle-antibody conjugates, which will react with a specific antigen as liquid form. The liquid is pulled through the strip by the capillary force and passes through a capture zone where the trapped nanoparticle accumulate in concentration until visually detectable and results can be obtained within 5-20 min. The most common used label is colloidal gold, while other detection systems are mainly based on fluorescent nanoparticles, dyed latex, and silver or carbon black used in improvement the detection limit (49, 50). The advantage of lateral flow assay includes rapid testing, easy to use, easy interpretation by naked eye, low cost and no instrument requirement. These features make the lateral flow assay ideal for implementing as rapid point of care testing, and field application.

Table 5 The application of lateral flow assay in infectious agent detection

Analyte	Method	Application	Sensitivity	References
PCR amplified product with dA tail of 23S ribosomal RNA *Brucella*-specific IgM antibodies in serum	Colloidal gold labelled oligo dT strands	Bacterial infections in arthroplasty	10 cells of *S. aureus*	(51)
Rotavirus in bovine faeces	Colloidal gold labelled primary antibody	Detection of Rotavirus infection	70% of positives	(52)
Satsuma *dwarf virus* in fruit extracts	Colloidal gold labelled antibody	Detection of virus infections		(53)
Influenza virus (H5N1)	Colloidal gold labelled antibody	Detection of H5N1 infection	1:1000 dilutions of allantoic fluid	(54)
Influenza virus type A and B	Colloidal gold labelled antibody	Detection of Influenza virus type A and B	94.4% compared with viral culture method	(55)
Antibody to *Trichinella* in swine serum	Colloidal gold labelled antigen	Trichinellosis	100% of positives	(56)
Recombinant nucleoprotein of Rabies virus	Colloidal gold labelled antibody	Diagnosis of rabies	95.5% compared with PCR method	(57)

CHAPTER IV

MATERIALS AND METHODS

Part I. Prevalence of HPV type 16 infection in precancerous and cancerous lesions

1. Clinical specimen

A total of 258 samples with histologically diagnosed as 191 CIN (136 CIN I, 23 CIN II, and 32 CIN III) and 67 squamous cell carcinoma (SCC) was recruited. These samples collecting from May 2012 to October 2013 were from the Department of Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All cervical cells samples were collected and stored in SurePath™ sample collection and kept at room temperature (RT) no longer than three months. They were further analyzed for HPV DNA detection and genotyping.

2. Extraction of DNA

DNA from cervical sample was extracted using High pure viral nucleic acid kit (Roche, USA) following manufacturer's instruction. In brief, one ml of cervical sample solution was placed in 1.5 ml microcentrifuge tube and centrifuged at 3000 rcf for 10 min. The cell pellet was resuspended in 200 µl of PBS, then was added with 200 µl of binding buffer supplemented with poly (A) and 50 µl of proteinase K. The suspensions were mixed immediately, incubated for 10 min at 72 °C, then 100 µl of binding buffer was added to with the suspension. The mixture was applied to High pure spin column and centrifuged for 1 min at 8,000 rcf. The spin column was washed with 500 µl of inhibitor removal buffer, centrifuged for 1 min at 8,000 rcf, and washed twice with 450 µl of wash buffer. After centrifugation (1 min at 8,000 rcf), it was centrifuged again for 10 sec at 13,000 rcf. The column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of

elution buffer was added onto the spin column, incubated at RT for 1 min and centrifuged for 1 min at 8,000 rcf. The eluate was kept at -20 °C until use.

3. HPV DNA detection and typing

HPV detection and typing was performed using CLART HPV2 Amplification kit (Genomica, Spain) based on the amplification of a 450 bp fragment within genotype specific HPV L1 region. Moreover, the reaction contains a primer set specific to a fragment of the human gene cystic fibrosis transmembrane conductance regulator (CFTR) for use as a genomic DNA control to confirm the sufficient quantity and quality of samples. The reaction also contains another primer set specific to a modified plasmid used as the amplification reaction control to check the ability of PCR reaction whether it contains DNA polymerase inhibitors. In the reaction, 5 µl of extracted DNA were used as template. The PCR program was started at 95 °C 5 min, one cycle and followed by 40 cycles of denaturing at 95 °C for 30 sec, annealing at 55 °C for 60 sec and extension at 72 °C for 90 sec. A cycle of extension 72 °C for 8 min was added at the end of the program. During amplification the PCR products were labeled with biotin. Prior to visualization, the PCR products were denatured at 95°C for 10 min. Hybridization was performed, using 10 µl of the denatured PCR products, between the amplicon and specific probes on CLART microarray. The results were obtained as an insoluble precipitate of peroxidase that occurred when a streptavidin conjugate bound to the biotin-labeled PCR products. The genotyping results were analyzed on the Clinical Array Reader (Genomica, Spain).

Part II. Preparation of HPV16 E6 and L1 recombinant proteins

1. Plasmid

Plasmid pGEX-3X-MT-E6 containing HPV 16 E6 fused with GST and pGEX-4T-2-L1 containing HPV 16 L1 fused with GST were kindly provided by Prof. Peter C Angeletti, Nebraska Centre for Virology, School of Biological Sciences, University of Nebraska Lincoln, USA. The plasmids were transformed in *E. coli* BL-21(DE3) by Kamonwan

Phlaingam of Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

2. Induction of recombinant proteins

The procedure is followed to Kamonwan (2009). In short, the *E. coli* BL-21(DE3) clones containing pGEX-3X-MT-E6 and pGEX-4T-2-L1 were streaked in Luria-Bertani (LB) agar plus 100 µg/ml ampicillin, and incubated at 37 °C overnight. A single colony was picked up and re-suspended in 20 ml LB broth containing 100 µg/ml ampicillin. The bacterial culture was incubated in shaking incubator at 37 °C overnight. Then, it was transferred into 2 liters of LB broth and continuously grown at 37 °C with shaking until the O.D. value reaches 0.6-0.8 at absorbance wavelength of 600 nm. The E6 recombinant bacteria were induced to express protein by adding 0.25 mM IPTG (isopropylthiogalactoside) and incubating for another 6 h at 25°C. Whereas L1 recombinant bacteria were induced at the condition 1.0 mM IPTG and incubated for 4 h at 37 °C. After that, the bacteria were then harvested by centrifugation at 10,000 rpm, 4°C for 5 min, washed with phosphate buffered saline (PBS), pH 7.4 twice, and kept frozen at -80°C until use.

3. Protein Extraction

The induced bacteria were thawed, resuspended in lysis buffer (see Appendix B) and let the suspension incubating for 20 min at RT, or until the suspension becomes turbid and viscously. Then the suspension was lysed by sonicator (High intensity ultrasonic processor VC505, SONICS) at amplitude of 35% with pulse on 30-sec and pulse off 50-sec for a total of 6 cycles. The supernatant and cell lysate were seperately collected by centrifugation at 17,000 rpm, 4°C for 30 min and kept at -20°C until use or further analysis by SDS-PAGE.

4. Protein Purification

Supernatant containing E6 was further purified by GST fusion protein affinity column purification kit (GE healthcare Co., USA). Before the sample was applied to the

column, it was filtered through a 45 µm filter. The procedure of E6 purification was followed the recommendation of the company. In brief, the column was equilibrated with 5 ml of binding buffer (see Appendix B), then the supernatant containing E6 was applied at a flow rate of 0.2 to 1 ml/min using a syringe fit to the luer connector onto the column. After that, column was washed with 5 to 10 ml of binding buffer at flow rate of 1 to 2 ml/min until no material appears in the effluent. In order to elute E6 protein, 5 to 10 ml of elution buffer (see Appendix B) was added at flow rate of 1 to 2 ml/min. In final step, Amicon® Ultra 15 ml filters [10 kDa molecular-weight cutoff (MWCO) Merck Millipore, USA] were used to concentrate the elution protein. The concentrated protein was collected from the filter device sample reservoir, mix proteinase inhibitor was added (see Appendix B), and kept at -80°C until use.

The L1 pellet was washed with buffer (see Appendix B) and solubilized in 8M urea solution (see Appendix B) at RT for 2 h. Debris was removed by centrifugation at 20,000 rpm, for 30 min at 20°C and supernatant containing L1 proteins was collected. The L1 proteins were desalted and buffer exchanged using Snakeskin dialysis tubing (Thermo scientific, USA) with 10kDa MWCO. After that, the dialysate was applied to Amicon® Ultra 15 ml filters [30 kDa MWCO, Merck Millipore, USA] to concentrate protein. The concentrated L1 protein was then suspended in a mix proteinase inhibitor (see Appendix B) and kept at -80°C until use.

5. Quantitation of proteins

Total proteins amount of E6 and L1 were determined by Quant-iT protein assay kit (Qubit fluorometer; Invitrogen Co., USA). The mixture of the E6 or L1 proteins and Quant-iT buffer were incubated at RT for 15 min. The concentration of E6 and L1 proteins were measured by Qubit fluorometer. The Qubit fluorometer generates concentration data based on the relationship between the three standards that used in calibration which are provided by manufacturer.

6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Purity of fusion proteins E6 and L1 were analysed by SDS-PAGE and Western blot using mouse anti-HPV 16 E6 and L1 monoclonal antibody (Santa cruz and Abcam), respectively. For SDS-PAGE, the solutions of proteins to be analyzed were mixed with 6X SDS-sample buffer (see Appendix B). Samples were heated for 5 min at 95 °C and separated using 10% SDS-polyacrylamide gel (see Appendix B) in running buffer (see Appendix). Following electrophoresis, the gels were either stained with Coomassie blue or transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, UK) by wet system (Biorad, USA) using transfer buffer (see Appendix B). Membrane were blocked with 3% bovine serum albumin (BSA) in Tris buffer saline (TBS) for 1 h at RT, washed three times with TTBS (TBS, 0.1% Tween 20) and incubated with primary antibody using 1:100 dilution of mouse anti-HPV 16 E6 monoclonal antibody (Santa cruz, USA) or 1:5000 dilution of mouse anti-HPV 16 E6 monoclonal antibody antibody (Abcam, USA) and 1:100 dilution of mouse anti-HPV 16 L1 monoclonal antibody (Santa cruz, USA) at 4°C overnight. After washing three times with TTBS, the membrane was incubated with secondary antibody using 1:5000 dilution of goat anti-mouse IgG labeled horseradish peroxidase (HRP) (Santa cruz, USA) at RT for one h. After washing three times with TTBS, proteins were detected by incubating with chemiluminescence (ECL) reagent (Prierce Biotechnology, USA) for 1 min and then exposed to X-ray film (Agfa Drystar DT2, Finemed, Belgium) according to the manufacturer's instructions. In case of L1, the proteins were analyzed by chromogenic detection using H_2O_2 substrate and 3, 3'-diaminobenzidine (DAB) to produce a brown precipitate at the precise site of enzymatic activity on the blot.

Part III. Preparation of control cell lines

1. Control cell lines and culture

CaSki and SiHa, human cervical carcinoma cell lines with about 600 copies and 1-2 copies of HPV 16 DNA were used as positive control cells for the E6 protein. C33A, a human cervical carcinoma cell line without HPV DNA was used as a negative control cell. Cervical HPV containing, except type 16 cell lines (HeLa, ME-180, MS751) and another HPV DNA negative laryngeal carcinoma cell (HEp-2) were used in specificity assay. Details of them were shown in Table 6. All of them were obtained from Virology Unit, Department of Microbiology, Chulalongkorn University. All cells were grown in Eagle's Minimum Essential Medium (GIBCO, USA) supplemented with 10% fetal bovine serum (PAA laboratories GmbH, Austria), 100 unit/ml penicillin, 100 µg/ml streptomycin (Bio Basic Inc., Canada) and 0.01 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) (Sigma Aldrich, USA). When the cells were grown in monolayer, subculturing was done. The culture media was removed and the monolayer cell was washed by PBS (see Appendix B) twice. Then, pre-warmed 0.25% trypsin-EDTA (see Appendix B) was added and incubated for 1-5 min at RT or until most of the cells shrinked and seperated under microscope followed by discarding of trypsin. Cells were detached by gently tapping and growth medium was added. The monolayer cell was subcultured at 2-3 days intervals with split-ratio of 1:3. The cells were cultured at 37 °C in 5% CO_2 air atmosphere.

Table 6 Standard cell lines used in control experiment

Cell lines	Type of cell line	HPV DNA
CaSki	Human cervical carcinoma	600 copies HPV16 DNA
SiHa	Human cervical carcinoma	1-2 copies HPV16 DNA
HeLa	Human cervical carcinoma	HPV18 DNA
ME-180	Human cervical carcinoma	HPV39 DNA, HPV18 DNA
MS751	Human cervical carcinoma	HPV18 DNA, HPV45 DNA
C33A	Human cervical carcinoma	Negative for HPV DNA
HEp-2	Human laryngeal carcinoma	Negative for HPV DNA

2. Cell lysis

Cells were detached from the culture flask by trypsinization and were washed with PBS. The cells were centrifuged for 5 min at 1500 rpm. Then the pellet of approximately 10^7 cells were resuspended with 300 µl of of Lysis-M reagent containing complete, EDTA-free protease inhibitor cocktail tablet (Roche, USA), incubated for 10 min on ice with gentle shaking. The lysate was centrifuged at 14,000 rcf for 10 min. The supernatant containing soluble protein was transferred to a new tube and kept at -20°C until use.

Part IV. Preparation of gold nanoparticles-antibody conjugate

1. Preparation of gold nanoparticles

Unconjugated AuNPs in diameter 10- and 40-nm used in this study are listed as follows.

I. 10-nm AuNPs were purchased from Sigma Aldrich, USA (cat G1527). All unconjugated gold colloids contain approximately 0.01% of $HAuCl_4$ suspended in 0.01% tannin acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide as preservative.

II. 40-nm in-house AuNPs was synthesized and kindly provided from Assist. Prof. Dr. Amornpun Sereemaspun, Laboratory of Nanobiomedicine, Faculty of Medicine, Chulalongkorn University.

III. 40-nm AuNPs were purchased from Cytodiagnostics, Canada (Cat G-40-20). The particles were suspended in 0.1 mM phosphate buffered saline (0.01 X PBS).

IV. 40-nm AuNPs from Arista biologicals, USA were kindly provided by Prof. Dr. Kavi Ratanabanangkoon, Laboratory of Immunology, Chulabhorn Research Institute. The particles were suspended in deionized (DI) water with no preservative.

2. Preparation of antibodies

The antibodies used in this study are listed in Table 7.

Table 7 List of antibodies used in this study

Type of antibody	Company	Concentration
Polyclonal goat anti HPV16 E6 (sc-1584)	Santa Cruz Biotechnology, USA	1-10 µg/ml gold or 1-2 µg/ line
Monoclonal donkey anti-goat (sc-2020)	Santa Cruz Biotechnology, USA	0.5-2 µg/ line
Monoclonal mouse anti HPV16 E6 (sc-460)	Santa Cruz Biotechnology, USA	1-10 µg/ml gold or 1-2 µg/ line
Monoclonal goat anti-rabbit (sc-2004)	Santa Cruz Biotechnology, USA	0.5-2 µg/ line
Monoclonal goat anti-mouse (Pierce 31160)	Pierce, USA	0.5-2 µg/ line
Monoclonal mouse anti HPV16 E6 (ab30716)	Abcam, UK	1-10 µg/ml gold or 1-2 µg/ line
Monoclonal mouse anti HPV16 E6 (ab70)	Abcam, UK	1-10 µg/ml gold or 1-2 µg/ line
Polyclonal rabbit anti HPV16 E6 (ABIN733448)	Antibodies-online.com, Germany	1-10 µg/ml gold or 1-2 µg/ line

3. Optimization of the amount of antibody binding to gold nanoparticles

First, the conditions of AuNPs and antibody were optimized. Flocculation curves were determined for each antibody in order to determine the minimum amount of antibody that stabilizes gold nanoparticle (58). One ml of AuNPs solution (10-nm and 40-nm) was adjusted to pH 8 with 0.2 M Na_2CO_3 and checked by pH paper or pH meter. A range of antibody amounts (final concentration 0 to 10 µg/ml gold) was added to 100 µl of AuNPs. After that, the mixtures were incubated for 10 min at RT, 10 µl of 10% NaCl were added to each tube. The solution was mixed rapidly and allowed to stand for 5 min before determining the optical density (O.D.) at 580 nm. The color of samples changes from brilliant red to blue as the concentration of antibody decreases. The optimum concentration of antibody for AuNPs labeling was the lowest concentration of antibody that did not change the color.

4. Preparation of antibody-conjugated gold nanoparticles

The gold nanoparticle labeled antibody was prepared according to the method of Yokota et al (1992) (59). One ml of AuNPs solution was adjusted by varying pH (pH 7, 8, 9) with 0.2 M Na_2CO_3. For preparing an AuNPs conjugate, the anti-HPV16 E6 antibody (Table 7) was added to the 1 ml of the 10-nm or 40-nm AuNPs solution at an optimal antibody concentration. After gently mixing for either 60 min or 30 min as indicated, at RT, 0.1 ml of blocking solution (either 10% BSA or 3% PEG in Milli Q water) was added to block the AuNPs surface. After gently mixing for either 30 min or 15 min as indicated, at RT, the mixture was centrifuged at 25,000 rcf for 10-nm AuNPs at 4 °C for 30 min and 6,000 rcf for 40-nm AuNPs at 4 °C for 15 min. The supernatant was discarded and the AuNPs conjugate was resuspended in storage buffer (20 mmol/L sodium borate, pH 8, 1% BSA and 0.1% sodium azide). The centrifugation and resuspension steps were repeated and the AuNPs conjugate was finally suspended in 0.1 ml storage buffer and then stored at 4 °C.

Part V. Lateral flow assay

The lateral flow device consists of four components: a sample pad (1.5 x 0.4 cm^2 cellulose fiber, Millipore), a conjugation pad (0.8 x 0.4 cm^2 glass fiber, Millipore), a nitrocellulose membrane (2.2 x 0.4 cm^2 NCM, Millipore), and an absorbent pad (1.5 x 0.4 cm^2). Most components were fixed on a backing pad (6 x 0.4 cm^2). A schematic diagram of the lateral flow device is shown in Figure 7. Primary antibody, anti HPV 16 E6 polyclonal or monoclonal antibody, and the secondary antibody (against the antibody on the AuNPs surface) were applied in a test line and a control line, respectively on a piece of NCM. The distance between the test line and control line was 0.5 cm. Various types of antibody, sizes of AuNPs, and blocking solution were optimized. After drying for 1 h in desiccator at RT, the membrane was blocked with 10% BSA and then dried, washed with Milli Q water twice, dried and stored under dry conditions.

The conjugate pad was prepared by pouring the 10 µl of AuNPs-antibody conjugate and then drying for at least one h in desiccator at RT.

Finally, the sample pad, conjugate pad, NCM, and absorbent pad were assembled sequentially on backing pad. Each segment overlapped 0.2 cm to facilitate the migration of solution during the assay. The 20 µl of samples were applied on the sample pad, and 30 µl of the running buffer (see Appendix B) was then added. The result was interpreted as positive when the color signals appeared at both the test and control lines whereas the negative result showed no signal on the test line and positive signal at the control line. The results were read as invalid when control lines did not give expected positive signals.

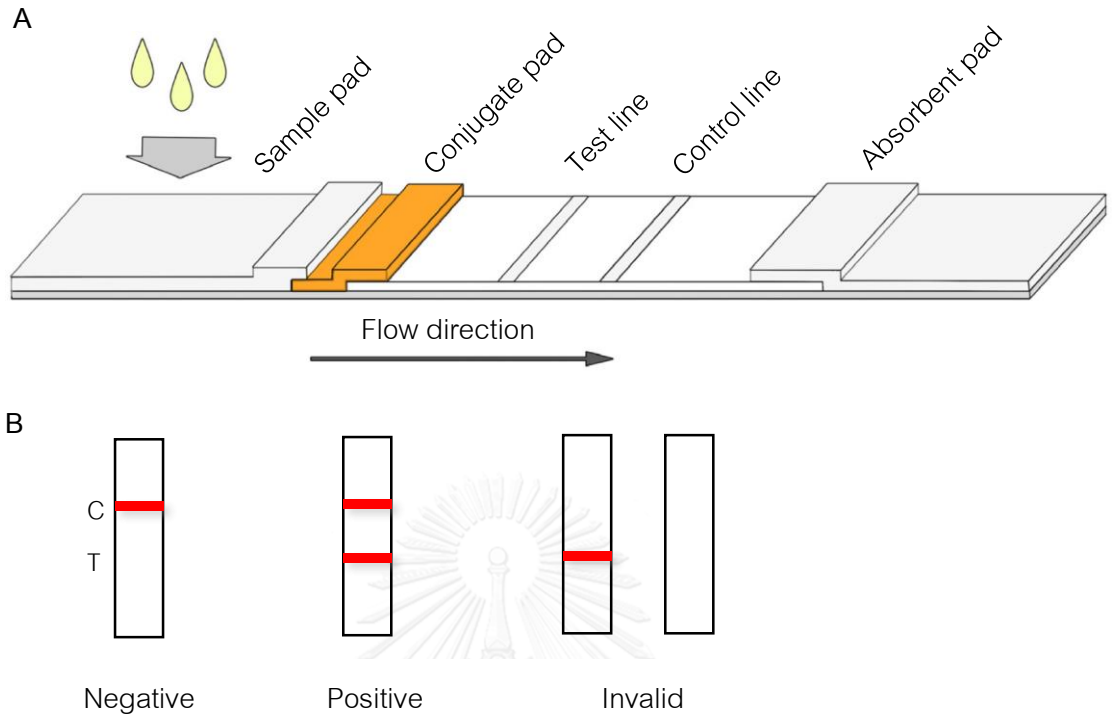

Figure 7 A schematic diagram of the lateral flow assay, A: The preparation and assembly of the lateral flow device (60), B: Result judgment of the strip (C, control line; T, test line)

CHAPTER V

RESULTS

Part I. Prevalence of HPV type 16 infection in precancerous and cancerous lesions

HPV detection and typing in clinical samples were performed by CLART Human Papillomavirus 2 kit based on PCR amplification of 450 bp fragment within the L1 region. The genotyping results were analyzed and automatically reported from Clinical Array Reader. All samples were positive for genomic DNA control and amplification reaction control. HPV DNA was detected in 185 (71.71%) of 258 clinical samples. The distribution of HPVDNA among each histological status, i.e., CIN I 90/136 (66.18%) CIN II 16/23 (69.57%), CIN III 26/32 (81.25%), SCC 53/67 (79.10%) was shown in Table 8. The prevalence of HPV type 16 infections per total cases was 20.16% (52/258) whereas the prevalence of HPV 16 among HPV DNA positive samples was 28.11% (52/185). The prevalence of HPV infection between CIN III (81.25%) and SCC (79.10%) was not different. The highest prevalence of HPV 16 infection was found in SCC (26/53, 49.06%).

Table 8 Histological classification and HPV positive

Histology	CIN I	CIN II	CIN III	SCC	Total
Number	136	23	32	67	258
Cases with HPV (+)	90	16	26	53	185
% HPV (+) per number	66.18%	69.57%	81.25%	79.10%	71.71%
Cases with 16 (+)	14	7	5	26	52
% cases per number	10.29%	30.43%	15.63%	38.81%	20.16%
% cases per HPV (+)	15.56%	43.75%	19.23%	49.06%	28.11%

Part II. Expression and purification of recombinant proteins

1. HPV16 E6 recombinant proteins

Recombinant HPV 16 E6 was successfully expressed in *E.coli* BL-21 (DE3) containing pGEX-3X-MT-E6 by inducing with 0.25 mM IPTG at 25°C for 6 h after that the protein was purified by GST affinity chromatography and further analyzed by SDS-PAGE and WB as shown in Figure 8. The E6 fusion protein band was about 43 kDa and the GST band was about 26 kDa.

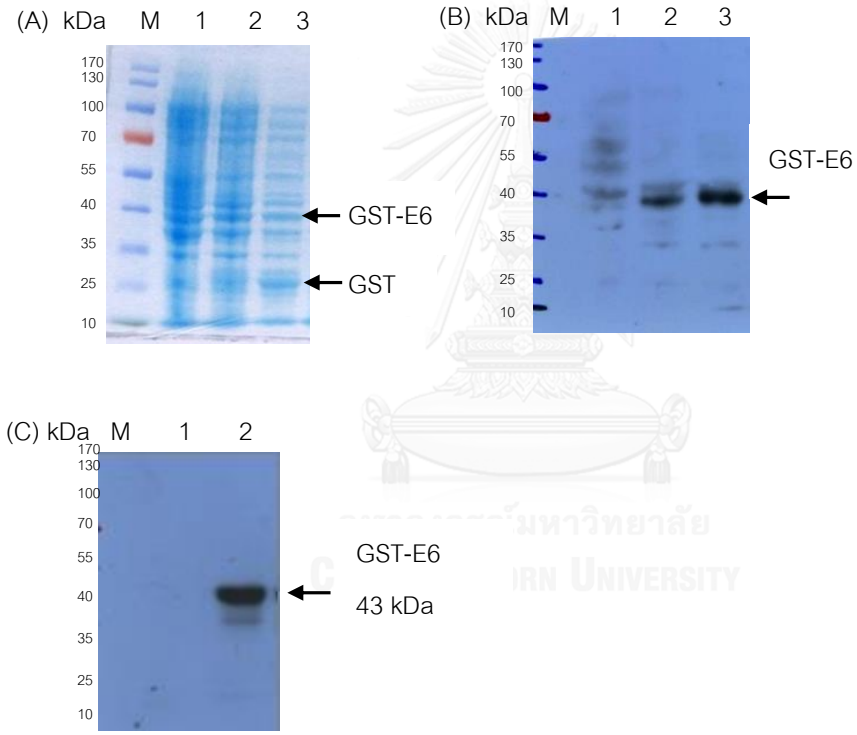

Figure 8 SDS-PAGE stained with coomasie blue (A) and western bolt determined by chemilluminescence (B) of GST-HPV 16 E6 from whole cell lysate (WCL) of E. coli BL-21 (DE3). Lane 1; non-induced WCL, lane 2 and 3; IPTG induced WCL from lot 1 and 2, respectively, (C) WB of proteins through GST affinity chromatography, lane 1; non-induced condition, lane 2; IPTG induced condition. M; molecular weight markers.
lane 1; WCL from E. coli BL-21 (DE3) non-induced by IPTG, lane 2; purified of GST-HPV 16 E6

2. HPV16 L1 recombinant proteins

The expression of *E.coli* BL-21 (DE3) containing pGEX-4T-2-L1 induced by 1 mM IPTG was at 37°C for 4 h. Since the recombinant HPV 16 L1 was found in inclusion bodies, the L1 in cell pellet of bacterial lysate was solubilized in 8M urea solution. The 8M urea solution protein containing recombinant HPV 16 L1 was analyzed by SDS-PAGE and Western blot showing the fusion protein band at about 80 kDa (Figure 9).

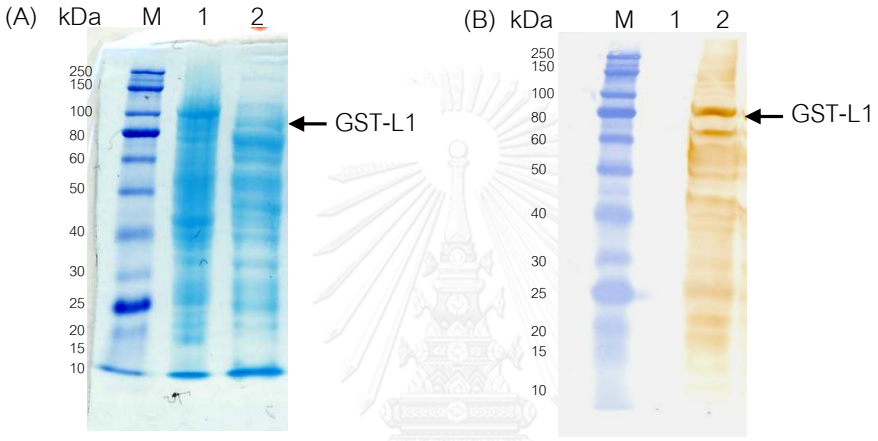

Figure 9 SDS-PAGE stained with coomasie blue (A) and western blot detected by chromogenic (DAB) reagent (B) of GST-HPV16 L1 after solubilized with 8M urea solution, lane 1; non-induced WCL, lane 2; IPTG induced WCL, M; molecular weight markers.

Part III. Lateral flow assay

1. Optimal concentration of binding antibody to gold nanoparticles

Prior to conjugation, the optimal amount of antibody that could stabilize AuNPs was determined. This can be obtained from a flocculation curve. 10% NaCl was added to each tube containing the mixture of various antibody concentration (0-10 µg/ml gold) and AuNPs solution. Flocculation was induced by addition of NaCl and absorbance was measured at 580 nm. The flocculation curve was generated by plotting the O.D. against the concentration of antibody (Figure 10). For anti-HPV 16 E6, the minimal antibody concentration that could saturate or stabilize the AuNPs was 6 µg/ml of AuNPs (arrow in Figure 10). In order to assure that the antibody was more than enough, the concentration of antibody used throughout this study was chosen at 10 µg per ml of gold solution. The color change of gold suspension was able to observe by naked eyes (Figure 11). The original color of the AuNPs suspension was pink and turned into blue when the antibody was inadequate. In contrast, the color remained stable when the antibody was enough or excess. From the picture, the blue color was shown at the concentration less than 6 µg/ml of AuNPs which corresponded to the results of flocculation curve.

Similar study was done with anti-HPV 16 L1 and the results were the same as anti-HPV 16 E6 (data not shown). Thus, the concentration of anti-HPV 16 L1 was at 10 µg per ml of gold solution as well.

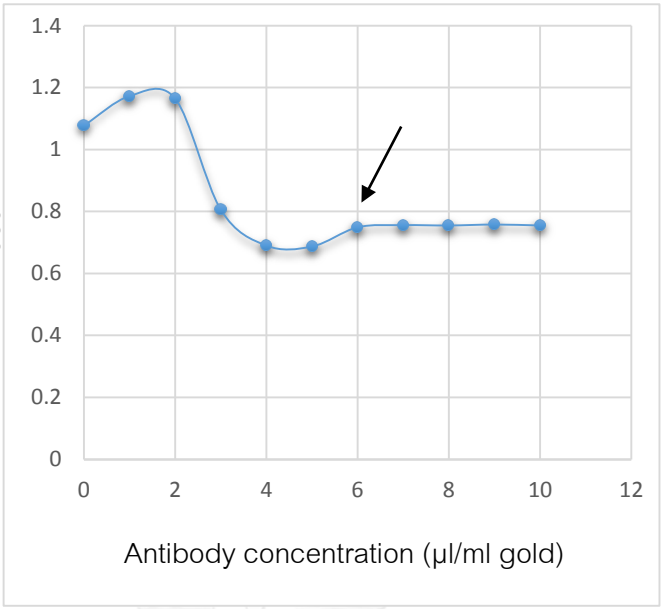

Figure 10 Example of Flocculation curve of the AuNPs conjugation with mouse monoclonal antibody against HPV 16 E6 at concentrations 0-10 µg/ml gold. The arrow indicates the minimal antibody (6 µg of antibody per 1 ml of gold solution) that was adequately bound to AuNPs.

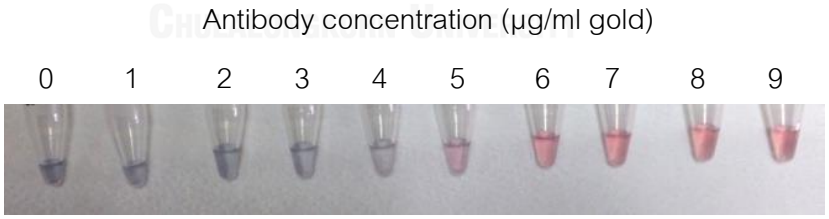

Figure 11 Photograph of the AuNPs conjugation with mouse monoclonal antibody against HPV 16 E6 at various concentrations (0-9 µg/ml gold).

2. Optimization of the lateral flow assay

The lateral flow assay to detect HPV 16 E6 or L1 antigen based on sandwich principle was developed and optimized. DW, Milli Q water or 0.1x PBS were used as a negative control while HPV 16 E6 or L1 recombinant protein was a positive control.

The lateral flow assay for HPV 16 L1 detection was optimized. The recombinant protein was applied on the lateral flow strip that goat polyclonal anti-HPV 16 L1 conjugated with 10-nm AuNPs and goat polyclonal anti-HPV 16 L1 was also immobilized on the test line and donkey anti-goat was on the control line. The result showed no signal on both test and control lines whereas the negative sample (Milli Q water) gave no signal at the test line and positive signal at the control line. This finding suggested the condition of 8M urea solution used to dissolve insoluble HPV 16 L1 recombinant proteins directly affected the immunological reaction on the strip. Then, the recombinant protein HPV 16 L1 was dialyzed serially to dilute out of the urea solution from 8M to 6M to 4M to 2M and finally Tris buffer, pH 8. It was found that L1 protein precipitated when the concentration of urea solution was less than 2M urea. However, HPV16 L1 solubilized in 2M Urea solution still showed no signal on both test and control lines indicating invalid results. Therefore, the development of lateral flow assay for detecting HPV 16 L1 was unsuccessfully attempted.

For development of lateral flow assay for detecting HPV 16 E6, the parameters that affected in the assay were optimized as follows.

2.1 Size of AuNPs.

Previous study of Kamolwan (14), 10-nm AuNPs was used in agglutination assay. So the study was continued using 10-nm AuNPs. However, several studies reported using 40 –nm size in lateral flow assay (48). Thus, in this study, both sizes of AuNPs have been compared. The AuNPs were obtained from both in-house and commercial AuNPs.

To evaluate the efficiency of gold particle size, the various setting conditions involving antibody conjugated gold and antibody used on the test and control lines were performed. The results were summarized in Table 9. Under optimized condition (Table 9; Attempt 9), both 10-nm and 40-nm AuNPs could be used. However, the 40-nm AuNPs gave better sensitivity of detection (5 μg of E6 recombinant proteins) than the 10-nm AuNPs (20 μg of E6 recombinant proteins).

Table 9 The results of optimization of the lateral flow assay conditions

Attempt	Size of AuNPs (nm)	Anti-E6 conjugated gold		Capture Ab Test line(anti-E6), T	Capture Ab Control line, C	Results			Interpre-tation
		Ab Conc. (ug/ ml gold)	Type			Sample	T	C	
1	10 40	2, 10 4, 6, 10	Goat polyclonal (sc-1584)	Goat polyclonal (sc-1584)	Donkey anti-goat (sc-2020)	DW	+	+	False positive
						Milli Q	+	+	
						0.1xPBS	+	+	
2	10, 40	10	Goat polyclonal (sc-1584)	Mouse monoclonal (sc-460)	Donkey anti-goat (sc-2020)	Milli Q	+	+	False positive
						0.1xPBS	+	+	
3	40	10	Mouse monoclonal (sc-460)	Goat polyclonal (sc-1584)	Goat anti-mouse (Pierce 31160)	Milli Q	+	+	False positive
						0.1xPBS	+	+	
4	40	10	Mouse monoclonal (sc-460)	Mouse monoclonal (sc-460)	Goat anti-mouse (Pierce 31160)	Milli Q	+	+	False positive
						0.1xPBS	+	+	
5	10, 40	10	Goat polyclonal (sc-1584)	Normal Goat IgG	Donkey anti-goat (sc-2020)	Milli Q	+	+	False positive
				Milli Q		Milli Q	-	+	
6	40	10	Mouse monoclonal (sc-460)	Normal Goat IgG	Goat anti-mouse (Pierce 31160)	Milli Q	+	+	False positive
				Milli Q		Milli Q	-	+	
7	10, 40	10	Mouse monoclonal (ab30716)	Mouse monoclonal (ab 70)	Goat anti-Mouse (Pierce 31160)	Milli Q	-	+	False Negative
						E6 protein	-	+	
8	10, 40	10	Rabbit polyclonal (ABIN733448)	Mouse monoclonal (ab 70)	Goat anti-mouse (Pierce 31160)	Milli Q	-	+	False Negative
						E6 protein	-	+	
9	10, 40	10	Mouse monoclonal (ab 70)	Rabbit polyclonal (ABIN733448)	Goat anti-Mouse (Pierce 31160)	Milli Q	-	+	Valid test
						E6 protein	+	+	

2.2 Type of antibodies used in Lateral flow device

Polyclonal and monoclonal from difference animal species (goat, mouse and rabbit) were used in this experiment as the AuNPs-labeled detection antibody and the capture antibody (on the test line). The antibody for control line was varied according to the antibody conjugated to AuNPs.

The 40-nm in-house AuNPs was first attempted to optimize the condition. The AuNPs were conjugated with goat polyclonal anti-HPV 16 E6 (sc-1584). Goat polyclonal anti-HPV 16 E6 (sc-1584) was also immobilized on the test line and donkey anti-goat (sc-2020) was on control line (Figure 12, T: test line, C: control line). After adding the samples (purified recombinant HPV 16 E6, Milli Q water and 0.1x PBS), false positive results occurred for all any given negative samples (Figure 12).

Figure 12 The false-positive results on lateral flow strip. Goat polyclonal anti-HPV 16 E6 (sc-1584) conjugated with 40-nm in-house AuNPs and goat polyclonal anti-HPV 16 E6 (sc-1584) was also immobilized on test line and donkey anti-goat (sc-2020) was on control line (T: test line, C: control line), A: 20 µg of HPV 16 E6 recombinant protein, B: Milli Q water, C: 0.1x PBS.

To characterize the AuNPs conjugate, the zeta potential measurement was used. Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential can indicate the stability of colloidal gold. The colloids with high zeta potential (more than ±30 mV) are electrically stabilized while colloids with low zeta potentials (from 0 to ±30) tend to aggregate. Besides zeta potential, the instrument can also calculate the mean size of particles. The zeta potential (-0.0553) and mean size of 40-nm in-house AuNPs conjugated goat polyclonal anti-HPV 16 E6 antibody (132 nm) were measured. The results indicated that the AuNPs conjugate was unstable and formed aggregation resulting to the false positive lines in lateral flow assay.

According to that result, several combination of the antibodies either monoclonal or polyclonal, were performed as followed.

(i) Polyclonal antibody-conjugated AuNPs, Polyclonal antibody-capture test line

(ii) Polyclonal antibody-conjugated AuNPs, Monoclonal antibody-capture test line

(iii) Monoclonal antibody-conjugated AuNPs, Polyclonal antibody-capture test line

(iv) Monoclonal antibody-conjugated AuNPs, Monoclonal antibody-capture test line

To confirm the presence of antibody on the test line, protein A/G, a recombinant fusion protein containing immunoglobulin Fc binding sites of both protein A and protein G, directly conjugated AuNPs was used to react with immobilized rabbit polyclonal or mouse monoclonal anti-HPV 16 E6 on lateral flow strip. The positive signal appeared on both strips confirmed the presence of antibody (Figure13).

A
B

Figure 13 The positive signal of protein A/G conjugated with 40-nm commercial AuNPs on lateral flow strip, A: rabbit polyclonal anti-HPV 16 (ABIN733448) and B: mouse monoclonal anti-HPV 16 E6 were immobilized.

Most of the results in those attempts (Table 9; 1 to 8) gave non-specific binding to test line indicating false positive. Moreover, false negative results were also demonstrated. Until the 9th attempt, mouse monoclonal anti-HPV 16 E6 (ab70) was used as the AuNPs-conjugated antibody and rabbit polyclonal anti- HPV 16 E6 antibody (1 mg/ml) was used at the test line. The valid results were seen as in Figure 14.

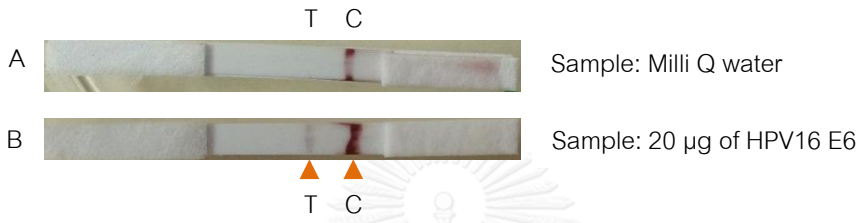

Figure 14 The valid results of lateral flow assay in condition of mouse monoclonal anti-HPV 16 E6 (ab70) conjugated with 10-nm commercial AuNPs. The rabbit polyclonal anti-HPV 16 E6 (ABIN733448) was immobilized on the test line and goat anti-mouse (Pierce 31160) on the control line (T: test line, C: control line), A: Milli Q water, B: 20 µg of HPV16 E6 recombinant protein

2.3 Blocking solution

Blocking solution was one factor so optimization between 10% BSA and 3% PEG was compared. From Figure 15, when 3% PEG was used as blocking solution in 10-nm AuNPs conjugation in control negative sample (Milli Q water), the result showed stronger positive signal compared to 10% BSA. In the meantime, using the 40-nm AuNPs conjugates, 10% BSA blocking solution gave more strongly positive signal than 3% PEG. The results indicated that 10% BSA was appropriate for 40 nm AuNPs, while 3% PEG was appropriate for 10 nm AuNPs.

Blocking solutions	10-nm AuNPs	40-nm AuNPs
10% BSA		
3% PEG		

Figure 15 The results of compared blocking solution between 10% BSA and 3%PEG

Finally, the condition for detecting HPV 16 E6 protein by the lateral flow assay was as followed

(i) Size 40-nm commercial AuNPs

(ii) Mouse monoclonal anti-HPV 16 E6 (ab70) (10 µg/ml AuNPs) as the AuNPs-conjugated Ab.

(iii) Polyclonal rabbit anti- HPV 16 E6 (ABIN733448) 1 µg at the test line

(iv) Goat anti-mouse immunoglobulin G (Pierce 31160) 0.5 µg at the control line

(v) 10% BSA as blocking solution

Under the optimized condition, recombinant HPV 16 E6 proteins were detectable at 5 µg of total proteins within 5 min after reaction whereas negative samples showed no signal until 30 min. All control lines gave completely clear. The test line was very faint signal but able to see by naked eyes.

3. Sensitivity of the assay

Sensitivity of the assay was determined using purified HPV 16 E6 recombinant protein at various amount of 5, 10, 15 and 20 µg. The assay using 40-nm commercial AuNPs indicated that at least 5 µg of purified HPV 16 E6 recombinant proteins was detectable by naked eye while at least 20 µg of total E6 proteins were detectable when 10-nm commercial AuNPs was used (Figure 14).

Since it showed quite low sensitivity of detection, Protein A/G precipitation assay was used to evaluate the binding conformation of antibody on the surface of the gold particles. Protein A/G was immobilized on the membrane of lateral flow strip. The AuNPs conjugated with mouse monoclonal anti-HPV 16 E6 (ab 70) and running buffer were applied on the strip. The result showed a positive signal suggesting that Fab part of the antibodies bind to the gold particles and inversely present Fc portion out of the surface (Figure 16). This was one possible explanation why the sensitivity of the assay was low.

Figure 16 The positive signal of immobilized protein A/G after binding with mouse monoclonal anti-HPV 16 E6 (ab70) conjugated 40-nm commercial AuNPs on lateral flow strip.

Consequently, to increase sensitivity, higher amount of polyclonal antibody was applied on the test line (from 1 µg to 2 µg). Unfortunately, no difference of signal was observed. In addition, the pH of system was also varied (pH 7, 8, 9), i.e., AuNPs conjugate solution and running buffer. As the results, the detection signal was still the same (faint positive signal in the test line) in all pHs.

Beside HPV 16 E6 recombinant protein, cervical cell lines containing HPV 16 for example CaSki and SiHa cell lines were used as positive samples to mimic the clinical

specimens used in the assay. The results found approximately $6.7x10^5$ of both CaSki and SiHa cell lysate gave positive signal on the test line by naked eye observations.

To examine that antibody-conjugated AuNPs can bind with HPV 16 E6 antigen on membrane directly, negative (Milli Q water and C33A cell line) and positive samples (recombinant HPV 16 E6,) were immobilized onto membrane and then the solution of antibody-conjugated gold was run through the membrane. As a result, no signal was detected in negative sample while the positive sample gave the obviously signal on the membrane and some result was shown in Figure 17. The detection signal from direct detection was more obviously observed than that from sandwich format.

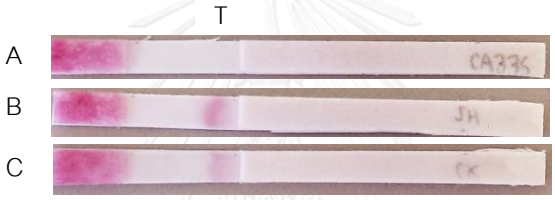

Figure 17 Direct detection of lateral flow assay, A: C33A, B: SiHa, C: CaSki protein were immobilized on membrane and mouse monoclonal (ab70) conjugated with 40-nm commercial AuNPs was applied on lateral flow assay (T: test line).

4. Specificity of the assay

The specificity of the developed lateral flow assay was tested against different human tumor cell lysate containing other HPV, i.e. Hela containing HPV type 18, ME-180 containing type 18 and 39, MS751 containing HPV type 18 and 45, C33A, and HEp-2 (human laryngeal carcinoma) without HPV DNA. No positive signal on the test line was observed indicating no cross-reactivity with at least HPV type 18, 39, and 45 (Figure 18).

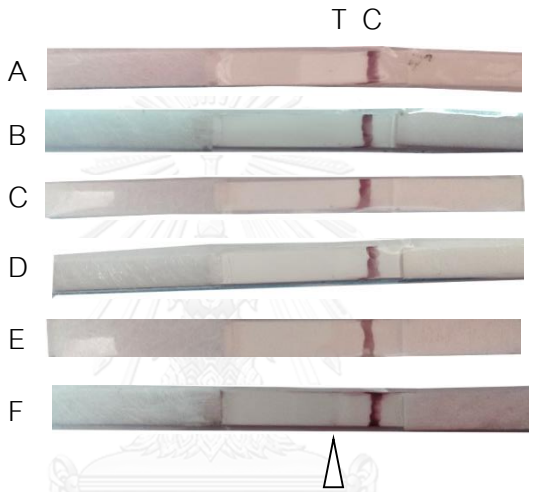

Figure 18 Lateral flow strip test with control cell lysates, A: HeLa, B: ME-180, C: MS751, D: C33A, E: HEp-2, and F: CaSki as positive control

5. Attempts for improvement

From the result, faint positive signal on the test line was the problem yet to be solved. The attempts were made to improve the sensitivity of the test. First, the principle of the assay was changed to competitive principle. Recombinant HPV 16 E6 proteins were immobilized at the test line. If the sample does not contain an excess of free antigen (HPV 16 E6), the antibody conjugated AuNPs can bind at the capture test line, giving a signal, whereas positive sample will block the binding sites on the antibodies, giving no signal. Nevertheless, this principle was not successful developed. There are always signal on the test line even with or without the antigen.

Moreover, an attempt to produce the polyclonal antibody specific to recombinant HPV 16 E6 protein was done using mouse model. Three injections was immunized using HPV 16E6 combining with complete Freund's adjuvant at the first time for a week followed by incomplete Freund's adjuvant twice 2 weeks apart. The blood sample was collected a week after the second and third immunization. The anti-HPV 16 E6 titer (2-fold dilution from 1:10-1:160) was determined in serum by immunofluorescence assay using CaSki cell line. The titer of both samples was 1:10. To concentrate the antibody, ammonium sulphate was used to precipitate the immunoglobulins from mouse serum. The concentrated antibody was then dialyzed in Tris buffer, pH 8 and applied as the captured test line. The result showed false positive signal.

CHAPTER VI

DISCUSSION

Cervical cancer worldwide is infected with specific types of HPV DNA mainly HPV 16 and HPV 18 (7, 8). In Thailand, the incidence of cervical cancer ranks top among cancers in women and is reported that HPV16 causes more than 50% of cervical cancer (8). In this study, among 258 clinical samples, HPV DNA was able to detect in 185 (71.71%) samples with CIN I (66.18%), CIN II (69.57%), CIN III (81.25%) and SCC, (79.10%). The recent information of the percentage of HPV infection in CIN I and CIN II was almost double time higher than previous study which reported that HPV infection was 33.3% in CIN-I and 36.8% in CIN-II (61). It suggested that the incidence of HPV infection increases with time among women. However, the percentage of HPV infection in CIN III and SCC in this study was not different from other studies, i.e., 74% CIN III (62), 75% CIN-III (61) and 82% in SCC (63). It is interesting that the highest prevalence of HPV 16 infection was found mostly in CIN II (43.75%) and SCC (49.06%) but not in CIN III (19.23%) which previously reported to be 48.65% by Lertworapreecha M, *et al,* 1998 (62). The reason of declining in HPV 16 prevalence might be due to the increase of another HPV type such as type 52 which was found up to 23.08% (data not shown). Some study showed that not only HPV 16 but also HPV 52 is the most common found in cervical cancer such as 16.3% (64) and 20.3% (65) in Taiwan and 15% in China (66). Moreover, Kantathavorn N, *et al,* in 2015 (67) from Thailand reported HPV type 52, 16, and 51 are the most common high risk genotypes found in 5906 Thai women who visit gynecology clinic for screening program. However, HPV 16 and 52 are classified in the same group.

Nowadays, the cervical cancer screening test is co-testing which based on cervical cytology screening and HPV testing (68). However, those tests cannot imply the stage of cancer development and also require for experience technician and expensive instrument. To early diagnosis of the cancer development, HPV 16 E6 is suggested to be a candidate marker. Since it is well known that HPV E6 oncoprotein is overexpressed in

cervical cancer cells, the presence of HPV E6 oncoprotein is a significant marker to early diagnosis of cervical cancer development. At present, HPV E6 mRNA detection kit is commercially available. Disadvantage of this assay is required laboratory and instrument. Therefore, an idea to detect HPV E6 protein instead of mRNA might solve the limitation since protein is more stable than mRNA. Additional with HPV L1 protein detection may have advantages for evaluating the HPV productive stage. In this study, a rapid lateral flow assay was developed to detect HPV 16 E6 and L1 proteins using antibody combining nanogold technology.

Recombinant HPV16 E6 and L1 proteins were used as positive control in the lateral flow assay development. Two recombinant fusion proteins, GST-HPV 16 E6 and GST-HPV 16 L1, were successfully prepared from *E. coli.* The method was followed by Kamonwan (14). The localizations of GST-HPV 16 E6 proteins was in soluble fraction and of recombinant HPV 16 L1 was in inclusion bodies which accordance with other reports (10, 69). Urea solution (8M) was used as solubilizer in extraction of recombinant HPV 16 L1 proteins. The yield of purified protein was very low. Urea in recombinant HPV 16 L1 protein directly affected the immunological reaction on the strip though it was dialyzed in Tris buffer. Thus, the development of lateral flow assay for detecting HPV 16 L1 was unsuccessfully attempted. The expressed GST-HPV 16 E6 proteins were easily purified by GST fusion protein affinity column purification kit. It can be further used in the lateral flow assay development.

To optimize the antibody required to coat on AuNPs, the flocculation curve followed Safenkova I, *et al.* (58) was created. The minimum antibody concentration was chosen in order to prevent the aggregation of AuNPs in the presence of excess salt (10 % NaCl) (70). Flocculation curve was generated between the absorbance at 580 nm in the presence of 10 % NaCl and the antibody concentration. The curve shows the characteristic behavior in agreement with flocculation curve previously reported (58) as expected. The region of the concentration dependence starting from a concentration of 6 µg/ml gold corresponds to the completion of stabilization of AuNPs by antibodies (arrow in Figure 10). However, the optimum antibody concentration (as estimated from the

flocculation curve) resulted in approximately 50-90 % coverage of the AuNPs surface (58). Choi DH, *et al.* (71) showed that the conjugated antibody concentration of 10 µg/ml gold could bind to many sizes of AuNPs (10, 20, 40 and 60 nm) without significant difference. Moreover, a study of Safenkova I, *et al.* (58) found that the concentration of 15 µg/ml gold demonstrated maximum binding with the analyte after varying the concentration of antibody from 10 to 100 µg/ml gold. However, higher concentration of antibody up to 23µg/ml gold has been reported (72). For these reasons, the excess concentration of antibody at 10 µg/ml gold was used in this further experiment to ensure the completeness of the reaction.

The factors which may affect the lateral flow reaction were examined. The size of the AuNPs influences the optical properties of the particle (73, 74). Several publications report on a comparison of the size (5 to 80 nm) of the nanoparticles (58, 75, 76). Safenkova I, *et al.* (58) showed the sensitivity using larger particles was observed. They found that the detection limit decreased from 80 to 3 ng/ml for a series of particles with a diameter from 6.4 to 33.4 nm. However, Laitinen MPA and Vuento M showed the instability of AuNPs decreased as the size increased (75). In this study, the AuNPs in diameter of 10 nm was used followed previous study of Kamolwan (14) and the AuNPs in diameter of 40 nm was another chosen since the size in diameter of 39 ± 5 nm provided best performance for gold nanoparticles in lateral flow assay (48). All attempts in this study used both 10-nm and 40-nm commercial AuNPs.

The antibody is another important factor that affects directly to the immunologic reaction on the strip. It should be high affinity and specific to provide a satisfying response. The type of antibody used in sandwich principle should be a combination of monoclonal antibodies in different epitopes for giving high specificity. However, the antibodies used in this study are purchases from commercial available. Thus, the limitation of this study is the selection of antibody epitope which was restricted and no different epitope monoclonal antibodies are in the market to purchase. Polyclonal and monoclonal antibodies from different animal species (goat, mouse and rabbit) were used in optimization. When preliminary attempts were performed to find the optimal

combination of antibodies using both polyclonal and monoclonal antibodies as indicated in Table 9, we found mostly false-positive results. One reason is that polyclonal antibody is produced by the immunization of whole antigens resulted in low specificity (77, 78). After optimization, the optimal antibody used to conjugate with AuNPs is mouse monoclonal antibody whereas the capture antibody on the test line is rabbit polyclonal antibody (Table 9; attempt 9). Interestingly, species of the animal produced antibody might affect the reaction as well.

In the first optimization of conjugated antibody, zeta potential measurements (-0.0553) revealed the antibody conjugated nanoparticles were unstable and the mean size of conjugated gold particles was 132 nm which was correlated to the false positive results of lateral flow due to aggregation of the particles. It is noted that the zeta potential and the mean size are not further used because the measurement requires a large number of conjugated gold particles (at least 1 ml).

Blocking solution between 10% BSA and 3% PEG were compared. From Figure 15, the results indicated that 3% PEG was suitable for 10-nm AuNPs blocking and 10% BSA was for 40-nm AuNPs blocking. There are 3 types of gold suspension solutions, i.e., Tannic acid solution (0.01% of $HAuCl_4$ suspended in 0.01% tannin acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide), DI and PBS. The difference of the gold solution also one factor affected types of blocking solution. Tannic acid solution was good with 3% PEG whereas DI and PBS were good with 10%BSA.

Finally, the optimal condition for detecting HPV 16 E6 protein was mouse monoclonal anti-HPV 16 E6 as conjugated AuNPs, polyclonal rabbit anti- HPV 16 E6 as captured antibody at the test line and goat anti-mouse immunoglobulin G at the control line. Under this condition, 40-nm commercial AuNPs gave the detectable positive line by naked eye at least 5 μg of recombinant HPV 16 E6 proteins while at least 20 μg of recombinant HPV16 E6 proteins was detectable when 10-nm commercial AuNPs was used. Then, the assay was tested with the cervical cell lines containing HPV 16 for example CaSki and SiHa to mimic the clinical specimens. Approximately $6.7x10^5$ of both

CaSki and SiHa cell lysate gave positive signal on the test line by naked eye observations. However, this developed test showed very low sensitivity of detection and may not be sensitive enough to detect HPV 16 E6 in clinical specimens. The sensitivity and limit of detection of lateral flow assay can be improved by various means, such as optimizing size of gold nanoparticles, adjusting concentration of antibody used for detection on the test line or conjugation with AuNPs, optimizing the conditions of the conjugation of AuNPs with the antibody and conditions of antigen-antibody binding (46, 79). Although we tried to improve the sensitivity of the assay, the results still showed faint positive signal on the test line.

From Figure 16, protein A/G was immobilized on the membrane to evaluate the binding conformation of antibody on the surface of the gold particles. The results showed a positive signal suggesting that Fab part of the antibodies bind to the gold particles and inversely present Fc portion out of the surface. This was one possible explanation why the sensitivity of the assay was low. After that the assay was determined for specificity using different human tumor cell lysates (Table 6). The results indicated that no cross-reactivity with at least HPV type 18, 39, and 45 (Figure 18).

An attempt to increase sensitivity was performed by changing the sandwich principle to competitive principle but it was not succeeded. In addition, another attempt to produce the mouse polyclonal antibody specific to recombinant HPV 16 E6 protein in-house gave very low anti-HPV E6 titer with high non-specific background. As expected, E6 is a poor immunogenic protein (80, 81).

In conclusion, our study could develop a sandwich lateral flow assay using gold nanoparticles. But it still could not apply to clinical samples due to very low sensitivity. However, this has just begun for the future improvement of the assay. The sensitivity and the specificity of the test will be further enhanced since the lateral flow assay is a promising tool as a point of care test for early and rapid screening test especially in the clinical field, low cost and no instrument requirement.

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APPENDICES

APPENDIX A

REAGENTS, MATERIALS AND INSTRUMENTS

A. Media and Reagents

Absolute ethanol	(Merck, Germany)
AccuGel™ 29:1 (40% Acrylamide)	(National diagnosis, USA)
Acetic acid	(Merck, Germany)
Amersham ECL Prime	(GE healthcare life science, UK)
Ammonium persulfate	(Bio Basic, USA)
Amplicilin	(Bio Basic, USA)
Bovine serum albumin	(Sigma Aldrich, USA)
Coomassie blue	(Sigma Aldrich, USA)
cOmplete Lysis-M	(Roche, USA)
CLART HPV2 Amplification kit	(Genomica, Spain)
DAB (3,3'-diaminobenzidine) Substrate Solution	(Sigma Aldrich, USA)
DTT	(Bio Basic, USA)
Donkey anti-goat IgG	(Santa Cruz, USA)
ECL reagent	(Prierce Biotechnology, USA)
EDTA	(Sigma-Aldrich, USA)
Fetal bovine serum	(GIBCO, USA)
Glutathione reduced	(USB, USA)
Goal colloidal solution (10 nm)	(Sigma Aldrich, USA)
Goal colloidal solution (40 nm)	(Cytodiagnostics, Canada)
Goat anti-mouse IgG conjugate HRP	(Santa Cruz, USA)
Goat anti-mouse IgG unconjugated	(Pierce, USA)
Goat anti-rabbit IgG conjugate HRP	(Santa Cruz, USA)
Guanidine hydrochloride	(Bio Basic, USA)
HEPES	(Sigma Aldrich, USA)

High pure viral nucleic acid kit	(Roche, USA)
HPV 16 E6 goat polyclonal antibody	(Santa Cruz, USA)
HPV 16 E6 rabbit polyclonal antibody	(Antibodies-online, Germany)
HPV 16 E6 mouse monoclonal antibody	(Abcam, UK)
HPV 16 E6 mouse monoclonal antibody	(Santa Cruz, USA)
HPV 16 L1 goat polyclonal antibody	(Santa Cruz, USA)
HPV 16 L1 mouse monoclonal antibody	(Santa Cruz, USA)
Hydrogen peroxide (H_2O_2) solution	(Sigma Aldrich, USA)
IPTG	(Bio Basic, USA)
LB broth powder	(Oxoid, UK)
LB agar powder	(Oxoid, UK)
Lysozyme	(Sigma Aldrich, USA)
MEM medium	(GIBCO, USA)
Methanol	(Merck, Germany)
$NaHCO_3$	(Merck, Geramany)
Penicillin G	(GIBCO, USA)
Polyethyleneglycon	(Sigma Aldrich, USA)
Potassium chloride	(Merck, Germany)
Potassium phosphate	(Bio Basic, USA)
Prestained protein ladder	(Thermo Scientific, USA)
Proteases inhibitor cocktail tablets	(Roche, USA)
Quant-IT protein assay kit	(Invitrogen, UK)
Sodium bicarbonate	(Bio Basic, USA)
Sodium chloride	(Merck, Germany)
Sodium dodecyl sulfate (SDS)	(Bio Basic, USA)
Sodium phosphate	(Bio Basic, USA)
Sodium tetraborate decahydrate	(Sigma Aldrich, USA)
Streptomycin	(Invitrogen, USA)
TEMED	(Bio Basic, USA)

Triton X-100	(Sigma Aldrich, USA)
Trypsin	(Bio Basic, USA)
Tris (hydroxymethyl) aminonethane (Trizma base)	(Sigma Aldrich, USA)
Tween 20	(USB, USA)

B. Materials

Absorbent pad	(Merck Millipore, Germany)
Affinity column GSTrap FF	(GE healthcare, USA)
Amicon® Ultra 15 ml filters	(Merck Millipore, Germany)
Centrifuge tube	(Labcon, Germany)
Conjugation pad (glass fiber)	(Merck Millipore, Germany)
Disposable cuvettes	(BIO-RAD, USA
Filter tip	(Sorenson, USA)
Microcentrifuge tube	(Sorenson, USA)
Nitrocellulose membrane	(Merck Millipore, Germany)
Polyvinylidene fluoride (PVDF) membrane	(GE Healthcare, UK)
Sample pad (cellulose fiber)	(Merck Millipore, Germany)
Snakeskin dialysis tubing	(Thermo scientific, USA)
SurePath sample collection kit	(BD, USA)
Tissue culture flask (T25 and T75)	(Nunc, Denmark)
X-ray film	(Agfa healthcare, Belgium)

C. Instruments

Autoclave (model-SX-700)	(Tomy, Japan)
ChemiDoc	(Bio-rad, UK)
Clinical Array Reader	(Genomica, Spain)
Centrifuge (Biofuge Stratos)	(SORVALLR, Germany)
CO_2 incubator	(Thermo Forma, USA)
Incubator	(Memmert, Germany)

Inverted microscope	(OLYMPUS, Japan)
Microcentrifuge (model: Froce 1418)	(Edison, USA)
Microscope	(OLYMPUS, Japan)
Mini Analytical Protein Electrophoresis Cells	(BIO-RAD, USA)
Mixer-vortex	(Scientific industrial, USA)
pH meter	(Accmet Basic, Singapore)
Qubit fluorometer	(Invitrogen™, UK)
Refrigerator	(Sanyo, Japan)
Spectrophotometer (SmartSpect™ 3000)	(BIO-RAD, USA)
Sonicator	(SONIC, USA)
Thermal cycler	(BIOER, China)
Trans-Blot® Cell	(BIO-RAD, USA)
Water bath	(Julabo, Gernany)

APPENDIX B

REAGENTS PREPARATION

Reagent for preparation of recombinant HPV 16 proteins

1. **Luria-Bertani broth**

LB broth powder	40	g
DW to	1	L

Sterilized by autoclave

2. **Luria-Bertani agar**

LB agar powder	30	g
DW to	1	L

Sterilized by autoclave

To pour plate, agar was allowed to cool about 50 °C and then amplicilin 100 mg/ml was added. After drying, plates were stored at 4 °C until used.

3. **Lysis buffer**

PBS (steriled)	9.65	ml
100 mM DTT	200	µl
Triton X-100	100	µl
200 KU Lysozyme	50	µl
Proteases inhibitor cocktail tablets	1	tabblet

Stored at 4°C

4. **Binding buffer**

NaCl	8	g
KCl	20	mg
Na_2HPO_4	1.1	g
KH_2PO_4	20	mg
DDW to	90	ml

Adjusted to pH 7.3 and adjusted volume to 100 ml with additional DDW

Sterilized by filtration (0.45 μm) and stored at room temperature

5. **Elution buffer**

Tris-HCl	1.21	g
Glutathione reduced	0.61	g
DDW to	190	ml

Adjusted to pH 8.0 and adjusted volume to 200 ml with additional DDW

Sterilized by filtration and stored at room temperature

6. **L1 washing buffer**

Tris	6.06	g
NaCl	5.84	g
DTT	0.15	g
EDTA	0.37	g
DDW (steriled) to	800	ml

Adjusted to pH 8.0 and adjusted volume to1000 ml with additional DDW (steriled) and stored at 4°C

7. **8M Urea solution**

Urea	240.24	g
NaH_2PO_4	6	g
EDTA	0.19	g
DTT	0.08	g
Tris	0.61	g
DDW(steriled) to	800	ml

Adjusted to pH 8.0 and adjusted volume to 1000 ml with additional DDW and stored at 4°C

Reagent for SDS-PAGE and western blot

1. **Resolving gel (10% Acrylamide)**

DDW	4.9	ml
1.5M Tris-HCl (pH 8.8)	2.5	ml
40% Acrylamide/bis 37.5:1	2.5	ml
10% SDS	100	µl
10% APS	50	µl
TEMED	20	µl

2. **Stacking gel (5% Acrylamide)**

DDW	2.4	ml
40% Acrylamide/bis 37.5:1	0.5	ml
0.5M Tris-HCl (pH 6.8)	1	ml
10% SDS	40	µl
10% Ammonium persulfate (APS)	40	µl
TEMED	4	µl

3. 1.5M Tris-HCl (pH 8.8)

Tris(hydroxymethyl)aminonethane-hydrochloride	18.5	g
DW	100	ml

Stored at room temperature

4. 0.5M Tris-HCl (pH 6.8)

Tris(hydroxymethyl)aminonethane-hydrochloride	3	g
DW	50	ml

Stored at room temperature

5. 10X Running buffer

Tris(hydroxymethyl)aminonethane	30.3	g
Glycine	144	g
SDS	10	g
DW	1000	g

Stored at 4°C

1X Running buffer

10X Running buffer	100	ml
DW	900	ml

Stored at room temperature

6. 10x Blotting buffer

Tris(hydroxymethyl)aminonethane	30.3	g
Glycine	144	g

Stored at 4°C

7. 1X Blotting buffer

10X Blotting buffer	100	ml
Methanol	200	ml
DW	800	ml

Stored at room temperature

8. 20X Tris buffer saline (TBS, pH 7.5)

Tris(hydroxymethyl)aminonethane	24.23	g
NaCl	175.32	g
DW	1000	ml

Stored at room temperature

9. 1X TBST

20X TBS	50	ml
Tween20	1	ml
DW	950	ml

Stored at room temperature

10. 6X Sample buffer

Bromophenol blue	0.002	g
SDS	1	g
Glyceral	3	ml
5% β-mercaptoethanol	0.5	ml
0.5M Tris-HCl pH 6.8	7	ml

Stored at -20^{o}C

11. 0.1% Bovine serum albumin (for dilute antibody)

Bovine serum albumin	0.05	g
1X TBST	50	ml

Stored at 4^{o}C

12. 3% Bovine serum albumin (membrane blocking reagent)

Bovine serum albumin	0.9	g
1X TBST	30	ml

This reagent should be fresh preparation

Reagent for cells cultivation

1. 1M HEPES

HEPES	23.83	g
DDW (steriled)	100	ml

Sterilized by autoclave and stored at 4°C

2. 10% Na_2HCO_3

Na_2HCO_3	10	g
DDW (steriled)	100	ml

Sterilized by autoclave and stored at 4°C

3. 2X MEM

MEM medium	20.8	g
DDW (steriled)	1000	ml

Sterilized by filtration and stored at 4°C

4. 10% MEM

2X MEM	100	ml
Fetal bovine serum	20	ml
1M HEPES	2	ml
Pen/Strep Antibiotic (5,000U/ml)	2	ml
10% Na_2HCO_3	3	ml
DDW (steriled)	73	ml

Stored at 4°C

5. 5% Trypsin

Trypsin	5	g
DDW (steriled)	100	ml

Sterilized by filtration (0.2 μm) and stored at -20°C

6. 0.25% Trypsin-EDTA

5% Trypsin (steriled)	5	ml
1% EDTA (steriled)	2	ml
1X PBS (steriled)	93	ml

Stored at 4°C

7. 1% EDTA

EDTA	1	g
DDW (steriled)	100	ml

Sterilized by autoclave and stored at 4°C

8. 10X PBS pH 7.4

NaCl	80	g
KCl	2	g
Na_2HPO_4	11.50	g
KH_2PO_4	2	g
DDW (steriled)	1000	ml

Sterilized by autoclave and stored at room temperature

9. 1X PBS

10X PBS (steriled)	50	ml
DDW (steriled)	450	ml

Stored at room temperature

Reagent for Lateral flow assay

1. **10% NaCl**

NaCl	1	g
DDW (steriled)	10	ml

Stored at room temperature

2. **10% BSA**

BSA	1	g
DDW (steriled)	10	ml

Stored at 4°C

3. **10% PEG**

PEG	1	g
DDW (steriled)	10	ml

Stored at 4°C

4. **Storage buffer**

Sodium tetraborate decahydrate ($Na_2B_4O_7$)	0.4	g
BSA	1	g
Sodium azide	0.1	g
Milli Q water	90	ml

Adjusted to pH 8.0 and adjusted volume to 100 ml with additional Milli Q water and stored at 4°C

5. **Running buffer**

0.1X PBS	99	ml
Tween 20	1	ml

Stored at room temperature

VITA

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